Immunochromic evidence that human apoB differs when expressed in rodent versus human cells


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Abstract  LDL from human apolipoprotein B-100 (apoB-100) transgenic (HuBTg+/+) mice contains more triglyceride than LDL from normolipidemic subjects. To obtain novel monoclonal antibody (MAb) probes of apoB conformation, we generated hybridomas from HuBTg+/+ that had been immunized with LDL isolated from human plasma. One apoE-specific and four anti-apoB-100-specific hybridomas were identified. Two MAbs, 2E1 and 3D11, recognized an epitope in the amino-terminal 689 residues of apoB in native apoB-containing lipoproteins (LpBs) from human plasma or from the supernatant of human hepatoma HepG2 cells, but did not react with LpB from HuBTg+/+ mice or LpB secreted by human apoB-100-transfected rat McArdle 7777 hepatoma cells. 2E1 reacted weakly and 3D11 reacted strongly with apoB from HuBTg+/+ mice after SDS-PAGE. The lack of expression of the 2E1 and 3D11 epitopes on native LpB from HuBTg+/+ mice did not solely reflect the abnormal lipid composition of murine LpB. Both epitopes were detected in all human plasma samples tested and in all human plasma LpB classes. Therefore, human apoB expressed by rodent hepatocytes or hepatoma cells appears to adopt a different conformation or undergoes different posttranslational modification than apoB expressed in human hepatocytes or hepatoma cells.—Wang, X., V. Chauhan, A. T. Nguyen, J. Schultz, J. Davignon, S. G. Young, J. Borén, T. L. Innerarity, H. Rutai, and R. W. Milne. Immunochromic evidence that human apoB differs when expressed in rodent versus human cells. J. Lipid Res. 2003. 44: 547–553.

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Lipoproteins containing apolipoprotein B-100 (apoB-100) (LpBs) are complex macromolecular structures that vary in size, hydrated density, electrophoretic mobility, and lipid and apolipoprotein composition. The physical and chemical heterogeneity modulates the conformation of apoB-100 and, as a consequence, its function (1–6). LDLs in human apoB-100 transgenic (HuBTg+/+) mice are enriched in triglyceride (TG) compared with LDL in human plasma (7). The metabolic basis for the abnormal LDL composition in the HuBTg+/+ mice is unclear (8–10). Because anti-apoB monoclonal antibodies (MAbs) can be sensitive probes of apoB conformation (1, 2, 4, 5, 11, 12), we hypothesized that anti-apoB MAbs could be specifically generated that could detect structural differences between LpB synthesized by humans and HuBTg+/+ transgenic mice.

Interspecies immunization generally results in the production of antibodies against species-specific epitopes that rarely discriminate between polymorphic forms of the antigen. In contrast, intraspecies immunization preferentially elicits an immune response against allogeneic or polymorphic epitopes. An example of the latter is the Ag-system of human apoB polymorphism that was originally defined by antibodies in the sera of multitransfused individuals and multiparous women (13). Similarly, the complex apoB polymorphisms of swine were identified with antibodies generated by allogeneic immunization (14). While interspecies immunization may be the protocol of choice for the production of antibodies specific for polymorphic epitopes in animals, ethical and technical limitations preclude its use in humans. The availability of HuBTg+/+ mice now offers the possibility for developing a strategy that simulates the conditions of intraspecies immunization while maintaining the advantages of using an-
imals for immunization. Here we demonstrate that MABs generated with spleen cells of HuBTg<sup>+</sup>/+ mice that had been immunized with human LDL can detect differences in the conformation or posttranslational modification between human apoB secreted by humans and mice.

MATERIALS AND METHODS

Preparation of lipoproteins

LDLs were prepared from plasma of healthy donors (Canadian Red Cross) or patients with familial defective apoB (FDB) who were heterozygous for the apoB-100<sub>5350</sub>Gln allele (Lipid Clinic of the Institut de Recherches Cliniques de Montréal). The plasma was supplemented immediately with EDTA (1.0 mM), sodium azide (0.02%), phenylmethanesulfonic fluoreide (0.5 mM), aprotinin (1 µg/ml), and butyld hydroxytoluene (20 nM). LDLs (1.019–1.063 g/ml) were isolated by sequential ultracentrifugation at 40,000 rpm for 18 h in a Beckman L5-65 ultracentrifuge with a 50.2 Ti rotor (Beckman Instruments, Palo Alto, CA) (15). Solvent densities were adjusted with KBr. The isolated LDLs were dialyzed against phosphate-buffered saline (PBS) (pH 7.4) containing 1 mM EDTA and 0.02% NaN<sub>3</sub> sterilized by ultrafiltration, and stored at 4°C in the dark. LpBs were isolated from mouse plasma by ultracentrifugation at 100,000 rpm for 3 h at a density of 1.065 g/ml in a Beckman Optima TL table-top ultracentrifuge. After centrifugation, the d < 1.063 g/ml fraction was removed and dialyzed as above.

Transgenic mice

The production and characterization of HuBTg<sup>+</sup>/+ (7), human apoB-100<sub>5350</sub>Gln (HuBT<sub>5350gln</sub> apoB<sup>+</sup>/+) (16), and human hepatic lipase human apoB (HuHLTg<sup>+</sup>/–HuBTg<sup>+</sup>/+) (17) transgenic mouse lines have been described. Human apoB-100 human cholesteryl ester transfer protein (CETP) transgenic mouse lines have been described. Human apoB-100 cholesteryl ester transfer protein (CETP) transgenic mice (HuBTg<sup>+</sup>/+, HuCETPTg<sup>+</sup>/+) were purchased from Taconic (Germantown, NY). LDL receptor knockout (LDL<sup>−/−</sup>) mice (Jackson Laboratory, Bar Harbor, ME) were bred with human HuBTg<sup>+</sup>/+ mice to obtain HuBTg<sup>+</sup>/+LDL<sup>−/−</sup> mice.

Generation of hybridomas

The HuBTg<sup>+</sup>/+ mice (7) were immunized by intraperitoneal injection of normal or FDB LDL (100 µg) in 200 µl of PBS containing 50 µg of the immunological adjuvant N-acetylglucamyl-t-alanyl-s-isoglutamine (Calbiochem, La Jolla, CA). The mice received at least two additional identical boosts at 3-week intervals. Serum antibody titers were monitored 1 week after each boost by a solid-phase radioimmunounmunoassay (RIA) with normal or FDB LDL as the immobilized antigen. Four days before the fusion and at least 3 weeks after the last boost, a final boost (50 µg of LDL in 100 µl of PBS) was administered by tail vein injection. The fusion of splenocytes from immunized mice with SP2/0 plasmacytoma cells has been described (18). Seven to 10 days after the fusion, when the clones occupied >10% of the surface of the well, supernatants were tested for the presence of antibodies by a solid-phase RIA. Positive hybridomas were recloned by limiting dilution, as described (18). The panel of anti-human apoB MAb that includes 1D1 and 4G3, and the antimouse apoB-100 MAb LF5, have been described (12, 19, 20).

Immunoassays

The solid-phase RIA and competitive solid-phase RIA have been described (18). Human apoB MAbs that includes 1D1 and 4G3, and the antimouse apoB-100 MAbs have been described (12, 19, 20).

Production and purification of MABs

Anti-human apoB-specific MABs were purified from hybridoma culture supernatants or from ascitic fluid of hybridoma-bearing mice by affinity chromatography on Protein A Sepharose (Amersham Pharmacia Biotech, Baie D’Urfé, PQ) (22).

Radioiodination of immunoglobulin G and Fc fragments

The goat anti-mouse immunoglobulin G (IgG) and goat anti-mouse IgG Fc-specific antibodies (Cedarlane Laboratories, Hornby, ON) were labeled as described (18). The specific activity of labeled IgG was 1–3 × 10<sup>4</sup> cpm/ng of IgG.

Electrophoresis

Lipoproteins were subjected to agarose electrophoresis (Lipogel, Beckman Instruments) and transferred to nitrocellulose membranes according to the manufacturer’s recommendations. Lipoproteins were also subjected to SDS-PAGE (3–10% gradient) and transferred to nitrocellulose membranes (24). The membranes were probed for human apoB, transgenic apoB, and mouse endogenous apoB with MABs specific for human (19, 23) or mouse (20) apoB and then 125<sup>I</sup>-anti-mouse IgG as described (19). For SDS-PAGE analysis of carboxy-terminally truncated apoB variants secreted from transfected McArdle 7777 rat hepatoma (McA7777) cells (25), human LpB from culture supernatants were immunoprecipitated with a rabbit anti-human apoB antisum (26) before being subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose membranes. The membranes were probed with mouse anti-apoB MABs and revealed by chemiluminescence after incubation with horseradish peroixidase-conjugated anti-mouse IgG (Amersham Pharmacia).

Measurement of protein concentration

Protein concentration was determined by a modified Lowry method (27) with BSA as a protein standard.

RESULTS

Despite multiple boosts with human LDL, immunization of HuBTg<sup>+</sup>/+ mice elicited only a weak immune response in the serum, and only five stable specific hybridomas were obtained from three separate fusions. One hybridoma secreted an MAb that was specific for an epitope in the N-terminal 22 kDa domain of human apoE (not shown). The four other hybridomas secreted anti-apoB-
specific MAbs, none of which could distinguish between LDL isolated from normal subjects and LDL isolated from FDB subjects. Thus, none of the MAbs were specific for epitopes whose expression was directly or indirectly modulated by the Arg3500→Gln. Two of the anti-apoB MAbs were subsequently shown to react with epitopes that are only exposed when LpBs are immobilized on a solid phase. The characterization of these latter MAbs is not described further.

We compared the reactivities of the two remaining MAbs, 2E1 and 3D11, with normal human LDL and LpB from nontransgenic mice, HuBTg+/+ mice, and HuB3500Q Tg+/+ mice. Both reacted well with human LDL but showed no reactivity with lipoproteins from nontransgenic, HuBTg+/+, or HuB3500Q Tg+/+ mice (Fig. 1), whereas the control anti-apoB-100-specific MAb 4G3 reacted with both the human LDL and LpB of the human apoB transgenic mice (Fig. 1). The same patterns of reactivity were seen with immunobLOTS of lipoproteins that had been separated by agarose gel electrophoresis (not shown).

The MAbs were then tested for reactivity with lipoproteins that had been separated by SDS-PAGE and transferred to nitrocellulose membranes (Fig. 2). MAb 2E1 reacted strongly with human apoB-100 and weakly with human transgenic apoB-100, whereas 3D11 reacted strongly with both. Thus, the specificity of MAbs for human LDL that was seen in the solid-phase RIA and after agarose electrophoresis was not apparent after SDS electrophoresis. The control anti-human apoB MAbs 1D1 and 4G3 recognized both human apoB-100 and human transgenic apoB-100. None of the MAbs recognized mouse apoB. Treatment of lipoproteins with PNGase F to remove N-linked sugars of apoB before SDS-PAGE did not alter apoB immunoreactivities (not shown). Likewise, the reactivities of the antibodies were the same when the electrophoresis was carried out under reducing or nonreducing conditions (not shown).

To determine if the MAbs recognize transgenic LpBs that were not adsorbed to plastic, we tested LpB samples for their ability to compete with immobilized human LDL for binding to MAbs in a competitive solid-phase RIA (Fig. 3). Human LDL and transgenic LpB competed efficiently for binding to 4G3; however, only human LDL and not transgenic LpB competed for binding to MAbs 2E1 and 3D11. VLDL and IDL isolated from human plasma also competed efficiently for binding to both 2E1 and 3D11 (not shown). Lipoproteins isolated from nontransgenic mice were ineffective competitors with all of the antibodies.

The lack of reactivity of MAbs 2E1 and 3D11 with human apoB expressed in human apoB transgenic mice could result from apoB genetic polymorphism (2E1), different posttranslational modification of apoB in the transgenic mice (2E1 and 3D11), or altered apoB conformation in the LpB of the transgenic mice (3D11). To see if the 2E1 and 3D11 epitopes were differentially expressed in different subjects, we tested plasma samples from five unrelated normolipidemic subjects for their reactivity with MAbs 2E1 and 3D11 and with the control anti-apoB

![Fig. 1. Reactivity of monoclonal antibodies (MAbs) 2E1, 3D11, and 4G3 with immobilized plasma lipoproteins from normal human subjects (h-LDL), HuBTg+/+ mice, HuB3500Q Tg+/+ mice, or wild-type mice (m-LpB). Lipoproteins immobilized on polystyrene microtiter wells were successively exposed to serial dilutions of antihuman apoB MAbs and 125I-anti-mouse immunoglobulin G (IgG). Results are expressed as radioactivity bound as a function of MAb dilution.](image-url)
Mab 4G3 in a competitive RIA. All of the plasma samples competed efficiently with the immobilized LDL for binding the 2E1 and 3D11 Mabs (Fig. 4). Similar results were obtained with an additional 21 subjects that were subsequently tested (not shown).

The 2E1 and 3D11 epitopes were detected on LpB from human apoB transgenic mice only after SDS-PAGE. The lack of reactivity with the native LpB from the human apoB transgenic mice only after SDS-PAGE. After electrophoresis, lipoproteins were transferred to nitrocellulose membranes and were exposed to Mabs 2E1, 3D11, 1D1, or 4G3, and then to 125I-anti-mouse IgG.

Fig. 2. Reactivity of MAb with lipoproteins from normal human subjects (lane 1), HuBTg+/ mice (lane 2), HuBR3500QTg+/ mice (lane 3), or wild-type mice (lane 4) after SDS-PAGE. After electrophoresis, lipoproteins were transferred to nitrocellulose membranes and were exposed to MAb 2E1, 3D11, 1D1, or 4G3, and then to 125I-anti-mouse IgG.

by a competitive solid-phase RIA. The introduction of the human hepatic lipase transgene onto the HuBTg+/ background did not result in expression of the 2E1 and 3D11 epitopes on the transgenic LpB (not shown). Moreover, incubation of LpB from the HuHLTg+/HuBTg+/ mice with plasma of HuCETPTg+/ mice did not lead to expression of the two epitopes. The cholesteryl ester-enriched LDL of LDLr−/−HuBTg+/ mice also failed to react with the 2E1 and 3D11 antibodies (not shown).

The HepG2 human hepatoma cell line secretes apoB-100 on particles that, like the LpB from HuBTg+/ mice, are TG-rich and have a hydrated density similar to that of LDL (28). We determined the 2E1 and 3D11 reactivity of LpB secreted from HepG2 cells. The LpB in the HepG2 culture supernatants competed effectively with LDL isolated from human plasma for binding to both antibodies (Fig. 5). In contrast, the LpB secreted by human apoB-100-transfected McA7777 rat hepatoma cells (29) were not recognized by MAb 2E1 or 3D11 (Fig. 5). The LpBs from both the HepG2 cells and the human apoB-transfected McA7777 cells competed effectively for the control of anti-human apoB MAb 1D1 and 4G3.

In an initial attempt to map the 2E1 and 3D11 epitopes, we tested a panel of 29 anti-apoB Mabs whose epitopes have been mapped within apoB primary structure (12) as well as 2E1 and 3D11 for their abilities to compete with 125I-2E1 and 125I-3D11 for binding to immobilized human LDL. While none of the 29 Mabs could compete with 125I-2E1 or 125I-3D11 for binding to LDL, 2E1 and 3D11 did show mutual competition (not shown). Thus, 2E1 and 3D11 appear to react with the same or adjacent epitopes. We next tested the reactivity of 2E1, 3D11 and control MAb 1D1 (epitope between residues 474 and 576) for their reactivities with a series of truncated apoB variants (25) that had been separated by SDS electrophoresis. Results are shown for 2E1 and 1D1 (Fig. 6). Both Mabs react with apoB-42, apoB-37, apoB-29, apoB-17, and weakly with apoB-15. Therefore, the epitopes are within the first 689 residues of apoB.

DISCUSSION

Immunization of HuBTg+/ mice with human LDL elicited an immune response that was weak but very specific for epitopes that differentiated the LDL used for immunization from the product of the human apoB transgene. This result was also reflected in the number of hybridomas obtained from the mice and in the specificity of the Mabs that they secreted. More importantly, as we had predicted, we obtained two Mabs (2E1 and 3D11) that distinguished between LpB synthesized in humans and in mice. In addition, one MAb was specific for apoE, a minor constituent of LDL isolated from human plasma. In the absence of an immune response against the majority of apoB epitopes, minor antigens of LDL, such as apoE, can become immunodominant. Two of the Mabs were specific for epitopes of human apoB that are only expressed when apoB confor-
mation is modified by adsorption of lipoproteins to polystyrene or a nitrocellulose membrane. As was the case for minor components of LDL, epitopes expressed on a subpopulation of denatured apoB molecules can become immunodominant in the context of the HuBTg+/− mouse.

Although some of the HuBTg+/− mice were immunized with LDL from FDB subjects, no MAbs were obtained that could distinguish between apoB-100 and apoB-100Arg3500→Gln. We considered the possibility that MAbs 2E1 and 3D11 detect other immunogenetic polymorphisms in apoB. Several observations suggest that this is not the case. Plasma from all individuals tested to date competed efficiently with human LDL for binding to 2E1 and 3D11 in a solid-phase RIA. Therefore, if 2E1 and 3D11 immunoreactivities were a function of apoB gene polymorphism, the haplotype represented by the human apoB transgene would occur infrequently in the normal population. Like LpB from HuBTg+/− tg mice, lipoproteins secreted by human apoB-100-transfected McA7777 rat hepatoma cells did not react with 2E1 and 3D11 in a competitive RIA. The apoB-100 construct used to transfect the McA7777 cells (29) and the clone used to generate the HuBTg+/− mice (7) were isolated from different li-

Fig. 4. Competitive solid-phase RIA of MAbs with plasma of six normolipidemic subjects. Dilutions of plasma were mixed with an appropriate dilution of the MAbs 2E1, 3D11, and 4G3 and were added to polystyrene microtiter wells to which normal human LDL had been adsorbed. After incubation, the bound MAb was detected with 125I-anti-mouse IgG. Results are expressed as the ratio of radioactivity bound in the presence of competitor (B) and radioactivity bound in the absence of competitor (B0).

Fig. 5. Competitive solid-phase RIA of MAbs with human plasma LDL and with lipoproteins that had been isolated from the culture supernatants of either HepG2 human hepatoma cells or McArdle 7777 (McA7777) rat hepatoma cells that had been stably transfected with human apoB-100 cDNA. Dilutions of lipoproteins were mixed with an appropriate dilution of MAbs 2E1, 3D11, and 4G3 and were added to polystyrene microtiter wells to which normal human LDL had been adsorbed. After incubation, the bound MAb was detected with 125I-anti-mouse IgG. Results are expressed as the ratio of radioactivity bound in the presence of competitor (B) and radioactivity bound in the absence of competitor (B0).

Fig. 6. Reactivity of MAbs 2E1 and 1D1 with truncated apoB variants. LpB in culture supernatants of McA7777 cells that had been stably transfected with human apoB-48 (A), apoB-42 (B), apoB-37 (C), apoB-34 (D), apoB-29 (E), apoB-17 (F), or apoB-15 (G) were immunoprecipitated with polyclonal anti-human apoB before SDS electrophoresis. The LpB were transferred to nitrocellulose membranes and tested for immunoreactivity with MAbs 2E1 and 1D1.
libraries. Thus, it is unlikely that the human apoB that is secreted in the two systems would represent the same rare apoB isoform.

Although LDL from HuBTg<sup>+/+</sup> transgenic mice are enriched in TG (7), it is doubtful that these accounts for their lack of 2E1 and 3D11 immunoreactivity. The introduction of the human CETP gene or the human hepatic lipase gene into the HuBTg<sup>+/+</sup> mice did not lead to the expression of the 2E1 or 3D11 epitopes on the LpB. Likewise, the cholesteryl ester-rich LDLs from LDL<sup>−/−</sup>-HuBTg<sup>+/+</sup> mice were not recognized by 2E1 and 3D11. The 2E1 and 3D11 immunoreactivity of the TG-rich LDL-like particles secreted by HepG2 cells also suggests that TG enrichment is not solely responsible for the lack of expression of the epitopes on LpB of HuBTg<sup>+/+</sup> mice. In fact, the similar immunoreactivities of human VLDL, IDL, and LDL show that the expression of the 2E1 and 3D11 epitopes is relatively independent of both LpB particle size and core lipid composition.

Since the 2E1 and 3D11 epitopes were detected on LpB secreted by human HepG2 hepatoma cells but not on LpB secreted by human apoB-100-transfected Mca7777 rat hepatoma cells, the differential immunoreactivity does not result from interspecies differences in the intravascular metabolism of LpB. Rather, we suspect that posttranslational modification of apoB differs in rodent and human cells and that this determines the expression of the 2E1 and 3D11 epitopes. Human apoB undergoes disulfide bond formation (30), glycosylation (31), phosphorylation (32), and covalent lipid modification (33) within the cell. The 2E1 and 3D11 MAbs might be specific for a modified residue or adduct. Alternatively, posttranslational modification of apoB could mask epitopes in native LpB of apoB transgenic mice. Within the amino terminal 689 amino acids of apoB that include the 2E1 and 3D11 epitope(s), there is a single site of N-glycosylation (Asn158) (34) and no free cysteine residues that undergo palmitoylation (35). It is unlikely that a more extensive glycosylation of Asn158 in human apoB expressed by rodent cells masks the 2E1 and 3D11 epitopes as an elimination of the N-glycosylation site in apoB-48 by site-directed mutagenesis (Asn158→Gln) did not alter the 2E1 and 3D11 immunoreactivity when tested by a competitive RIA (Nguyen, Vukmirica, Yao, and Milne, unpublished observations). The similarity in the reactivities of native and reduced apoB-100 indicates that the differential epitope expression is not due to differences in disulfide bonds.

The restricted repertoire of antibody specificities that we obtained with immunized <i>hapolB</i><sup>+/+</sup> transgenic mice as a source of spleen cells to generate hybridomas demonstrates the potential of this immunization protocol. Immunization of transgenic mice allows the targeting of the immune response to minor components present in the antigen preparation, to subtle conformational differences between the immunogen and the product of transgene, and potentially to sites in the antigen that show genetic polymorphism. This approach could be used to produce MAbs against any antigen for which appropriate transgenic mouse lines exist. An obvious example in the field of lipoproteins would be MAbs specific for apoE isoforms. Two of the MAbs we generated with this protocol allowed us to identify immunochemical differences between human apoB secreted by human cells and human apoB secreted by rodent cells. We are currently investigating other possible mechanisms responsible for these differences as well as the potential functional consequences.

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