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Platelet Activation Releases Megakaryocyte-Synthesized Apolipoprotein J, a Highly Abundant Protein in Atheromatous Lesions

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Apolipoprotein J (apoJ) is an abundant glycoprotein in many biological fluids, and its constitutive high level synthesis is characteristic of many epithelial cells exposed to harsh fluids such as urine, bile, and gastric secretions. In addition, dramatic induction of apoJ occurs in cells surrounding several kinds of pathological lesions. Because platelets and circulating inflammatory cells represent critical elements in numerous pathological processes, we evaluated bone marrow cells for the presence of apoJ. Based upon messenger RNA* in situ *hybridization and immunofluorescent protein detection, high-level apoJ gene expression and protein accumulation occurred exclusively in mature megakaryocytes. Our results indicate that apoJ is stored in platelet granules and is released into extracellular fluid following platelet activation. Because atheromatous plaque development involves platelet aggregation and activation, we looked for and found abundant apoJ protein in advanced human atheromatous lesions. Thus, platelet sequestration and activation may lead to the rapid deployment of apoJ into sites of vascular injury. We hypothesize that platelet-derived apoJ participates in both short-term wound repair processes and chronic pathogenic processes at vascular interfaces. (Am J Pathol 1993, 143:763–773)

Apolipoprotein J (apoJ) is an abundant secreted glycoprotein found in most physiological fluids, including seminal plasma, urine, breast milk, cerebrospinal fluid, and blood[1,2] (for review see refs. 3 and 4). The protein has been purified by numerous investigators and, based upon its varied biochemical properties, has been given a number of designations (clusterin, complement lysis inhibitor, SP-40, 40, SGP-2, TRPM-2). Highly conserved homologues have been identified in a wide range of vertebrates. The protein is synthesized as a preproprotein that, following signal peptide removal, is cleaved to two subunits, each containing amphipathic helix domains and, depending on species, two to four N-glycosylation sites. The chains are cross-linked through five pairs of highly conserved cysteines. ApoJ has a general capacity to interact with hydrophobic molecules. In blood, the protein is tightly associated with both lipid and apoAI in high- and very high-density lipoproteins.[5–8] Whereas Northern analysis reveals that most adult and embryonic tissues contain apoJ messenger (m)RNA, *in situ* hybridization analysis shows that only specific subpopulations of cells within those tissues show expression.[4,9,10] Most frequently, these high-expressing cells are epithelial cells that line fluid compartments and either secrete or are exposed to various surface-active components of the extracellular environment.[10] We have proposed that these cells form a novel group of tissue-fluid barrier cells.

Circulating inflammatory cells and platelets play a key role in tissue injury response and promote repair processes by the release of a variety of biological effectors. These include growth factors, cytokines, chemotactic signals, coagulation factors, and proteins that mediate cellular and molecular adhesion. To evaluate whether apoJ expression could play a role

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in platelet or inflammatory cell functions, perhaps at sites of tissue injury or cellular damage, we surveyed human bone marrow cells for evidence of apoJ production. Of the cell types surveyed, only megakaryocytes synthesized substantial apoJ. We show that the protein is stored in platelet granules, released into extracellular fluid following activation, and abundant in atheromatous lesions.

Materials and Methods

In Situ *Hybridization*

Human bone marrow aspirates were collected in heparin-coated syringes, separated on a Ficoll column, and the band containing the mononuclear cells and megakaryocytes was collected. Ficoll-purified cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 C overnight and cytocentrifuged onto slides coated with 3-amino propyltriethoxysilane (Histology Control Systems, Glen Head, NY). Mouse bone marrow was flushed from adult femurs and tibias with PBS that contained 100 U/ml of heparin, fixed, and cytocentrifuged as above. All of the following tissues were fixed in 4% paraformaldehyde in PBS, embedded in optimum cold temperature compound (Miles Laboratories, Elkhart, IN), and cryosectioned onto coated slides. Mouse embryos were obtained from timed pregnant mice. Human atherosclerotic arteries were obtained from surgical amputation specimens of three adult patients with advanced atherosclerosis. Nonatherosclerotic vascular tissue was obtained from proximal aortic tissue surgically removed during repair of coarctation of the aorta in two infants and from two adult femoral arteries obtained following traumatic amputations. Sense and anti-sense complementary RNA probes were labeled with [^{35}S]rUTP, using a commercially available kit (Stratagene, La Jolla, CA). Human probe was transcribed from an 827-bp fragment from the 3′ end of the human apoJ complementary DNA clone λ1–3[5] inserted into pBluescript SK+ (Stratagene). Control human artery specimens were hybridized with sense and anti-sense probes of human β-actin mRNA. For the mouse tissue studies, apoJ probe was transcribed from a pBluescript vector that contained a 1.3-kb mouse apoJ complementary DNA insert. Hybridizations were performed as previously described[11] with a total of 5×10^5 cpm in a final volume of 30 µl per slide. The sections were hybridized overnight at 45 C, treated with 50 µg/ml of RNAse A (Sigma Chemical Co., St. Louis, MO) and 100 units/ml of RNAse T1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 minutes at 37 C, and washed to a final stringency of 0.1× standard citrate saline at 50 C. Slides were dipped in NTB2 emulsion (Kodak) that was diluted 1:1 with 0.6 mol/L ammonium acetate, exposed for 10 to 14 days, and developed in D19 developer (Kodak). Sections were counterstained in hematoxylin and eosin and photographed under dark- and bright-field illumination.

Immunohistochemistry

Fresh human bone marrow aspirates were collected as described above. The unfixed cells were washed and cytocentrifuged onto silane-coated slides, air-dried, fixed in acetone for 10 minutes, and permeabilized for 10 minutes in 0.1% Triton X-100 in PBS. Cryostat sections of unfixed snap-frozen atherosclerotic arteries and nonatherosclerotic vessels were obtained as described above. The sections were fixed in acetone for 10 minutes before immunostaining. Nonspecific binding was blocked with 5% nonfat dried milk in PBS. ApoJ protein was localized with a mouse monoclonal antibody (MAb), MAb11[6] against human apoJ, diluted 1:100. The primary antibody was detected with fluorescein-conjugated anti-mouse F(ab′)2 fragment (Dako, Carpinteria, CA). Coverslips were applied over mounting media containing propidium iodide (Oncor Laboratories, Rockville, MD) to counterstain the nuclei. Control slides were incubated with normal mouse serum and the secondary F(ab′)2 antibody. All slides were examined with a Zeiss epifluorescence microscope.

Platelet Activation Studies

Human platelet-rich plasma was prepared from whole blood collected from normal volunteers in acid citrate dextrose buffer.[12] The platelet-rich plasma was centrifuged at 850*g* for 10 minutes, and the pellet was resuspended in platelet buffer (137 mmol/L NaCl, 2 mmol/L KCl, 5.5 mmol/L glucose, 1 mmol/L MgCl, 5 mmol/L (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic]), pH 7.4)[13] with 300 ng/ml of prostaglandin I_2 (Sigma) to prevent activation and washed three times (10 ml volumes) in this buffer. For activation studies, platelets were resuspended at a final concentration of 10^6/µl in platelet buffer without prostaglandin I_2. Platelets were activated by incubating 225 µl of stirred platelet suspension with 100 µg/ml of adenosine diphosphate (ADP, Sigma), 0.5 IU/ml of thrombin (Ortho, Raritan, NJ), or 2.5 µg/ml collagen (Helena, Beamont, TX). All incubations were performed

for 10 minutes at 37 C. Platelet aggregation was monitored with a Chrono-log whole blood Lumi-aggregometer (Chrono-log Co., Havertown, PA). Control samples were incubated in platelet buffer without activating agents.

Following activation, samples were centrifuged at 850*g* for 15 minutes. ApoJ concentration was determined in the supernatants by competitive enzyme-linked immunosorbent assay, as described by Jenkins et al.[14] For electrophoretic evaluation of apoJ, supernatants were concentrated by ultrafiltration, using a Centricon (Amicon, Beverly, MA) 30,000-MW cutoff filter, and brought to equivalent volumes with gel sample buffer (8 mol/L urea, 60 mmol/L Tris, 2% sodium dodecyl sulfate, 10% glycerol, pH 7.0) with and without dithiothreitol, and loaded onto 11% polyacrylamide minigels. All samples were heated for 30 minutes at 60 C. Electroimmunoblot analysis was performed with a rabbit polyclonal antibody[15] at 1:10,000 dilution. The primary antibody was detected with horseradish peroxidase conjugated anti-rabbit immunoglobulin G (BioRad Laboratories, Richmond, CA) at 1:5,000 dilution and visualized by an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

Immunoelectron Microscopy

Fresh human platelets were collected and washed three times in acid citrate buffer with prostaglandin I_2 and centrifuged onto gelatin-coated glass slides. The platelets were permeabilized in acetone and 0.1% Triton X-100, blocked as described above, and incubated with MAb11 or control mouse serum. The primary antibody was detected with a colloidal gold-labeled anti-mouse antibody (Amersham, 10-nm particles) and postfixed in 1% osmic acid. Following graded alcohol dehydration, inverted bean capsules were placed over the platelets, filled with plastic embedding media, and polymerized at 60 C. The capsules were then popped off the slides, and the surfaces were sectioned for electron microscopy. Thin sections were counterstained in uranyl acetate and viewed on a Phillips 400 electron microscope.

Platelet Microvesiculation Studies

Fresh washed human platelets were incubated with MAb11 or normal mouse serum in acid citrate buffer, washed, and detected with fluorescein isothiocyanate-conjugated secondary antibody. Platelets were then washed and some of the samples were subjected to activation by thrombin (1 IU/ml). The samples were analyzed on an EPICS (Coulter, Hialeah, FL) flow cytometer to determine the distribution of fluorescein-positive particles.

In a second set of experiments, washed human platelets were activated with thrombin and separated into three fractions by differential centrifugation. The pellet from low-speed centrifugation (850*g* for 10 minutes) contained large aggregated platelet clumps; the supernatant was then centrifuged at a higher speed (20,000*g* for 22 minutes) to collect the microparticle fraction.[16] Each fraction was subjected to electroimmunoblot analysis for apoJ, as described above. To control for efficiency of fractionation, identical blots were probed with a mouse monoclonal antibody directed against P-selectin (αGMP-140, Dako).

CHRF-288 Cell Line Studies

To evaluate apoJ synthesis and processing by megakaryocytes, the human megakaryocytic cell line CHRF-288[17] was employed as a model system. Cells were grown as suspension cultures in T-75 flasks in Fisher's medium supplemented with 10% horse serum (Gibco Bethesda Research Laboratories, Gaithersburg, MO), penicillin, and streptomycin and passaged approximately every 7 days. Metabolic labeling and immunoprecipitation of apoJ were done as previously described for HepG2 cells.[15] ^{35}S-labeled protein was visualized by fluorography of dried gels by using Enlightening (DuPont, Boston, MA). CHRF-288 cells were also treated with 1×10^{-8} phorbol 12-myristate 13-acetate (PMA) to induce maturation.[17] Treated and untreated cells were fixed, cytocentrifuged, and evaluated for apoJ expression by *in situ* hybridization as described for bone marrow cells above.

Results

Intense Accumulation of apoJ mRNA in Megakaryocytes

Using *in situ* hybridization to localize apoJ mRNA, we observed three positive cell populations in mouse fetal liver. As previously observed in adult liver,[10] strong signal was evident in bile duct epithelial cells, and moderate signal was present throughout parenchymal hepatocytes. Unlike what was previously seen in adult liver, intensely positive cells

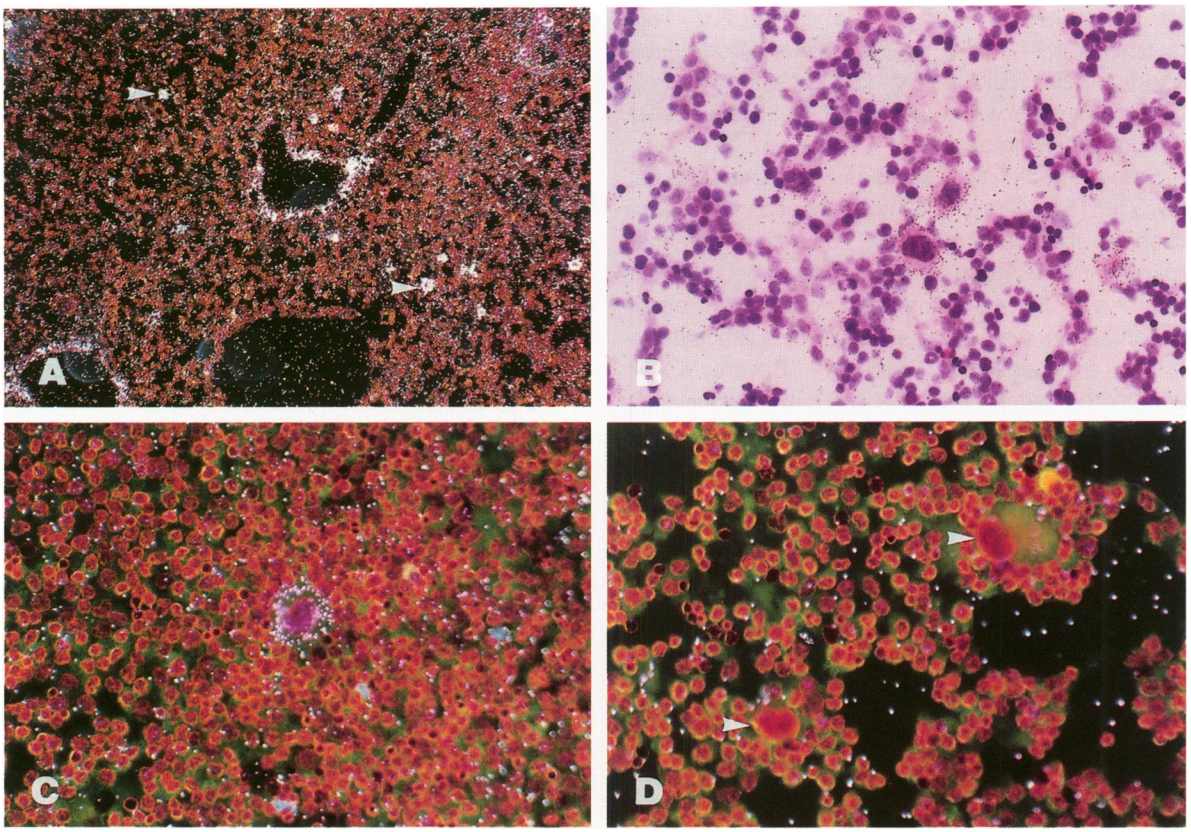

Figure 1. *High-level expression of apoJ mRNA in megakaryocytes.* A *to* D: In situ *hybridization with* ^{35}S*-labeled apoJ probes.* A: *liver from a day-17 mouse embryo hybridized with mouse anti-sense probe. In portal areas, intense signal (bright white grains) is in bile duct epithelial cells. There is also weaker signal in hepatocytes throughout the lobule. Isolated cells that are intensely positive (arrowheads) and scattered throughout the hepatic lobule are mature megakaryocytes.* B: *Same as* A, *but high magnification with bright-field illumination. Megakaryocytes located in sinusoidal spaces show positive signal as black grains. There is no signal from other hematopoietic cells in the sinusoids.* C: *Human bone marrow aspirate sample with anti-sense probe. A single mature megakaryocyte has strong hybridization signal (white grains), indicating the presence of apoJ mRNA.* D: *Same as* C *but hybridized with the sense strand probe. No signal is present in the megakaryocytes (arrowheads) or other bone marrow cells. (Magnification:* A *110×;* B, C, D *445×).*

were scattered throughout the fetal liver lobule (Figure 1A). These cells were identifiable as mature megakaryocytes in the sinusoidal space (Figure 1B). None of the other hematopoietic cells in the fetal liver showed apoJ expression, and no mature megakaryocytes were observed that failed to express apoJ. Similarly, in adult mouse bone marrow, megakaryocytes with mature cytological features were the only cell type that exhibited detectable apoJ mRNA (not shown). An identical result was observed in human bone marrow (Figure 1C). Hybridization with the sense probe was negative for all cells, including megakaryocytes (Figure 1D).

Previous studies have shown that the megakaryocytic tumor-derived cell line CHRF-288 shows little differentiation beyond the megakaryoblast stage, but upon the addition of PMA undergoes partial differentiation toward a more mature megakaryocyte form.[17] Immunoprecipitation analysis of metabolically labeled proteins synthesized by this cell line indicated that CHRF-288 cells synthesized and se-

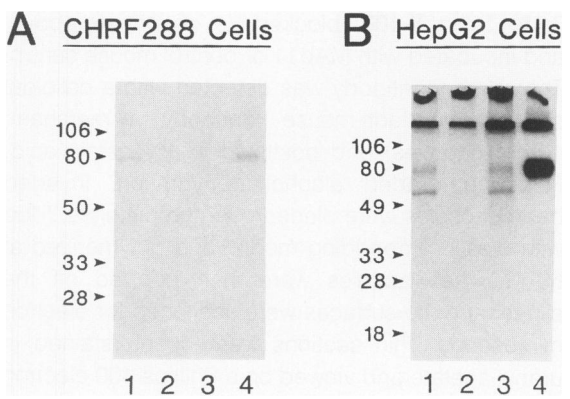

Figure 2. *Biosynthesis of apoJ by a human megakaryocyte line. Megakaryoblastic leukemia cells CHRF-288* (A) *and HepG2 human hepatoma cells* (B) *were metabolically labeled with [*^{35}*S]methionine and analyzed by immunoprecipitation for secreted apoJ.* A, lanes 1, 2, *and* 4: *Culture media from labeled cells were precipitated with rabbit polyclonal anti-apoJ;* lane 3 *shows the result of precipitation with preimmune serum.* Lane 2 *contains purified human apoJ as a cold competitor;* lane 4 *contains albumin as competitor.* B, lanes 1 *to* 3: *HepG2 cell lysates were immunoprecipitated with rabbit anti-apoJ in the presence of no competitor, purified unlabeled human apoJ, or albumin, respectively.* Lane 4: *media from HepG2 cell was immunoprecipitated with rabbit anti-apoJ.*

creted apoJ protein, although at low levels as compared to human HepG2 liver cells (Figure 2, A and B). Analyzed by *in situ* hybridization, uninduced CHRF-288 cells expressed no detectable apoJ mRNA (Figure 3A). In contrast, cultures induced with PMA contained occasional cells that showed both strong apoJ signal and the cytological features of highly differentiated megakaryocytes (Figure 3B).

Megakaryocytes and Platelets Store ApoJ Protein

To determine if and where apoJ protein accumulates in megakaryocytes, a MAb directed against human apoJ was used to localize the protein. Strong immunofluorescence was seen in megakaryocytes but not in any other marrow cells. As shown in Figure 4, there was intense granular signal throughout the megakaryocyte cytoplasm and in all platelets. Samples that were incubated in nonimmune mouse serum were uniformly negative.

Platelet Activation Releases ApoJ

Cell types characterized thus far have indicated that apoJ is continuously secreted.[4] However, we questioned whether platelets might instead store apoJ in their granules. As shown in Figure 5, platelets activated with either ADP, thrombin, or collagen all released significant apoJ to the fluid phase. Released apoJ was present as a distinct doublet around 35 kd when electrophoresed under reducing conditions. In contrast, under these conditions, plasma apoJ migrated as a single broad band (Figure 5B) corresponding to the faster migrating platelet apoJ species.

To determine the amount of apoJ present in platelets and plasma, quantitative enzyme-linked

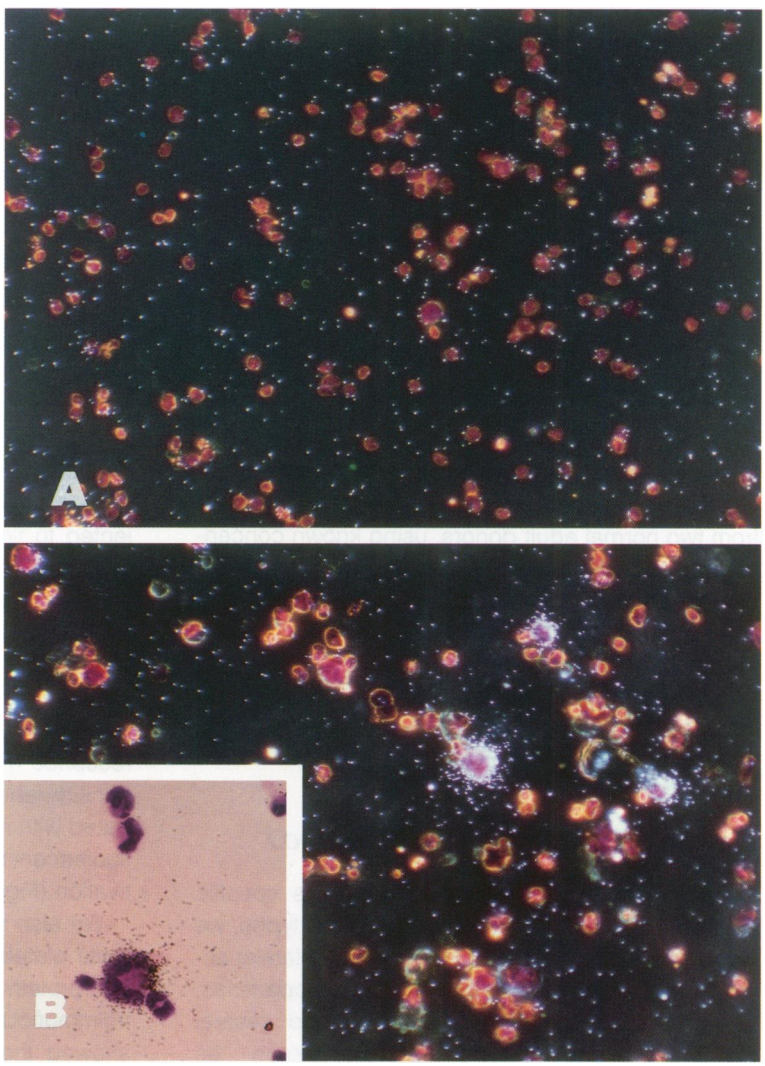

Figure 3. *in situ detection of apoJ mRNA in CHRF-288 cells.* **A:** *Uninduced culture showing no detectable mRNA signal.* **B:** *Induced culture showing strong signal in more mature cells that occur occasionally with induction. Inset: bright-field illumination of an intensely positive cell showing large, well-differentiated megakaryocyte. Note the absence of signal in the smaller immature cells. (Magnification:* **A**, **B** *445×, inset 600×).*

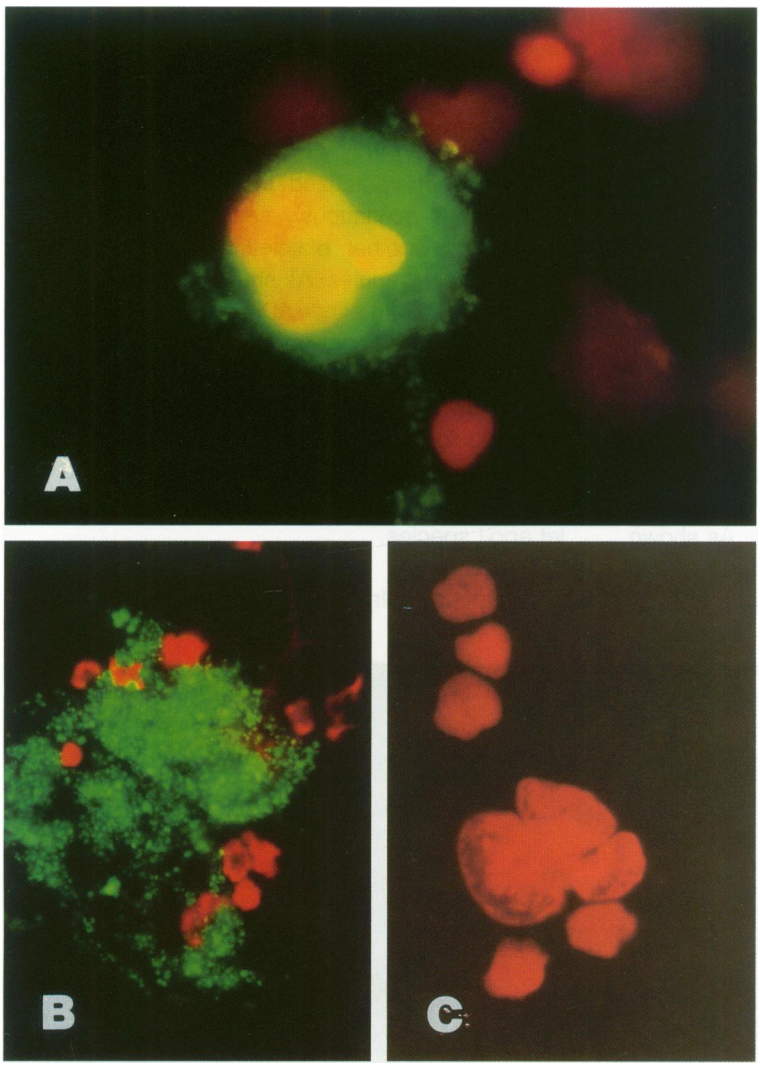

Figure 4. *High-level apoJ protein in megakaryocytes and platelets.* A: *Human bone marrow aspirate stained with anti-human apoJ antibody and detected with fluorescein isothiocyanate-conjugated secondary antibody (green). The nuclei are counterstained with propidium iodide (red). The megakaryocyte in the center of the photomicrograph shows strong fluorescence in a granular pattern throughout the cytoplasm. Numerous intensely stained platelets can be seen at the surface of the megakaryocyte.* B *shows strong apoJ staining of a large aggregate of platelets from a bone marrow aspirate.* C: *human bone marrow cells stained with nonimmune mouse serum. No fluorescent signal is present in the megakaryocyte or other bone marrow cells. (Magnification: 900×).*

immunosorbent assays were performed with citrate-treated platelet-poor plasma and washed platelets from two normal adult donors, using known concentrations of purified plasma apoJ as a standard. Assuming equivalent immunoreactivity of plasma and platelet apoJ, our results indicate that 20 to 40% of the apoJ in whole blood (70 to 90 μg/ml) is sequestered in platelets and that 0.6 to 2.0% of protein released by activated platelets is apoJ (range of two individuals, data not shown).

Platelet Microparticles Exclude ApoJ

Because apoJ has been shown to have specific lipid and protein interactions in some cell types, we wanted to determine if apoJ released by platelet activation is associated with platelet microparticles, which are rich in lipid, cholesterol, and several membrane-bound platelet proteins such as P-selectin.[18–21] Platelets were stained for apoJ with mAb11 antibody, activated with thrombin, and subjected to flow cytometric analysis to resolve platelets from microparticles. Unactivated platelets appeared as a single apoJ-positive population (Figure 6A). Following thrombin activation, based on forward light scatter (y axis), the platelet population became bimodal in size with a significant fraction of the small particles exhibiting little or no apoJ fluorescence (Figure 6B). The larger particles retained considerable apoJ fluorescence. Platelets incubated with nonimmune serum showed no significant fluorescent signal in the absence or presence of activation (Figure 6, C and D).

We also utilized differential centrifugation to separate platelet aggregates, microparticles, and soluble protein released by platelet activation. Electroimmunoblot analysis of these fractions (Figure 7) showed the presence of apoJ in the aggregated

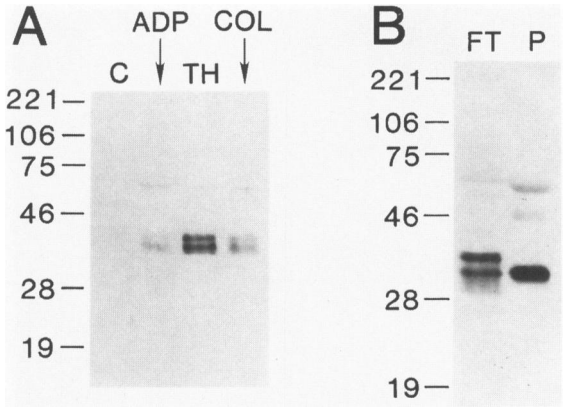

Figure 5. *Platelet activation releases apoJ protein. Electroimmunoblot analysis of platelet supernatant fractions.* **A:** *Purified platelets were activated for 10 minutes with either ADP, thrombin (TH), or collagen (COL). Equivalent portions of supernatant fractions obtained following 850g centrifugation were analyzed by electroimmunoblotting under reducing conditions. Unactivated platelets were handled identically* **(lane C). B:** *Electroimmunoblot comparison of platelet and plasma apoJ.* **Lane FT:** *Supernatant from platelets subjected to three cycles of freeze-thaw.* **Lane P:** *0.1 μl of platelet-poor plasma sample from platelet donor.*

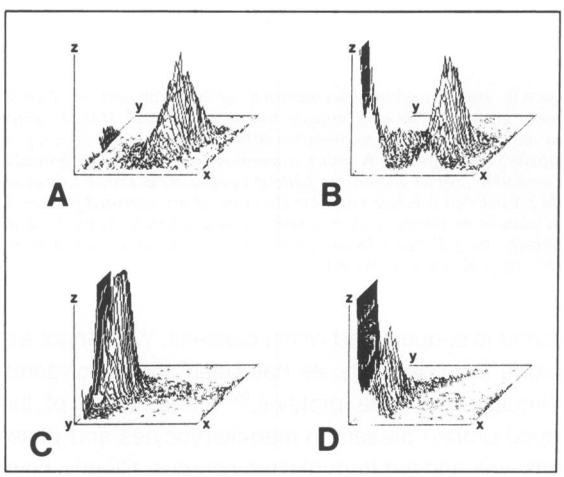

Figure 6. *Flow cytometric analysis indicates the absence of apoJ in activated platelet microparticles. Platelets were stained with MAb11 antibody* **(A** *and* **B)** *or normal mouse serum* **(C** *and* **D).** *Platelets were thrombin-activated* **(B** *and* **D),** *or not activated* **(A** *and* **C),** *and subjected to flow cytometric analysis to resolve platelets from microparticles. Y axes represent forward light scatter that is proportional to size. X axes represent fluorescence intensity. Z axes are proportional to the number of particles. The most numerous fraction of small particles from activated platelets had only background fluorescence and were clustered around the origin in* **B.**

platelets in the low-speed centrifugation pellet (P1) and in the soluble high-speed centrifugation supernatant (P3). However, apoJ was not present in the pellet from high-speed centrifugation (P2), which contained platelet microparticles. This fraction was confirmed to contain platelet microparticles based on detection of P-selectin protein (Figure 7). These results confirm that apoJ is not present in platelet microparticles and that platelet activation causes

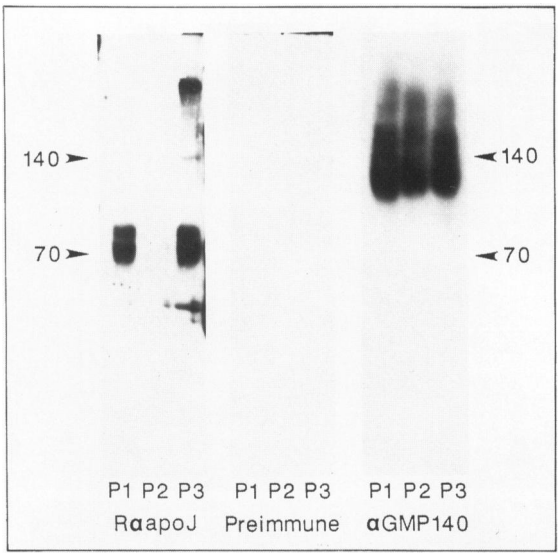

Figure 7. *Platelet microparticles exclude apoJ. Platelets were activated with thrombin and resolved by differential centrifugation into three fractions. The pellet from low-speed centrifugation contains aggregated platelets (P1). The low-speed supernatant was subjected to high-speed centrifugation; and the resulting pellet contains microparticles (P2), and the supernatant contains soluble protein (P3). The presence of apoJ, nonspecific, and GMP-140 immunoreactivities were assessed in parallel immunoblots with rabbit anti-apoJ (RαapoJ), preimmune rabbit serum, and mouse monoclonal anti-GMP140 (αGMP140), respectively. Bound antibody was detected by means of enhanced chemiluminescence.*

soluble-phase release of apoJ. Also, platelet aggregates may retain some apoJ.

Immunoelectron Microscopy Localizes apoJ to Platelet Granules

To determine the subcellular localization of apoJ within platelets, immunogold labeling of human platelets fixed with acetone and Triton X-100 was performed. As shown in Figure 8, permeabilized platelets incubated with apoJ antibody showed most colloidal gold particles in granules (A) and larger vacuolar profiles (B) within the platelet cytoplasm. Only rare gold particles were seen on the surface membrane, and occasional isolated particles were present in the platelet cytoplasm. No signal was present in the cytoplasm of samples incubated with nonimmune mouse serum (Figure 8C).

Atheromatous Plaque Lesions Contain Abundant apoJ

Because the formation of atheromatous plaque lesions has been shown to depend upon the presence of platelets, we sought to determine if apoJ protein is present in atherosclerotic lesions. As shown in Figure 9, immunohistochemical analysis

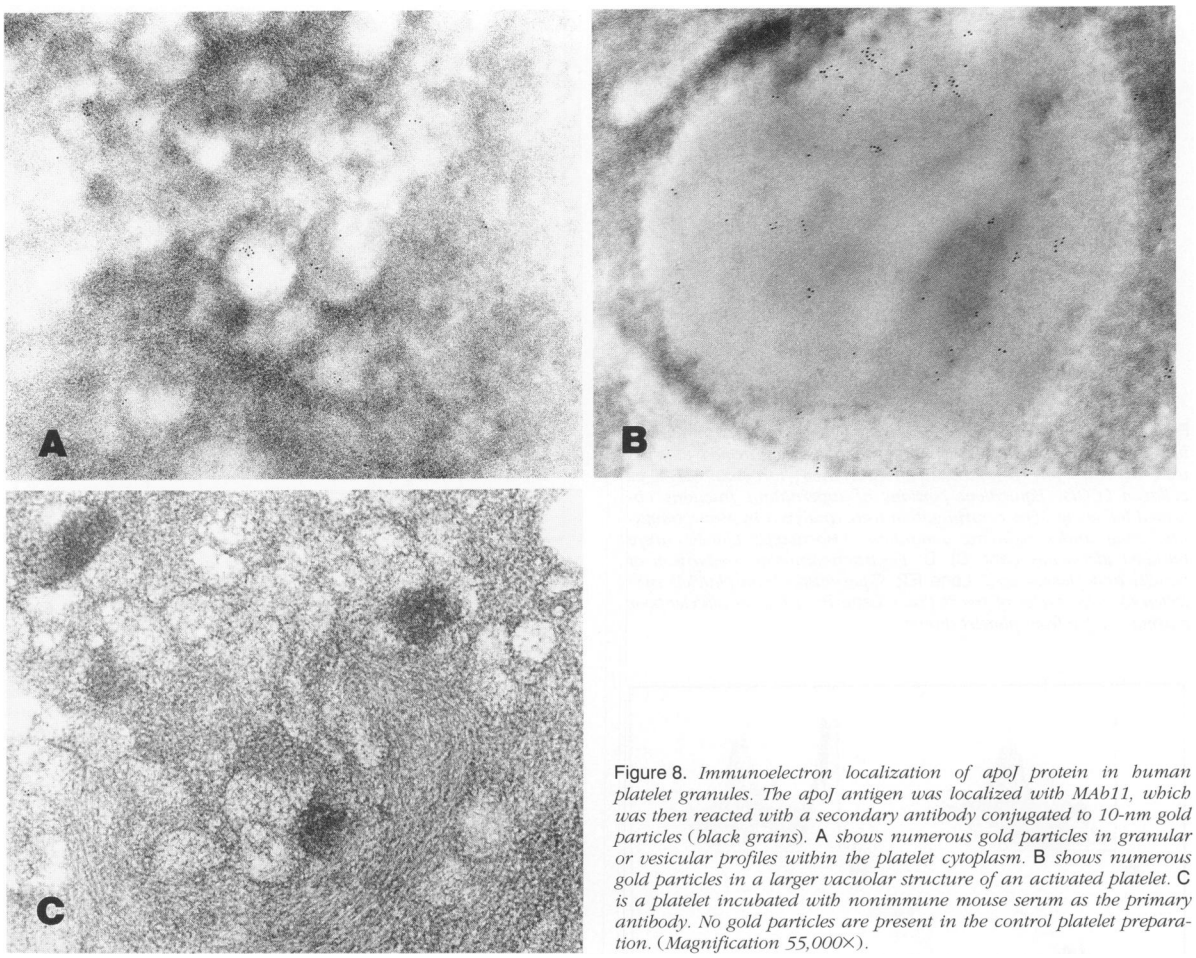

Figure 8. *Immunoelectron localization of apoJ protein in human platelet granules. The apoJ antigen was localized with MAb11, which was then reacted with a secondary antibody conjugated to 10-nm gold particles (black grains).* A *shows numerous gold particles in granular or vesicular profiles within the platelet cytoplasm.* B *shows numerous gold particles in a larger vacuolar structure of an activated platelet.* C *is a platelet incubated with nonimmune mouse serum as the primary antibody. No gold particles are present in the control platelet preparation. (Magnification 55,000×).*

for the presence of apoJ in an artery with advanced atherosclerosis shows intense fluorescence signal in the intimal plaque (A). Sections made from adult nonatherosclerotic arteries (Figure 9B) or infant aorta (not shown) showed no apoJ protein accumulation. Sections of atherosclerotic lesions incubated with nonimmune mouse serum were also negative (not shown). In contrast, *in situ* hybridization of the atherosclerotic arteries showed no apoJ mRNA in the intimal plaque despite easily detectable β-actin mRNA in the same sample (Figure 9, C and D, respectively).

Discussion

We have shown that apoJ mRNA is present in significant amounts in megakaryocytes and that the protein is stored in platelet granules from which it is released into the extracellular compartment after activation. Based on enzyme-linked immunosorbent assay analysis, 20 to 40% of apoJ circulating in the blood is sequestered within platelets. We cannot exclude the possibility, as has been shown for some circulating plasma proteins,[22,23] that some of the apoJ protein present in megakaryocytes and platelets was derived from plasma sources. Clearly, however, abundant platelet-specific apoJ exists and can be distinguished from plasma apoJ by its altered electrophoretic mobility under reducing conditions. Moreover, *in situ* hybridization analysis indicates that apoJ mRNA is synthesized in abundance within megakaryocytes and metabolically labeled apoJ protein was immunoprecipitated from the megakaryocytic cell line CHRF-288.

In mouse and human bone marrow, the only cell types in which apoJ mRNA was detected or in which the protein accumulated were morphologically well-differentiated megakaryocytes. This suggests that the activation of apoJ gene expression occurs late in megakaryocyte maturation. This was further indicated in the *in vitro* studies of the CHRF-288 cell line. Grown under standard culture conditions, this cell line

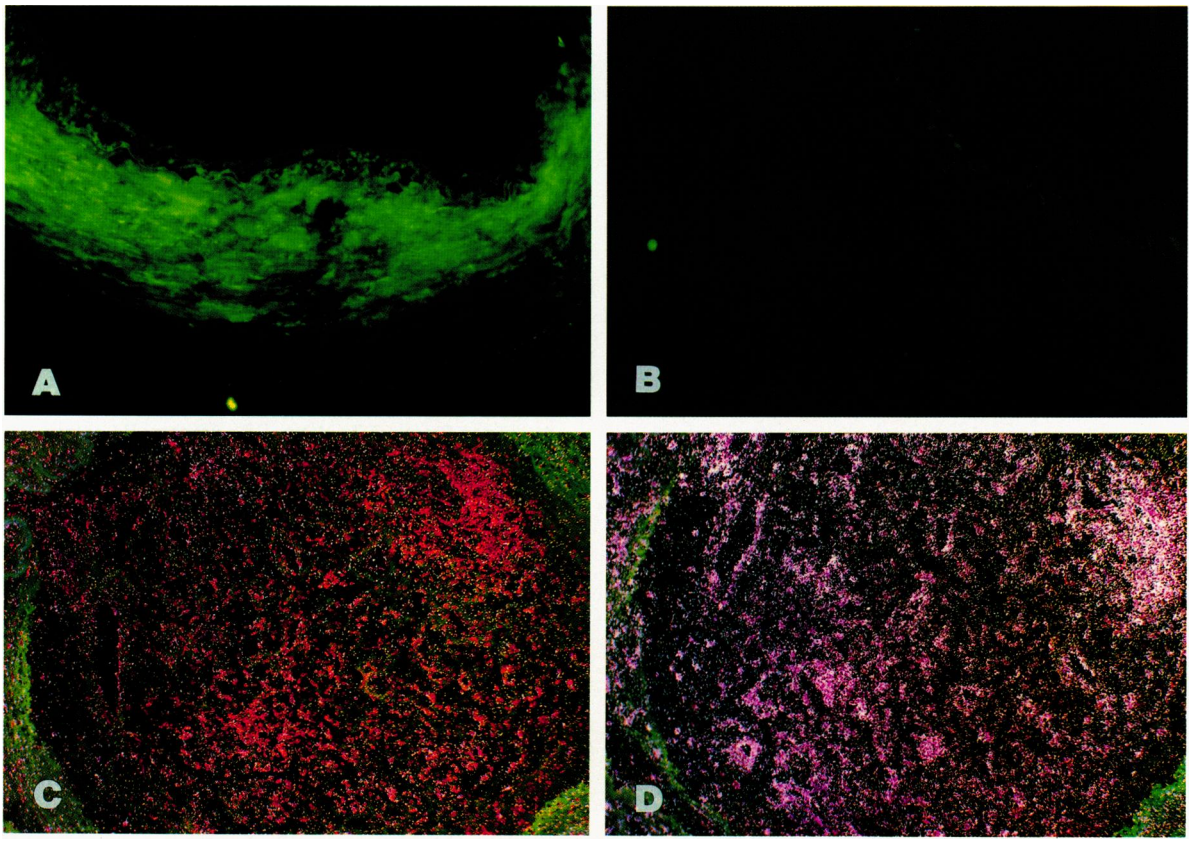

Figure 9. *Intense accumulation of apoJ in atheromatous plaque lesions. Cryostat sections of human femoral artery with advanced atherosclerosis* **(A)** *or adult nonatherosclerotic femoral artery* **(B)** *were stained with the anti-apoJ MAb11 and a fluorescein-conjugated secondary anti-mouse antibody. Sections were counterstained with propidium iodide (appears as red dots) to demonstrate nuclei.* **C** *in situ hybridization for apoJ mRNA in atheroma.* **D** *In situ for human β actin. (Magnification* **A** *to* **D:** *110×).*

is arrested at the megakaryoblast stage.[17] Low-level apoJ synthesis by these cells was only detectable by metabolic labeling and immunoprecipitation, but not by *in situ* hybridization or immunofluorescence analyses. PMA treatment of the culture induced the formation of a small but distinct subpopulation of much more mature cells that expressed abundant apoJ mRNA that was easily detectable by *in situ* hybridization analysis.

Thrombin, collagen, and ADP all triggered the release of apoJ from platelets, consistent with α-granule storage. Electron microscopic immunolocalization showed apoJ within platelet cytoplasmic granules and larger vacuolar structures. Formation of vacuolar structures has been attributed to either fusion of α-granules or dilatation of the open canalicular system during activation and is involved in the secretory process.[24,25]

Several classes of compounds are released by activated platelets. Because apoJ has been shown to associate with lipid,[4,6,7] we considered the possibility that apoJ is released in lipid-rich platelet microparticles that have procoagulant activity.[19,20,26] However, apoJ was found only in the soluble phase or the platelet aggregate and was not found in association with microparticles. This suggests that apoJ is differentially associated with platelet activation fractions. It remains to be determined whether apoJ is associated with other proteins in the soluble platelet fraction.

The function of apoJ is unknown. A variety of hypothetical intracellular and extracellular roles have been proposed and reviewed.[3,4] Most of the cell types that synthesize apoJ constitutively secrete it without storing it. Storage of apoJ does occur in bovine adrenal medullary cells where the bovine homologue of apoJ, GPIII, has been shown to be tightly associated with the chromaffin granule membrane and specific proteins. Palmer and Christie[27] speculate that the function of apoJ in this case is to stabilize components of the granules such as carboxypeptidase H and other glycoprotein proteases. Similar apoJ interactions could be envisioned in platelet granules or in the release of platelets from

megakaryocytes. An alternative hypothesis is that the key function(s) of megakaryocyte-synthesized apoJ occurs after platelet activation either in the soluble phase or when bound to the matrix of the platelet aggregate.

In light of its cell and tissue expression pattern and its biochemical ability to interact with a variety of hydrophobic moieties, we have recently proposed that apoJ has an important extracellular role in protecting cells at fluid-tissue interfaces.[4,10] The presence of apoJ in platelets is consistent with this hypothesis if it serves a protective role at sites of vascular tissue injury. Regulation of complement activity represents one example of an activity capable of delimiting injury. ApoJ inhibits complement-mediated cell lysis *in vitro*.[1,7] A number of other membrane-bound platelet factors that regulate the complement system and affect the activity of other inflammatory mediators[26,28–31] have been identified. Thus, a large bolus of apoJ delivered by platelets could significantly modify the activities of complement or other biological agents at sites of vascular injury.

ApoJ is present in abundance in atherosclerotic plaque. Similar accumulation of apoJ has been shown in senile dementia plaques and in glomerulonephritic deposits.[32,33] The significance of apoJ in either of these lesions is unknown. Platelets have been strongly implicated in the development of atherosclerotic plaque lesions,[34–36] and their release of growth factors and cytokines has been shown to stimulate vascular smooth muscle cell proliferation (for review, see ref. 37). Because *in situ* hybridization analysis did not show local apoJ gene expression within the intimal lesion, its presence in the atheromatous plaque may be a consequence of platelet deposition or infiltration from plasma. In either case, the accumulation of apoJ is likely to modify the development of atherosclerotic vascular lesions. It is now important to determine the targets of apoJ interaction in the lesions and whether apoJ is acting to promote or restrict the development of atherosclerotic injury.

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