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Apolipoprotein J/Clusterin Induction in Myocarditis

A Localized Response Gene to Myocardial Injury

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The function of apolipoprotein J (apoJ) is unknown, but it has been hypothesized to be cytoprotective. In the normal heart, abundant apoJ mRNA and protein are expressed in atrial myocytes; no expression is detected in ventricular myocytes. To provide clues about the role of apoJ in the heart, the response of apoJ to heart disease, including three models of myocarditis and two models of* in vivo *pressure overload hypertrophy, were examined. In the disease model studied extensively, myosin-induced myocarditis,* in situ *hybridization detected induction of apoJ mRNA in ventricular myocytes immediately before histological evidence of injury. ApoJ message in ventricular myocytes reached high levels as myocarditis became more severe. Evidence of early apoJ induction, before inflammation and injury, also occurred in viral myocarditis. ApoJ mRNA was not present in the inflammatory or interstitial cells during myocarditis. In areas of severe inflammation and myocardial fiber degeneration, apoJ showed a gradient of expression, with highest levels in myocytes immediately surrounding the lesion and diminishing with increasing distance. ApoJ protein also accumulated in myocytes at the interface between degenerated myocardial tissue and the surrounding cardiac tissue. During cardiac hypertrophy that occurred without associated inflammation or cell damage, ventricular apoJ mRNA was not detected. When ischemic damage accompanied hypertrophy, apoJ was induced in the ventricular myocytes near the lesion borders. The correlation of apoJ induction with ventricular tissue damage, but not hypertrophy, suggests that apoJ is a repair response protein. We propose that apoJ functions to limit tissue injury and/or promote tissue remodeling. (Am J Pathol 1996, 148:1971–1983)

The physiological role of apolipoprotein J (apoJ; also designated clusterin, SGP-2, SP40,40, complement lysis inhibitor, and TRPM-2), a highly conserved secretory glycoprotein,[1,2] remains a subject of speculation. Its patterns of inducibility and deposition associated with a variety of physiological and pathological processes suggest that it is active at sites of tissue remodeling during development and in response to stress. ApoJ expression is, for example, dramatically increased during mammary gland regression,[3] prostate involution,[4] renal injury,[5,6] and neurodegeneration.[7–9] Both Alzheimer disease[10–12] and atherosclerotic vascular disease[13,14] are characterized by accumulation of apoJ protein in degenerative lesions. In the absence of obvious inductive stimuli, apoJ is also expressed constitutively, predominantly in epithelial cells, which serve as critical barriers between harsh biological fluids and tissues.[15]

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One exception to this constitutive pattern of epithelial boundary apoJ expression occurs in the heart. In hearts of adult mice, apoJ is constitutively expressed in myocytes of the right and left atrium, with no detectable expression in ventricular myocytes.[15] In contrast, during cardiogenesis, apoJ expression is restricted to prevalvular and valvular cell types.[16] Early during valve morphogenesis, myocytes of the muscle wall underlying the endocardial cushions in the atrioventricular canal and truncus arteriosus express apoJ mRNA. The regression of this muscle layer coincident with the sculpting of valves from cushion tissue is associated with induction of apoJ mRNA in stromal-like cells constituting both valve leaflets and annuli. The adult pattern of atrial apoJ expression is established at birth, with low level apoJ mRNA persisting in the valve annuli.

The developmental switches that control apoJ expression in cardiac cell types are intriguing and suggest that the mouse heart will be ideal for defining apoJ's function. Cardiac function becomes crucial early during embryonic development and can be studied in the adult *in vivo* or, in the perfusion system, in isolation from hormonal and other physiological influences. The availability of cardiac-specific promoters, eg, the cardiac α-myosin heavy chain promotor,[17] makes it possible to evaluate the consequences of manipulating a gene in the heart of a live animal. Furthermore numerous heart disease models have been established and characterized in the mouse, enabling us to determine how apoJ expression is altered by pathological perturbation to provide important clues about its function. We focus here on the cardiac expression of apoJ in the normal adult mouse, in response to myocarditis and pressure overload hypertrophy. Atrial myocytes express apoJ message and protein in healthy mice and in mice with myocarditis or ischemic injury. In contrast, ventricular myocytes, which do not express detectable apoJ message constitutively, express abundant apoJ when cardiac inflammation or injury is evident. ApoJ expression is most pronounced in those ventricular myocytes that border areas of tissue injury. Based on apoJ's known biochemical properties and its pattern of regulated expression in the heart, we propose that apoJ serves to separate injured from healthy tissue, similar to its constitutive potential to act as a barrier between healthy tissue and membrane-active biological fluids, to maintain tissue integrity and to enhance tissue repair processes.

Materials and Methods

Tissues

Mice were sacrificed by asphyxiation in CO_2. Hearts and kidneys were excised and rinsed in sterile phosphate-buffered saline (PBS). Tissues for *in situ* hybridization were fixed for 24 hours in 4% (w/v) paraformaldehyde (EM Science, Cincinnati, OH) in PBS, pH 7.4, cryoprotected in 30% sucrose in PBS, and embedded in OCT (Miles Laboratories, Elkhart, IN). For immunohistochemistry, organs were washed in PBS and embedded directly in OCT.

Myocarditis

Transforming growth factor (TGF)-β1-deficient mice were obtained from Drs. Tom Doetschman and Ron Diebold (Cincinnati, OH). Paraformaldehyde-fixed hearts and kidneys from A/J mice infected with Coxsackie B3 (CB3) for 5 or 14 days were obtained from Dr. Noel Rose (Baltimore, MD). Myocarditis was induced in FVB/N (Jackson Laboratory, Bar Harbor, ME) mice by injection with cardiac myosin, essentially as described.[18] Briefly, 100 μg of purified mouse cardiac myosin[19] in 50 mmol/L sodium pyrophosphate (pH 7.3) was emulsified in complete Freund's adjuvant (CFA) and injected twice subcutaneously into mice, 1 week apart. On the day of the first injection, 500 ng of pertussis toxin (List Biologicals, Cambell, CA) was also injected intraperitoneally. Control mice were injected with pertussis toxin and 50 mmol/L sodium pyrophosphate emulsified in CFA. The mice injected with myosin developed a severe inflammatory response in the heart 18 to 25 days after the first injection. No gross or histological changes were noted in control mice.

Cardiac Hypertrophy

Pressure overload cardiac hypertrophy was induced in mice by two methods: transverse aortic constriction (TAC)[20] and pulmonary artery banding (PAB).[21] Briefly, transverse aortic constriction was established in anesthetized, intubated mice (8-week-old C57BL/6XSJL, Jackson Laboratory) by exposing the transverse aorta through a small incision in the chest wall at the second intercostal space. A 7–0 suture ligature was placed around the transverse aorta, which was briefly tied down against a 27-gauge needle to result in a 0.4-mm narrowing of the vessel when the needle is removed. This technique has previously been shown to produce a reproducible constriction of the aorta of 65 to 70%, associated

with the characteristic features of cardiac hypertrophy.[20] For PAB, mice were anesthetized and intubated, and a lateral thoracotomy was performed at the third intercostal space. A suture ligature was placed around the pulmonary artery and tied against a 25-gauge needle, which was then rapidly removed. The chest was closed, the pneumothorax was evacuated, and the animals were allowed to recover.[21] Analysis was performed 1 to 2 weeks after constriction. Sham-operated animals underwent similar operations except for vessel constriction.

Histological Evaluation of Tissues

Hearts and kidneys were evaluated histologically for the presence of inflammation and/or tissue damage. Cryostat sections were stained with hematoxylin and eosin (H&E) and graded on a sale of 0 to 4+, depending on the percentage of tissue that was either actively involved in an inflammatory response or showed evidence of tissue damage; 200 μm of each heart was evaluated by examining at least six 5-μm sections representing the entire organ. The following scale was set: 0, no involvement; 1, 1 to 10% involvement; 2, 10 to 30% involvement; 3, 30 to 50% involvement; 4, 50 to 70% involvement; 4+, >70% involvement. Grades wee assigned in two ways, with identical differences between comparative sets (diseased *versus* control). First, the grade was based on the section that indicated the most severe tissue injury. Alternatively, grades for all sections of the same heart were considered to obtain the average grade.

In Situ *Hybridization*

Mouse apoJ sense and antisense RNA probes were synthesized by using a commercially available transcription kit (Stratagene, La Jolla, CA) with ^{35}S-labeled rUTP (Amersham Life Sciences, Arlington Heights, IL) from a plasmid that contained the 0.8-kb mouse apoJ 5′ cDNA insert from clone 5–1.[13,22] Atrial natriuretic factor (ANF) riboprobes were similarly transcribed from a plasmid containing the rat ANF cDNA.[23] Cryostat sections (5 to 7 μm) were air dried on 3-aminopropyltriethoxysilane-coated slides (Sigma Chemical Co., St. Louis, MO) post-fixed, prehybridized, hybridized with riboprobe (1×10^6 cpm), digested with ribonuclease, and washed, as previously described,[24] using 0.1X standard saline citrate at 55°C (final stringency). Slides were exposed to NTB2 emulsion (Kodak) for 7 to 10 days and developed in D19 developer (Kodak, Rochester, NY). Sections were counterstained with H&E and photographed under dark- and bright-field illumination.

Immunohistochemistry

Analysis was performed, essentially as described by Witte et al.[16] Briefly, sections were washed and blocked with 5% nonfat dry milk in wash buffer (50 mmol/L Tris-HCl, 257 mmol/L NaCl, 0.1% Triton X-100, pH 7.3). The sections were then incubated for 1 hour with either 1 μg of anti-rat SGP-2 (apoJ) IgG[25] or 1 μg of rat anti-mouse leukocyte common antigen (LCA) IgG (Pharmingen Research Products, San Diego, CA), which reacts with all cells of hematopoeitic origin except erythrocytes. Negative control sections were treated identically, except the primary antibody was nonimmune rabbit or rat IgG. The secondary antibody was peroxide conjugated and detected with 0.05% diaminobenzidine (Sigma) and 1% nickel sulfate (Malinckrodt, Pittsburgh, PA). All sections were counterstained with Fast Red.

Electroimmunoblot Analysis

Mouse atrium and ventricle tissues were homogenized in 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and 0.02% sodium azide. Atrial, ventricular, or plasma samples, solubilized in 10 mmol/L Tris-HCl, pH 6.8, containing 10% glycerol and 2% SDS, were analyzed by gel electrophoresis through 10% polyacrylamide in the presence of SDS.[26] Electrophoresed samples were transferred for immunostaining to polyvinylidene fluoride at 100 mA for 16 hours at 4°C in 25 mmol/L Tris-HCl, pH 8.3, 192 mmol/L glycine, and 20% methanol. The polyvinylidene fluoride was incubated for 2 hours with primary antibody, either anti-SGP-2 at 1/100 dilution or anti-apoAI (The Binding Site, San Diego, CA) at 1/10,000 dilution, in blocking buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.5% nonfat dry milk, 0.01% (v/v) antifoam A (Sigma)). The blots were washed thoroughly in blocking buffer, incubated for 1 hour with horseradish-peroxidase-conjugated secondary antibody (1/20,000), and visualized by enhanced chemiluminescence (Amersham).

Results

ApoJ Is Localized to Atrial Myocytes

To determine the relationship between sites of apoJ synthesis and deposition, the location of apoJ mRNA and protein was determined in hearts of healthy adult (6 to 12 weeks) mice by *in situ* hybridization, using an

antisense cRNA probe and by immunohistochemical analysis using a polyclonal rabbit antibody raised against rat apoJ (SGP-2) that cross-reacts with mouse apoJ.[22,25] ApoJ mRNA (Figure 1a) and protein (Figure 1c) were co-localized to the right and left atrium, specifically in atrial myocytes (Figure 1c inset). Little apoJ message or protein was evident in ventricular myocytes or in interstitial stromal cells of either the atria or ventricles. There was, however, apoJ mRNA signal in the smooth muscle cells of the larger coronary arteries, as we reported previously.[15] The specificity of the reagents diagnostic for apoJ was indicated by the absence of apoJ signal when the tissue was hybridized with sense apoJ cRNA (Figure 1b) or incubated with nonimmune purified IgG (Figure 1d).

To confirm the apoJ distribution pattern, protein was extracted separately from atrial and ventricular tissue and evaluated for apoJ by electroimmunoblot analysis (Figure 2). Immunoreactive apoJ was detected in atrial but not ventricular extracts. The atrial apoJ was similar in size to plasma apoJ under nonreducing conditions. It is unlikely that the apoJ in the atrial samples originated from blood contamination, as apoAI, evident in plasma samples, was not detected in the tissue homogenates.

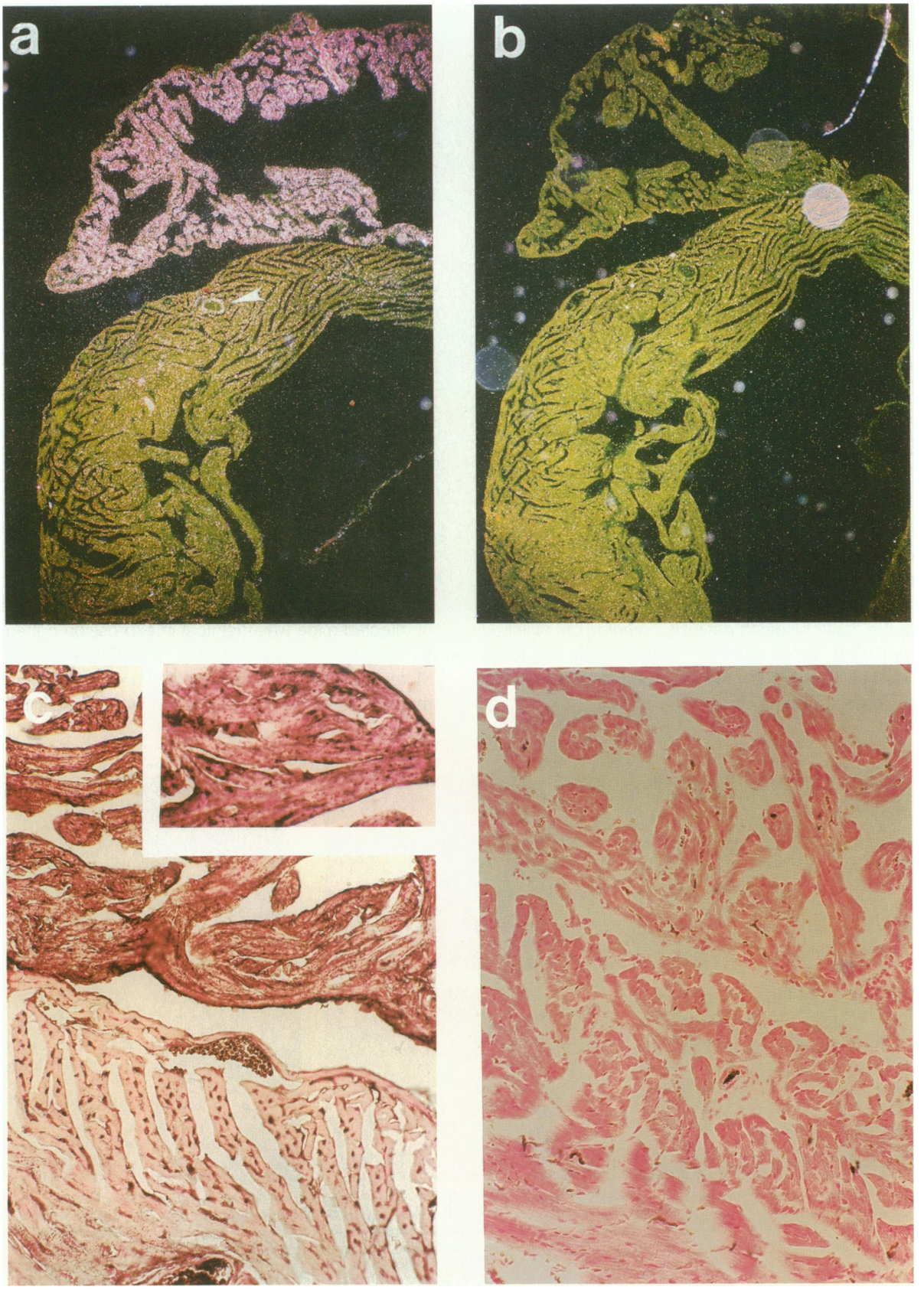
ApoJ Expression in Ventricular Myocytes Is Dramatically Increased in Myocarditis

To provide clues about the function of apoJ in the heart, we asked whether its expression pattern is altered in mouse models of heart disease. Myocarditis was induced in mice by inoculation of pertussis-toxin-challenged mice with cardiac myosin in an emulsion of CFA. The consequent autoimmune disease resulted in an inflammatory response that, at its peak, destroyed the myofibers of the heart. In a typical example shown in Figure 3, A–C, approximately 60% of the myocardium (myocarditis score of 4) was involved in a diffuse interstitial inflammatory response. In contrast to the atrial myocyte restriction of apoJ gene expression in control mice, myocarditis dramatically induced apoJ expression in the ventricular myocytes (Figure 3, A and B). ApoJ-expressing myocytes were most prominent in apparently viable myofibers bordering areas of inflammation and myofiber atrophy (Figure 3B). Consistent with apoJ message expression, apoJ protein was also present in these ventricular myocytes (Figure 3C), appearing as granular immunoreactive deposits (arrowheads) concentrated in myocytes at or near the boundaries of the lesions. Inflammatory cells, visualized with antibody to LCA, were detected in these same areas (not shown). No apoJ protein was detected in the stromal connective tissue that replaced damaged myocardial fibers of the heart. Induction of apoJ expression during myocarditis appeared to be specific for the heart. ApoJ mRNA in the kidney, for example, was restricted to the distal convoluted tubular epithelium, the normal site of constitutive expression,[15] and was identical to the expression pattern in kidneys of littermate controls.

In situ hybridization analysis of mouse hearts excised at varying times after myosin injection was undertaken to delineate the timing of induction of apoJ expression relative to that of leukocyte infiltration and morphological evidence of tissue injury. ApoJ mRNA accumulated in a subset of ventricular myocytes (Figure 3D) before evidence of an inflammatory response (Figure 3F) and obvious tissue injury (Figure 3E). As myocarditis became more pronounced, the number of apoJ-expressing cells increased until much of the healthy tissue had been replaced by scar tissue, which contained no apoJ-expressing cell types. There was no evidence of apoJ induction in the ventricular myocytes of hearts from control animals, injected with CFA and pertussis toxin only, at any time period. As expression of the ANF gene in ventricular myocytes is considered diagnostic of stress-associated cardiac hypertrophy,[27] we asked whether ANF expression in ventricular myocytes is also altered during myocarditis. Unlike apoJ expression, ANF expression was not induced in ventricular myocytes during myocarditis over the time investigated.

It should be noted that this is the first report of myosin-induced myocarditis in FVB/N mice, which are $H2^q$ haplotype.[28] Previously, it has been demonstrated that susceptibility to myocarditis is haplotype

Figure 1. *ApoJ is restricted to atrial myocytes in the normal adult mouse heart.* **a:** In situ *hybridization with a ^{35}S-labeled antisense apoJ riboprobe. Atrial myocytes (oriented toward the top of the panel) show intense signal (bright white grains), with no detectable expression in ventricular myocytes. Smooth muscle cells of the larger coronary arteries* (**arrowhead**) *also express apoJ mRNA.* **b:** In situ *hybridization with the control sense probe shows no signal in the atrial or ventricular myocytes.* **c:** *Immunohistochemistry with an antibody that detects mouse apoJ. Diaminobenzidine, enhanced with nickel sulfate, was used for detection to yield a black reaction product. ApoJ protein is restricted to atrial myocytes, with little or no apoJ evident in ventricular myocytes.* **Inset:** *Higher magnification of a heart similar to that seen in* **C** *illustrates the granular apoJ localized to atrial myocytes.* **d:** *Serial section to* **C** *stained with nonimmune serum as a negative control. The brown reaction product present in the ventricular myocardium was also detected in the antibody in the antibody control (no primary antibody), indicating that it is due to diaminobenzidine reaction with erythrocyte endogenous peroxidase. Magnification, ×50,* (**a** *and* **b**)*; ×120,* (**c** *and* **d**)*; ×300,* (**inset** *in* **c**).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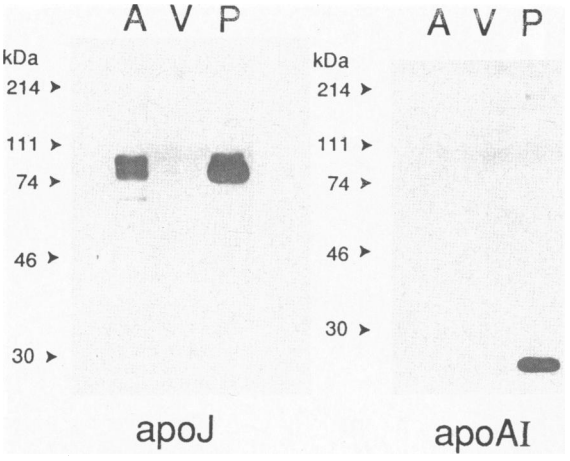

Figure 2. *Electroimmunoblot to detect apoJ. Atrial (A) or ventricular (V) homogenates or mouse plasma (P) samples were analyzed under nonreducing conditions for the presence of apoJ protein. ApoJ (80 kd) was detected in atrial but not ventricular homogenates. To exclude the possibility that the source of apoJ in the atria is plasma contamination, apoAI was used as a control. ApoAI (28 kd), present in plasma at a level approximately 10 times that of apoJ, is not in atrial or ventricular homogenates.*

dependent. However, development of myocarditis in mice of several haplotypes, including $H2^q$, indicates that susceptibility is not strictly controlled by genes of the major histocompatibility complex but is linked to at least one other locus.[29] Interestingly, this locus has been mapped to mouse chromosome 14 in the region of the apoJ gene.[13,30]

We next asked whether myocarditis induced in mice by CB3 virus infection[31,32] was also associated with an apoJ response. Tissues representing the two phases of viral myocarditis, the acute stage of viral replication (5 to 7 days after infection) and the chronic autoimmune phase of inflammation (10 to 20 days after infection), were evaluated for apoJ expression. During acute myocarditis, apoJ expression was restricted to atrial myocytes (Figure 4a), a pattern identical to that of uninfected and mock-infected littermate controls. Although CB3 actively replicates in the myocytes during acute myocarditis,[33] little tissue damage was evident and very few inflammatory cells were present. During the autoimmune phase of myocarditis, however, apoJ mRNA was abundant in ventricular myocytes, at levels comparable to those in atrial myocytes (Figure 4b). This induction occurred as early as 14 days after infection in the absence of obvious tissue damage, although inflammatory cells were evident. At this early time point, the autoimmune disease had not resulted in myocardial fiber loss or fibrosis. No apoJ mRNA was detected in the inflammatory or interstitial stromal cells. As in myosin myocarditis, induction of apoJ appeared to be specific for the heart; apoJ mRNA in the kidneys of infected mice was identical in expression pattern to kidneys of mock-infected littermate controls. Moreover, no ANF mRNA was detected in ventricular myocytes, in contrast to apoJ mRNA accumulation.

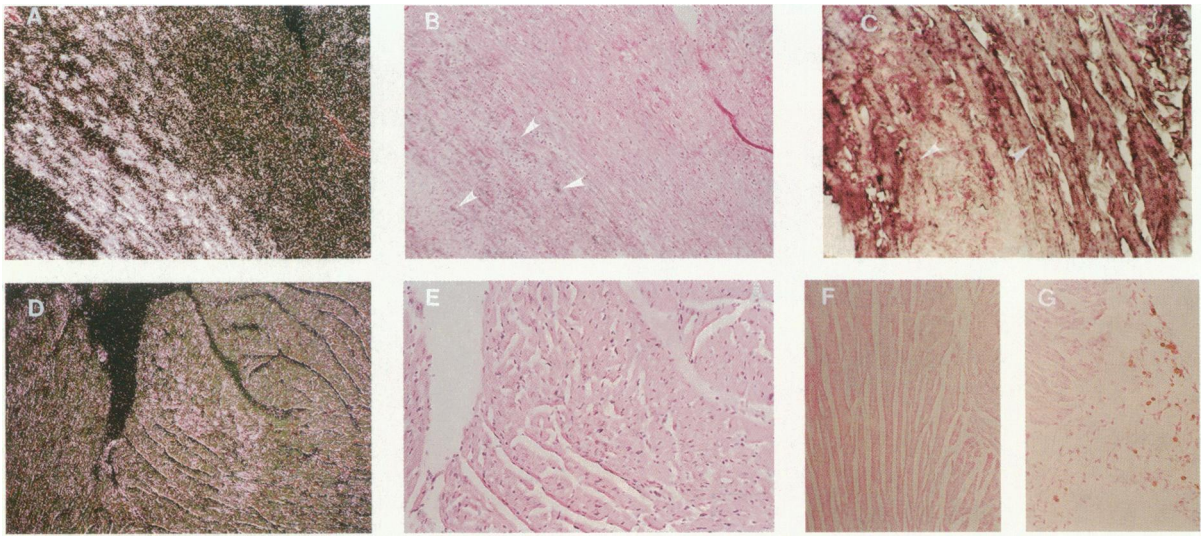

Figure 3. *ApoJ is induced in ventricular myocytes in myosin myocarditis.* **A:** In situ *hybridization with apoJ antisense riboprobe shows that autoimmune myocarditis (score = 4), induced by the injection of myosin, is associated with marked induction of apoJ mRNA in the ventricular myocytes (n > 20).* **B:** *Same as* **A,** *but bright-field illumination.* **Arrowheads** *indicate areas with inflammatory cell infiltrates and myocardial fiber degeneration.* **C:** *ApoJ protein is localized to the ventricular myocytes* (**arrowheads**) *surrounding areas of damage and inflammation (score = 4).* **D:** *ApoJ mRNA can be detected in ventricular myocytes before histological evidence of tissue injury (n = 6). Shown here are representative sections obtained 8 days after myosin inoculation.* **E:** *Bright-field image of* **D,** *showing no evidence of inflammation of myocardial fiber degeneration.* **F:** *Heart shown in* **D** *stained for leukocytes with anti-LCA. No inflammatory infiltrates are identified.* **G:** *Epicardial tissue with LCA-stained inflammatory cells as a positive control. Magnification, ×120* (**A, B, D, F,** *and* **G**) *and ×240* (**C** *and* **E**).

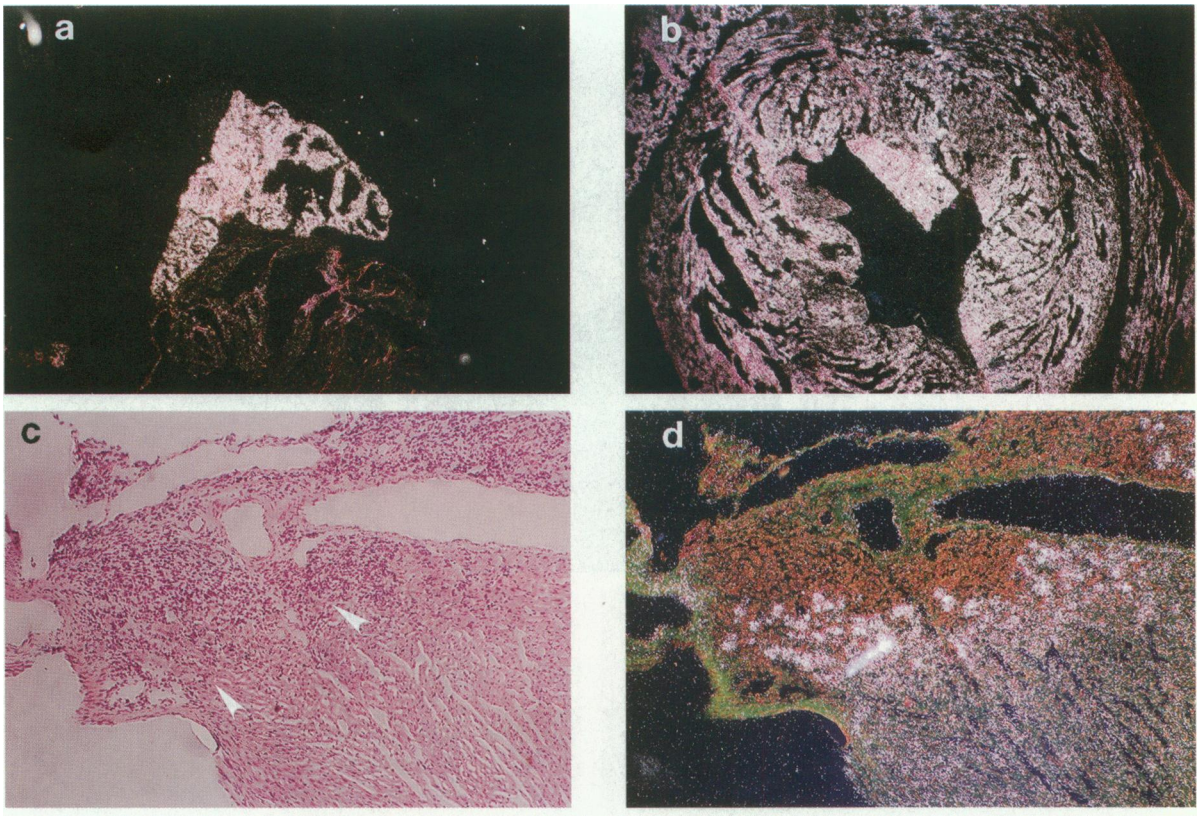

Figure 4. *ApoJ is expressed in ventricular myocytes in autoimmune myocarditis.* **a:** *ApoJ expression is not induced in ventricular myocytes during the acute phase of CB3-induced myocarditis (n = 2, score = 0).* **b:** *ApoJ mRNA is highly induced in the ventricular myocardium during the later autoimmune phase (score = 1) of CB3-induced myocarditis (n = 2).* **c:** *Hematoxylin and eosin (H&E) staining of a section of heart obtained from a mouse homozygous for TGF-β1 deficiency (n = 4) shows a severe inflammatory lesion* (**arrowheads**; *score = 3).* **d:** In situ *hybridization of the same heart as in* **c** *shows a gradient of ventricular apoJ induction surrounding the lesion; the cells expressing the highest level of apoJ mRNA are those nearest the inflammatory foci, with the level of expression decreasing with increasing distance from the lesion. There is no expression in the inflammatory infiltrate. Magnification, ×50* (**a** *and* **b**); *and ×120* (**c** *and* **d**).

TGF-β1 deficiency also altered the pattern of apoJ mRNA expression in the heart. As is shown in Figure 4c, and previously described by Shull et al,[34] mice genetically deficient in TGF-β1 develop severe myocarditis. As in other models of myocarditis, apoJ mRNA was dramatically elevated in ventricular myocytes (Figure 4d). Atrial apoJ expression was roughly equivalent in hearts of TGF-β1-deficient (−/−) and TGF-β1-sufficient (+/+) mice. In the ventricles of TGF-β1 (−/−) mice, apoJ message was particularly pronounced in myocytes immediately surrounding the inflammatory lesions. The gradient of apoJ mRNA was striking, with signal intensity in myocytes decreasing with increasing distance from lesions. No apoJ mRNA was detected in inflammatory or interstitial stromal cells. TGF-β1-sufficient littermates showed no inflammatory foci and no ventricular apoJ mRNA. The kidneys of TGF-β1-deficient mice were histologically normal, with no evidence of inflammation, and the pattern of kidney apoJ mRNA expression in TGF-β1 (−/−) mice was identical to that in TGF-β1 (+/+) mice.

Cardiac Stress without Tissue Damage Does Not Induce ApoJ Expression

In an attempt to determine whether apoJ is induced in noninflammatory forms of cardiac disease associated with myocardial stress, cardiac hypertrophy was induced by TAC or PAB. These models produce a uniform hypertrophic response in the involved ventricle without myocardial fiber injury or loss except in rare foci of ischemic lesions in the severely hypertrophied heart. TAC induces cardiac hypertrophy but not cardiac failure.[20] TAC-induced hypertrophy of the heart shown in Figure 5 was not associated with tissue damage (Figure 5A) or with up-regulation of ventricular apoJ mRNA (Figure 5B). This heart weighed 134% more than hearts of sham-operated littermates, characteristic of the hypertrophic response in mice.[20,35] In addition, we found that ANF mRNA, normally localized to the atria only, showed the expected induction in the ventricular myocytes of the hypertrophic hearts in response to the stress of pressure overload.[27] It was interesting that ANF

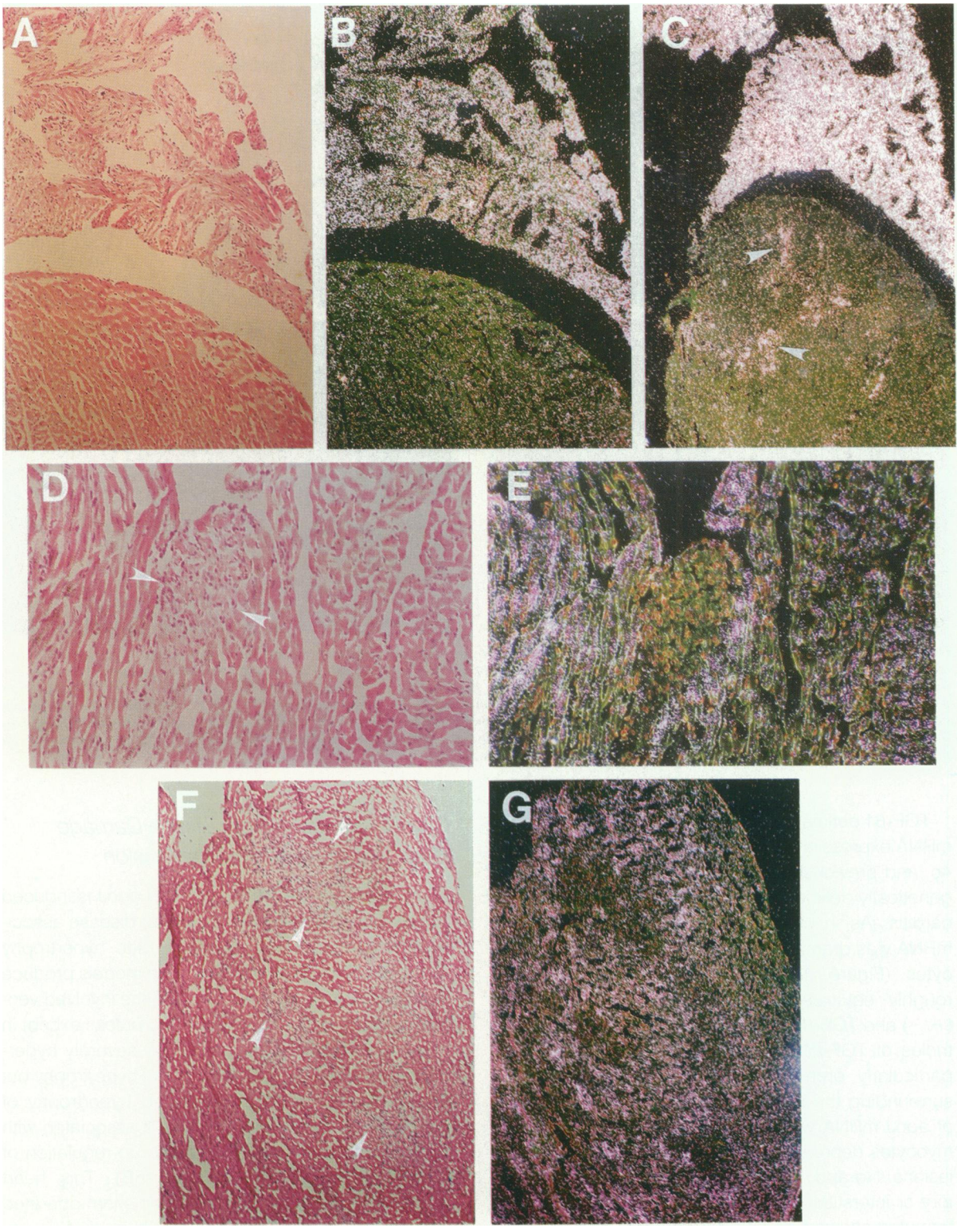

Figure 5. *Ventricular hypertrophy alone does not induce apoJ expression.* **A:** *H&E staining of a hypertrophied heart shows no morphological evidence of tissue damage or inflammation.* **B:** In situ *hybridization of the same section as* **A** *with apoJ riboprobe shows apoJ expression in atrial myocytes only, with none detected in ventricular myocytes.* **C:** In situ *hybridization with an ANF riboprobe shows high expression in atrial myocytes and sporadic expression in ventricular myocytes* (**arrowheads**) *in a pressure-overload hypertrophied heart.* **D:** *H&E-stained section of a severely hypertrophied heart with focal areas of tissue damage* (*area between* **arrowheads**; *score = 1*). **E:** In situ *hybridization with an apoJ antisense probe shows apoJ mRNA induction in ventricular myocytes near the areas of focal tissue damage* (*same section as* **D**). **F:** *H&E-stained section of TAC hypertrophied heart shows multiple areas of ischemic damage* (*score = 1;* **arrowheads**), *around which ANF signal* (**G**) *is most intense. Magnification,* ×*120* (**A** *to* **C**, **F**, *and* **G**) *and* ×*220* (**D** *and* **E**).

message was not uniformly expressed among the cardiomyocytes; only selected myofibers in the heart expressed ANF mRNA (Figure 5C).

Constriction of the thoracic aorta or pulmonary artery can cause focal ischemic cardiac muscle damage. Thus, 55% (5 of 9) of the TAC mice developed small localized foci of cardiac damage (0 to 5% of the heart affected); 67% (4 of 6) of the PAB mice developed slightly larger lesions (0 to 20% of the heart affected). An example of a heart from a mouse with TAC-induced hypertrophy in which tissue damage was evident is shown in Figure 5D. Areas of myocardial fiber atrophy and fibrosis, presumably as a result of focal ischemic injury, appear as fibrous stromal tissue. There were no inflammatory infiltrates evident histologically in this section at this time, but the myocardial fibers at the edge of the lesions showed mild nuclear pleomorphism. Immunohistochemical analysis for leukocytes, using the sensitive anti-LCA antibody, however, indicated the presence of inflammatory cells in the ischemic lesions of other hypertrophied hearts (not shown). As noted for myosin-induced myocarditis and TGF-β1 deficiency, for which defined lesions were evident, the induction of ventricular apoJ mRNA was pronounced in those myocytes bordering the injury (Figure 5E). In myocytes residing in areas of the hypertrophied heart without evidence of ischemic injury, no apoJ mRNA was detected. ANF, like apoJ, was expressed at the highest levels in myocytes bordering focal areas of ischemic damage, as determined by *in situ* hybridization (Figure 5, F and G); ANF signal was present but diminished in hypertrophied areas of the heart distal to the ischemic lesions. Similar results were obtained when right ventricular hypertrophy was induced by PAB. Constriction itself did not induce ventricular apoJ expression; however, if cardiac tissue were secondarily injured, apoJ mRNA was induced in the myocytes surrounding the areas of tissue damage. No apoJ or ANF mRNA was detected in the ventricles of hearts of sham-operated littermate control animals.

Discussion

The identification of regionally restricted genes in the heart has been a major goal of research in the cardiovascular field as a prerequisite to understanding how the heart develops and functions and how it responds to disease. The primary cell type that expresses the apoJ gene in the adult heart is the myocyte. We have not detected apoJ mRNA in pericardial, endocardial, or interstitial cells or in cell types derived from the blood. ApoJ gene expression is, moreover, highly compartmentalized. In hearts of normal adult mice, the apoJ gene is predominantly expressed in atrial myocytes, with no apoJ mRNA or protein evident in ventricular myocytes. However, ventricular myocytes proximal to sites of ischemic and/or inflammatory tissue injury express abundant apoJ message. We demonstrate here that ventricular apoJ induction occurs in three distinct myocarditis models and in two pressure overload hypertrophy models. In each model, high level apoJ expression in ventricular myocytes was associated with the presence of inflammatory cells and/or tissue injury. Only apparently viable ventricular myocytes bordering the damaged myocardial tissue express apoJ message; no apoJ mRNA or protein was detected in severely damaged cardiomyocytes. Although the number of animals examined in each model of heart disease varied (from 2 to 20), the results were consistent; whenever inflammatory cells or tissue damage were evident in the ventricular myocardium, apoJ mRNA accumulated in ventricular myocytes. The absence of altered apoJ expression in the kidneys or other organs of mice that show ventricular myocyte apoJ expression indicates that the apoJ response after cardiac perturbation reflects a local response in the heart and does not result from or cause a systemic signal that affects apoJ expression in other tissues.

It is significant that not all forms of ventricular perturbation result in apoJ induction. Pressure overload of the heart, as shown here, can cause ventricular hypertrophy in the absence of an apoJ response when tissue injury does not occur. We (D. P. Witte and B. J. Aronow) also have found no evidence of apoJ induction in dramatically dilated hearts from mice treated with phenylhydrazine, which induces acute high output heart failure without inflammation or myofibril degeneration. The association of ventricular cardiomyocyte apoJ mRNA and protein with inflammation and tissue injury implies that cardiac stress alone does not affect apoJ gene expression. Maximal apoJ induction appears to require a product associated with the presence of inflammatory cells and/or injured myocardium. The induction of ventricular apoJ mRNA may denote an early sign of incipient cell damage, as a careful analysis of the temporal dependence of myosin myocarditis indicated induction of apoJ mRNA before histological evidence of inflammation or myocardial injury. In addition, we noted apoJ mRNA accumulation before obvious tissue injury in our CB3-induced myocarditis study.

ApoJ gene induction in previously nonexpressing cell types, such as ventricular myocytes, appears to

be a marker of impending and actual tissue injury/inflammation in a variety of tissues. ApoJ message is markedly up-regulated in epithelial cells in the kidney obstructed by ureteric ligation but not in the contralateral kidney, which undergoes compensatory hypertrophic cell growth only.[36,37] During ischemic injury of the kidney, like the heart, apoJ protein accumulates in the periinfarct zone only, not in unaffected areas.[5,6] Similarly, using neurotoxins that injure different areas of the brain, Rozovsky et al[38] established that apoJ gene induction occurs proximal to the site of injury. In fact, apoJ accumulates in many degenerative lesions such as atherosclerotic plaque.[13,14] In the aortic valve lesions of mice fed a high fat diet, infiltrating macrophage-like cells express the apoJ gene.[13] In the central nervous system, apoJ protein accumulates in amyloid deposits[10,11] and is co-localized with vitronectin in senile plaques.[11] In other inflammatory lesions of the central nervous system, eg, multiple sclerosis, cerebral infarcts, and AIDS-related lesions, apoJ protein is localized in or near the lesions.[12] Many of these lesions have been shown to share the feature of localized membrane attack complex (MAC) of complement.

Complement components accumulate at sites of tissue damage, apparently independent of damage etiology.[39–41] ApoJ is a potent inhibitor of complement-mediated cell lysis *in vitro*[42] and has been proposed to regulate MAC assembly and function *in vivo*.[43,44] That complement regulation may be important in the heart is indicated by the co-localization of terminal complement components with apoJ in acute myocardial infarcts in humans.[45] However, we can now dissociate apoJ induction from terminal complement complex activation in the heart. Myocarditis in our study was elicited in strains of mice (FVB/N and A/J) that are deficient in the ability to secrete C5,[28,46] one of the components necessary for MAC assembly, precluding any direct regulation of apoJ expression by activated terminal complement components. Nevertheless, signals that initiate terminal complement deposition may also regulate apoJ expression, such that apoJ is induced wherever conditions favor local MAC assembly, regardless of whether assembly itself is possible. The signal(s) in these cardiac disease models responsible for apoJ induction may be a product of activated or injured cells, consistent with the association established over time between injury boundaries and myocytes expressing the highest levels of apoJ mRNA. Consistent with this possibility, Nath et al[47] determined that muscle homogenates can induce apoJ gene expression in renal tubular epithelial cells as an *in vitro* model of rhabdomyolysis.

Although the results presented here do not unequivocally define the physiological role of apoJ in the heart, they provide significant insight. The gene product first appears early in the development of chronic autoimmune myocarditis, signaling a change in cellular environment before histological evidence of tissue damage. At this stage, only a few isolated cells are likely to be injured, hence the unremarkable histology. We propose that these sites of submicroscopic injury are indicated by apoJ-expressing myocytes. ApoJ may bind released cytotoxic constituents and mediate their inactivation and/or catabolism. The apoJ-cytotoxin complex can subsequently be removed from the environment. ApoJ is a ligand for gp330,[48] a member of the low density lipoprotein receptor family of transmembrane proteins, which deliver cholesterol and other lipids to cells and also catabolize protease-antiprotease complexes.[49–51] This postulated function of apoJ in clearing tissue debris predicts that the expression of gp330 is also up-regulated in proximity to injury sites, and studies to test this prediction are underway. If the magnitude of the injury response exceeds the capacity for tissue repair, it is possible that the local concentration of apoJ becomes sufficiently high to cause its self-association, accounting for the immunopositive bands of apoJ at lesion boundaries. In preliminary studies of the behavior of apoJ's surface monolayer properties, we (G. Retzinger and J. A. K. Harmony) have evidence suggestive of a conformational change in apoJ as the distance between molecules is decreased. The apoJ boundary, resembling an apoJ monolayer, may serve as a physical barrier to sequester the cellular breakdown products derived from the lesions. Such a function has been previously proposed for fibrinogen-fibrin.[52]

On the other hand, our results do not provide unequivocal evidence that apoJ's role is protective rather than deleterious. The deposition of other secretory products, including fibrin,[53] can prove harmful as well as beneficial. Enhanced tissue damage by apoJ seems unlikely in view of recent data[44] that indicate that apoJ limits tissue injury in the isolated perfused kidney during passive Heymann nephritis. In this model, kidneys perfused with plasma depleted of apoJ are injured more severely than kidneys perfused with apoJ-containing control plasma, supporting a protective function of apoJ. Additionally, apoJ is constitutively expressed at many tissue-fluid interfaces and is present in virtually all biological fluids and is therefore unlikely to be a harmful molecule.

Our proposed model, in which apoJ serves as a cytoprotective barrier at dynamic biological interfaces does not account for the abundance of apoJ message and protein constitutively expressed in atrial myocytes. Cells of the heart and coronary arterial vasculature, critical to life, are unique in their constant exposure to pressure and flow changes. Understanding the cellular requirements or metabolic responses that are common to myocardial cells, whether they are exposed to a potentially cytotoxic extracellular environment in inflammatory-mediated cardiac lesions or during the rapid growth and remodeling of embryonic cardiac tissue during development, will provide insight into specific apoJ functions and, in addition, into the stresses experienced by these cells during normal cardiac contraction. The results presented here demonstrated a correlation between induced apoJ expression and tissue damage. Although the precise function of apoJ during cardiac disease is unknown, it will now be possible to use transgenic mice that either over- or underexpress cardiac apoJ to define this function.

Our findings have implications for heart disease in humans. With the exception of the TGF-β1-deficient mouse, the disease models used here produce phenotypes in the mouse similar to human disorders. The most common cause of myocarditis in humans is CB3 infection, and mouse and human CB3-induced myocarditis are histologically and immunologically similar.[33,54] In addition, TAC- or PAB-induced hypertrophy can cause ischemic damage, similar to that found in human heart disease caused by aortic stenosis[55,56] and other models of cardiac hypertrophy. It is therefore predictable that ventricular myocyte apoJ expression occurs during cardiac disease in man. We predict that apoJ will serve as a clinical marker of pathological injury of the heart and other tissues, particularly during inflammatory conditions, including graft rejection in organ transplant patients. It is not known how a normal immune response to tissue injury can progress to a self-destructive pathological process of scarring and functional degeneration. The accumulation of apoJ message and protein may be important in this progression. It is therefore critical to identify the signals that regulate apoJ's response to myocarditis to better understand the mechanisms used by myocardial tissue in response to injury.

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