Cardioglobulin

TISSUE LOCALIZATION AND PLASMA ACTIVITY WITH SPECIAL REFERENCE TO CARDIOVASCULAR DISEASE AND LUPUS ERYTHEMATOSUS

By Edward J. Leonard, M.D., T. J. Maximin, M.D., and Stephen Hajdu, M.D.

With the Technical Assistance of Wade Jefferson

ABSTRACT

Two experimental approaches were made to gain insight into the physiological significance of cardioglobulin. The first was to determine whether cardioglobulin was localized to a particular organ or tissue, since earlier studies had suggested that the system might function as a regulator of myocardial contractility. By fluorescence localization of antibodies to rat cardioglobulin-C, cardioglobulin-C was found not only on the surface of heart muscle cells, but in other extracellular locations which, broadly speaking, were either cell surfaces or basement membrane. The second phase of the study was measurement of plasma activity of cardioglobulin-A, -B, and -C in human diseases. The previously discovered increased overall activity in patients with elevated left ventricular systolic pressure was due to increased cardioglobulin-A. Decreased activity in patients with idiopathic myocardial failure was due to decreased cardioglobulin-C. Patients with advanced cirrhosis and evidence of impaired protein production also had low cardioglobulin-C. Low plasma cardioglobulin was found in 22 of 24 patients with systemic lupus erythematosus. Cardioglobulin was normal in all of 17 patients with other connective tissue diseases. The new tissue and clinical findings may provide a clue in the search for the functional significance of this recently discovered protein system.

ADDITIONAL KEY WORDS
plasma proteins idiopathic heart failure hypertension connective tissue diseases immune fluorescence cardiotoxic human

Cardioglobulin is a complex system of proteins that occurs in mammalian plasma and can be identified by its effect on the contractility of isolated frog hearts. The various components which make up the system have been described in an accompanying paper (1) and the cellular basis for the action of these proteins on the amphibian assay tissue has been elucidated (2-4). Despite this accumulated knowledge, the physiological significance of the system has remained unknown. Its action on the frog heart and the changes in its plasma concentration in some patients with cardiovascular disease gave rise to the hypothesis that the system is involved in the regulation of myocardial contractility (5).

Recently we designed a group of experiments to determine whether cardioglobulin was localized specifically on myocardial cell surfaces, in accordance with the hypothesis. We produced an antibody to rat cardioglobulin-C and determined on what tissue sites the antibody became fixed when layered onto sections of various rat organs in vitro. The antibody was found not only on myocardial
cell surfaces but in many additional noncardiac locations that correspond to basement membrane or other intercellular component. This led to a clinical survey of cardioglobulin in a wide variety of diseases which included not only cardiovascular groups but also patients with "connective tissue" disease. The results of both the tissue studies and the clinical assay will be presented in this paper.

Methods

Production of Antibody to Rat Cardioglobulin.—The starting material was serum from 1-year-old germ-free Sprague-Dawley rats. The use of germ-free animals with low gamma globulin and the elimination of fibrinogen by clotting reduced the amount of protein behaving similarly to cardioglobulin-C on gel filtration. Separation of a fraction containing cardioglobulin-C was done on Sephadex columns according to the procedure already outlined (1), except that barium chloride (needed for stabilizing cardioglobulin-A) was not used and both the serum and eluting solution contained 1 mg/ml ATP and ascorbic acid. The fraction was collected, divided into 1-ml aliquots and stored at −78°C. The purpose of this crude fractionation was to reduce the amount of noncardioglobulin protein in the immunizing preparation. Two NIH strain guinea pigs, after being bled for control serum, received seven weekly 1-ml intramuscular injections of a suspension of equal volumes of rat cardioglobulin-C fraction and complete Freund's adjuvant (Difco Laboratories). A week after the last injection the animals were bled for immune serum. Subsequently they were periodically given a booster injection of antigen and bled for additional serum. The criteria for antcardioglobulin activity in the immune guinea pig sera will be presented in the Results.

Detection of Antibody Fixation to Tissues.—This was done by standard immunofluorescence techniques (6). Sections 3 μ thick were cut from quick-frozen sections of exsanguinated female Sprague-Dawley rats, layered on microscope slides, and air dried. The slides were placed in glass racks on a Dubnoff shaker and gently agitated in three 10-minute changes of a solution of 138 mM NaCl containing .01 M phosphate buffer at pH 7.0. This series of washes was intended to remove traces of unbound plasma proteins in the sections. The slides were wiped dry, covered with dilutions of guinea pig antiserum and allowed to equilibrate at 25°C for 30 minutes. They were washed again on the same schedule as the first time, dried, and equilibrated for another 30 minutes with a 1:60 dilution of fluorescein-conjugated guinea pig antiserum.1 The slides then went through a third washing series, and a drop of buffered glycerol (1 part of pH 8.5, 0.15 M phosphate buffer plus 9 parts of glycerol) was put onto each tissue section and a cover slip was placed. The slides were stored at 4°C and were examined under a Leitz fluorescence microscope, usually within several hours. Photographic records were made of almost all the experimental material, using Ansocchrome 200 film and the Leitz automatic camera.

Absorption of Antiserum.—The preparation of frog hearts with cardioglobulin-C bound to the membrane surface has been described in the accompanying paper (1). In summary, if the Ringer's solution perfusing a cannulated frog heart is replaced successively by an approximately 10-fold dilution of human plasma, then a solution of rat cardioglobulin-C, and is finally washed thoroughly with Ringer's solution, the result is a so-called cardioglobulin-B-C heart, which has rat cardioglobulin-C bound to the cell surface by criteria of both biological activity and chemical measurement (4). It was possible to absorb out cardioglobulin-C antibody by equilibrating the antisera in a series of cardioglobulin-B-C hearts. This was done by setting up a series of four to six such hearts stimulated to contract at 20/minute. Into the cannula of the first heart was placed 1.5 ml of a 10-fold dilution of the guinea pig antiserum to rat cardioglobulin-C. After an equilibration of 15 minutes the solution was transferred to the second heart, and so on successively for the series. After the solution was removed from the last heart, it was stored frozen for use on rat tissue sections as described in Results. The frog hearts were washed thoroughly, frozen, sectioned and layered with fluorescein-conjugated guinea pig antisera to test for fixation of antibody to heart. Successive hearts showed fluorescence intensity progressively diminishing to zero, an indication of complete absorption of antibody from the solution.

Human Plasma Samples for Assay.—The clinical assays reported in this paper were done between January and May 1966, according to methods described in the accompanying paper (1, Table 1). Because of the possibility that over a period of time minor variations could occur in the sensitivity of frog hearts or in the potency of stock cardioglobulin standards, the experimental design provided for simultaneous evaluation of all the clinical groups under study. We collected 17 ml of human venous blood from each subject.

---

1Horser origin, produced by Progressive Laboratories, Inc.; distributed by Roboz Surgical Instrument Co., Washington, D. C.
CARDIOGLOBULIN IN DISEASE

529

as described previously (1). No blood was used unless flow was satisfactory from a good venipuncture, and little deviation from the 17-ml volume was allowed in order to keep heparin concentration reasonably constant. When samples were drawn from patients in other hospitals or clinics, portable equipment from our laboratory was used for separation and freezing of plasma.

Results

I. TISSUE LOCALIZATION OF CARDIOGLOBULIN

These studies were done with serum of guinea pigs immunized with a rat cardio-
globulin-C fraction. Antibody in the guinea pig serum was evaluated in three ways: immuno-electrophoresis, inhibition of cardiglobulin activity in the frog bioassay, and antibody binding to cardiglobulin-B-C hearts determined by immunofluorescence.

By immuno-electrophoresis the guinea pig antiserum produced five or six faint precipitin lines with whole rat serum in addition to two

---

TABLE 1

Plasma Cardioglobulin in Normal Subjects and in Patients with Various Diseases

<table>
<thead>
<tr>
<th>Group</th>
<th>No. subjects</th>
<th>Plasma cardioglobulin (units/ml plasma)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>25</td>
<td>12.1 (4.6)</td>
<td>6.3 (4.1)</td>
<td>33.1 (18)</td>
<td></td>
</tr>
<tr>
<td>Hypertension-aortic stenosis</td>
<td>11</td>
<td>20.1* (4.5)</td>
<td>6.7 (3.4)</td>
<td>44.0 (24)</td>
<td></td>
</tr>
<tr>
<td>Heart failure, idiopathic</td>
<td>14</td>
<td>12.2 (3.8)</td>
<td>5.6 (3.0)</td>
<td>19.3 (†)</td>
<td></td>
</tr>
<tr>
<td>Heart failure, valvular</td>
<td>9</td>
<td>13.9 (5.9)</td>
<td>6.2 (3.2)</td>
<td>43.9 (28)</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis, mild to moderate</td>
<td>8</td>
<td>13.8 (5.3)</td>
<td>9.0 (5.4)</td>
<td>35.0 (34)</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis, severe</td>
<td>8</td>
<td>8.7 (5.9)</td>
<td>5.4 (2.1)</td>
<td>7.7* (6.0)</td>
<td></td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>24</td>
<td>5.8* (3.7)</td>
<td>4.5 (3.5)</td>
<td>7.5* (9.6)</td>
<td></td>
</tr>
<tr>
<td>Other connective tissue disease</td>
<td>17</td>
<td>11.5 (4.3)</td>
<td>6.9 (5.5)</td>
<td>29.2 (19)</td>
<td></td>
</tr>
</tbody>
</table>

*Mean (so).
†Six of the 14 subjects had cardioglobulin-C below the lowest value found among the normals. See text.
‡Mean values significantly different by t-test (7) from normal (P < .001).

Circulation Research, Vol. XXII, April 1968
major ones (Fig. 1, control antiserum). As shown later in this section, one of the faint lines represented antibody to cardioglobulin-C.

The effect of the immune serum on the biological activity of cardioglobulin-C was tested on cardioglobulin-B-C hearts that required only the addition of cardioglobulin-A to complete the system. Before the addition of cardioglobulin-A the hearts were equilibrated for 15 minutes with 1 ml of guinea pig antiserum that had been diluted from 10 to 10,000 fold. The guinea pig serum was then removed, the hearts were washed with Ringer's solution, and cardioglobulin-A was added in excess to determine whether the B-C hearts had been affected by the guinea pig serum. The immune serum completely inhibited cardioglobulin activity in dilutions varying from 10 to 1000 fold. Nonimmune guinea pig serum had no effect. Therefore it appeared that the antiserum contained an antibody which combined with and inhibited cardioglobulin-C known by independent chemical evidence to be present on B-C hearts.

Antibody to rat cardioglobulin-C could also be demonstrated on the surface of cardioglobulin-B-C frog hearts by fluorescent staining. Frozen sections were made of B-C hearts, and guinea pig antiserum was layered over the sections. The results of a typical experiment are shown in Figure 2. By bioassay, rat cardioglobulin-C is bound only to the B-C heart (Fig. 2a), and it is only in this case that intense fluorescent staining occurs, indicating the presence of antibody to rat C. Since prior application of cardioglobulin-B is required for binding of rat C, the heart which received rat C alone should not have bound rat C and therefore would not be expected to stain. Thus

**FIGURE 2**

Localization of guinea pig antiserum on frog hearts, (a) B-C hearts; (b) C hearts; (c) B hearts; (d) hearts not equilibrated with any mammalian protein prior to guinea pig antiserum. See text for description. Original magnification 220X. Line in upper right corner is 25μ.
the various controls which showed little or no staining included hearts exposed only to rat C (Fig. 2b), B hearts without rat C (Fig. 2c), hearts exposed to no cardioglobulin fraction (Fig. 2d) and B-C hearts followed by nonimmune guinea pig serum (not shown). Since intense staining occurred in the B-C hearts but in none of the controls, these results confirm the presence of antibody to rat cardioglobulin-C in the guinea pig antiserum.

The next step in the study was a survey of rat tissues to determine where the guinea pig antibody to rat cardioglobulin-C became bound. Intense fluorescence was observed between muscle fibers of rat heart (Fig. 3a) and skeletal muscle (Fig. 3b). Variable fluorescence was seen between smooth muscle fibers. In no instance was fluorescence found within muscle cells. Fluorescence was not confined to muscle. It could be seen outlining Bowman's capsule and the capillaries of the glomerular tuft (Fig. 3c) and vessels of the choroid plexus (Fig. 3d), suggesting a basement membrane distribution. Fluorescence in relation to cell surfaces was also found in kidney tubules (Fig. 4a), secretory cells of stomach (Fig. 4b), thymus, spleen (Fig. 4c), and adrenal medulla (Fig. 4d).

The liver was the only organ to show fluorescence within cells. A diffuse brightness not seen in control sections occurred throughout the cytoplasm.

Fluorescence was by no means characteristic of all extracellular tissue. Although it was striking in choroid plexus and glomerulus, fluorescence in other vessels was neither intense nor constant. Collagen bundles (from rat tail) did not stain, although a fine reticular outlining between the bundles was often seen. Connective tissue of brain showed no
fluorescence, except for occasional bright flecks which might represent small vessels. And although fluorescence in the spleen was extracellular, the pattern was honeycomb, outlining cells rather than reticular fibers. Thus the immune fluorescence studies appear to be identifying a protein which has a widespread extracellular distribution that corresponds to no system or structure hitherto described.

Experiments were performed to determine whether the observed fluorescence represented antibody to cardioglobulin or whether it was antibody to noncardioglobulin plasma protein. Antibody to cardioglobulin-C was removed from the guinea pig antiserum by absorption with a series of cardioglobulin-B-C hearts until fluorescence staining of B-C hearts could no longer be detected (see Methods). By immunoelectrophoresis, the absorbed antiserum showed absence of one precipitin line (Fig. 1, absorbed antiserum). Since the line persisted in frog hearts not exposed to rat protein, it was likely that disappearance of the line was due to specific absorption of antibody to cardioglobulin-C by heart-bound rat C (and incidentally established that rat C moved electrophoretically with the beta-globulins). All of the rat tissue sites that showed fluorescence with guinea pig antiserum had markedly diminished or no fluorescence when tested with absorbed antiserum. Several controls indicated that absorption had eliminated antibody to cardioglobulin-C and only this antibody. Antiserum equilibrated with fresh frog hearts showed no binding of antibody to frog heart (by fluorescence assay) and stained rat tissues comparably to standard guinea pig antiserum. Since it had been shown (4) using 45Ca-labeled cardioglobulin-C that this protein became bound to a B heart but not to a fresh heart, guinea pig antiserum was absorbed with a series of C hearts (i.e. hearts exposed to cardioglobulin-C without B, which after washing would retain no cardioglobulin-C). The antiserum thus treated stained rat tissues comparably to the unabsorbed serum. Thus, absorption of antiserum by hearts known to have bound cardioglobulin-C resulted in disappearance of one antibody precipitin line by immunoelectrophoresis and in disappearance of all tissue-fixing capacity of the serum. Absorption by hearts without bound cardioglobulin-C did not remove tissue-fixing capacity. It is reasonable to conclude, therefore, that the fluorescence pattern observed is due solely to antibody to cardioglobulin-C.

Circulation Research, Vol. XXII, April 1968
II. CARDIOGLOBULIN IN HUMAN DISEASE

Clinical studies based on the assay for cardioglobulin published in 1961 produced two major findings (5). Patients with long-standing hypertension or aortic valvular stenosis, diseases characterized by increased left ventricular systolic pressure, had higher concentrations of plasma cardioglobulin than those of normal controls. Among patients with congestive heart failure due to myocardial disease of unknown cause was a group with plasma cardioglobulin which fell below the range found in normals or in patients with congestive failure secondary to valvular disease. Since the assay employed at that time probably reflected the combined activity of all three cardioglobulin components, the study on these patient groups was repeated with the improved methods by which the separate components of cardioglobulin could be measured.

The results of this re-evaluation are presented in Table 1. Patients in the hypertension-aortic stenosis group were selected according to criteria previously described (5). Cardioglobulin-A is higher than in normal controls. There is a tendency for increased cardioglobulin-C in the group but it is not statistically significant. There are no differences in cardioglobulin-B. Among the various diagnostic categories studied, cardioglobulin-A was increased only in the hypertensive group.

Second, we studied 14 patients with myocardial failure of unknown cause. If cardioglobulin is an important determinant of myocardial contractility, cardioglobulin deficiency might lead to congestive heart failure, and patients with this deficiency would be classified clinically as having myocardial disease of unknown cause. The patients were selected as previously described (5). No significant abnormalities of cardioglobulin-A or B occurred in this group (see Table 1) but among the 14 patients there were 6 whose cardioglobulin was less than the lowest value of 10 units/ml found among the 26 normals. These were 8.3, 7.1, 6.7, 6.7, 5.0 and 5.0 units/ml. The data were suggestive of two populations with respect to cardioglobulin-C activity, as found previously (5). Cardioglobulin-C values in controls with valvular failure were distributed normally with a mean of 43.9 units/ml (Table 1).

Three of the six patients with idiopathic failure and low cardioglobulin-C were alcoholic. However they had no evidence of liver disease, and 14 alcoholic patients without cardiac failure or severe liver disease had no abnormalities of cardioglobulin. Therefore the low values in idiopathic failure were probably not due to alcoholism.

To determine whether impaired protein synthesis might lead to low cardioglobulin, we studied eight patients with far advanced liver disease. All had significant elevation of prothrombin times (21 seconds or more; control, 15) and had one or more serious clinical manifestations such as coma, massive ascites, or cachexia. Table 1 shows a significant depression of cardioglobulin-C in the group. Although congestive failure was not prominent among these patients, it may be significant that of the three patients with the lowest cardioglobulin-C values one developed a ventricular diastolic gallop at bed rest in the hospital, one had congestive failure of unknown cause and one, before admission, had peripheral edema, the cardiac origin of which was not entirely excluded. In contrast to these patients, no abnormal values were found in a group of eight alcoholic patients with mild or moderate liver disease (Table 1).

The apparent distribution of rat cardioglobulin-C in intercellular or basement membrane sites prompted the assay of cardioglobulin in patients with "connective tissue disease." The results with the first few patients with systemic lupus erythematosus were so striking that a large number were collected for study. The results are presented in Table 2, the patients being arranged approximately by guest on April 12, 2017 http://circres.ahajournals.org/ Downloaded from
in the order of increasing cardioglobulin abnormality. Group means for comparison with normals are shown in Table 1. Only two patients out of the 24 had all three cardioglobulin components in the normal range, whereas 17 of 24 had abnormal values of at least two components. It can also be seen that abnormal cardioglobulin can occur in systemic lupus despite negative LE tests and normal serum complement. With one exception (case 24), this group of patients was ambulatory and treated with either steroids or antimalarial drugs.

Equally striking were the results on 17 patients with "connective tissue" disease other than systemic lupus. The diseases represented were: scleroderma, 4; rheumatoid arthritis, 6; Sjögren's syndrome, 2; Reiter's disease, 1; polyarteritis, 1; polymyositis, 2; and sarcoid with joint pain and erythema nodosum, 1. No value of any cardioglobulin component was outside the normal range except for a cardioglobulin-A of 5.0 units/ml in a patient with polymyositis.

It is unlikely that administration of steroids accounted for the low cardioglobulin activity in patients with lupus. Among seven patients without systemic lupus who were receiving LE tests and serum complement measurements, when done on plasma samples drawn within 1 week of collection for cardioglobulin assay, are also given. Of the 10 patients with negative LE tests at the time of cardioglobulin assay, all had positive tests during the previous course of their disease. Serum complement was measured in Dr. Lawrence Shulman's laboratory at Johns Hopkins Hospital. The mean and standard deviation for normals is 38 ± 4. LE tests were performed either in Dr. Shulman's laboratory or in the hematology laboratory at the National Institutes of Health.

*Cardioglobulin values below the lowest figure obtained in normals. The bottom of the range for normals was: A, 7.7 units/ml; B, 2.0 units/ml; C, 10.0 units/ml.
steroids, plasma concentration of all three cardioglobulin components was normal, with the exception of one cardioglobulin-A value of 5 units/ml in a patient with polymyositis. Furthermore, five of the lupus patients with low cardioglobulin (cases 3, 7, 8, 18, 19) were not receiving steroids, whereas the two patients with normal cardioglobulin (cases 1 and 2) were both taking steroids.

Discussion

Before the present investigations were undertaken it was thought that the function of cardioglobulin was regulation of myocardial contractility. This belief was derived from the increase in contractility caused by addition of the system to the isolated frog heart and the increased plasma cardioglobulin activity in hypertension and aortic stenosis and a decreased activity in certain patients with idiopathic myocardial failure. These clinical observations were explained by assuming that the level of activity in the plasma reflects activity at the tissue site where the system is thought to work, that myocardial contractility is enhanced by increased cardioglobulin, that a compensatory increase occurs in conditions characterized by increased development of left ventricular systolic tension, and that a primary pathological decrease in activity could lead to myocardial failure (5).

Implicit in this hypothesis is the idea that the action of cardioglobulin at the tissue site is a simple function of its concentration and therefore plasma concentration would be an indication of tissue activity. An alternative basis for control would be a regulating mechanism at the site of action. In this case, plasma concentration might vary inversely with tissue activity. If utilization at the cell level is so high that its rate cannot be matched at the site of synthesis, the result will be a lowered steady-state plasma concentration. More information is needed to decide whether altered plasma concentrations reflect increased or decreased tissue activity of the system.

Whatever the basis for control of cardioglobulin, impairment of synthesis should be reflected in decreased plasma levels and the results in patients with far advanced cirrhosis could thus be explained. The other conditions with plasma cardioglobulin abnormalities are relatively few in number: high cardioglobulin-A in the hypertension-aortic stenosis group, low cardioglobulin-C in about one-third of patients with idiopathic congestive failure, and multicomponent abnormalities in systemic lupus.

As a result of these studies, the role of cardioglobulin has to be considered in the light of two contrasting sets of facts. On the one hand are the clinical correlations in cardiovascular disease and the action of cardioglobulin on cellular function in the model system, the isolated frog heart. All this is consistent with the original simple hypothesis. On the other hand are the markedly decreased plasma cardioglobulin components in patients with systemic lupus without detectable cardiovascular dysfunction, and also the widespread distribution in the normal rat of cardioglobulin in noncardiac extracellular sites, generally in relation to cell surfaces. These findings do not fit readily into the framework of the original hypothesis and are leading to study of cardioglobulin in systems other than cardiac muscle.

The plasma alterations in systemic lupus could be dismissed as one more incidental abnormality in this disease were it not for two reasons. The first is the widespread tissue distribution of cardioglobulin in sites at least superficially similar to those which may be involved in systemic lupus. The second is that many of the observed serological abnormalities in the disease occur in only a fraction of the patient population (for example,

\( \text{Correlation Research, Vol. XXII, April 1968} \)
positive Coombs' test in 10%; platelet antibodies, 75 to 80%; circulating anticoagulants, infrequently; positive serology, 5 to 15%; rheumatoid factor, 30%; antithyroglobulin, 20% [8]). In contrast to the irregular occurrence of these changes, low activity of at least one cardioglobulin protein was found in 22 out of 24 lupus patients (92%). This is despite the fact that the group as a whole represented an outpatient population with the disease under relatively good control. Furthermore, among patients in the broad category of connective tissue disease, low cardioglobulin was characteristic of only those with lupus. The findings of low concentrations of cardioglobulin in lupus plasma may provide a clue in the search for the functional significance of this newly discovered protein system.

Acknowledgment
For the studies on tissue localization of cardioglobulin we thank Maurice Mufson for generously giving much of his time to instruct us in immunofluorescent techniques, John Fahey and Harriet Cranger for running many immunoelectrophoreses, and Arthur Strauss for use of his fluorescent microscope and automatic camera. We appreciated Leon Sokoloff's instructive review of the tissue section transparencies. The clinical study could not have been done without the enthusiastic cooperation of many physicians. We would especially like to thank Frank Marcus, Rashid Massumi, James Bacos, Gerald Shugoll, Thomas Mattingly, Proctor Harvey, Eugene Braunwald, Glenn Morrow and their staffs and also Janet Holder of the Georgetown myocarditis study for their referrals of patients. George Burch and John Walsh kindly made available their patients with myocardial failure in New Orleans. Our study of patients with systemic lupus and related diseases was greatly helped by John Decker and his staff at NIH and by Lawrence Shulman and Mary Stevens at Johns Hopkins Hospital.

References
Cardioglobulin: Tissue Localization and Plasma Activity With Special Reference to Cardiovascular Disease and Lupus Erythematosus

EDWARD J. LEONARD, T. J. MAXIMIN and STEPHEN HAJDU

Circ Res. 1968;22:527-536
doi: 10.1161/01.RES.22.4.527

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1968 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/22/4/527

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at: http://circres.ahajournals.org/subscriptions/