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Membrane attack complex of complement and 20 kDa homologous restriction factor (CD59) in myocardial infarction

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Abstract In order to investigate the mechanism of deposition of the complement membrane attack complex (MAC) in cardiomyocytes in areas of human myocardial infarction, the 20 kDA homologous restriction factor of complement (HRF20; CD59) and complement components (C1q, C3d and MAC) were analysed immunohistochemically using specific antibodies. Myocardial tissues obtained at autopsy from nine patients who died of acute myocardial infarction were fixed in acetone and embedded in paraffin. The ages of the infarcts ranged from about 3.5 h to 12 days. In cases of myocardial infarction of 20 h or less, MAC deposition was shown in the infarcted cardiomyocytes without loss of HRF20. Where the duration was 4 days or more, the cardiomyocytes with MAC deposition in the infarcted areas also showed complete loss of HRF20. Outside the infarcts, HRF20 in the cardiomyocytes was well preserved without MAC deposition. The present study suggests that the initial MAC deposition in dead cardiomyocytes can occur as a result of degradation of plasma-membrane by a mechanism independent of complement-mediated injury to the membrane. Loss of HRF20 from dead cardiomyocytes may not be the initial cause of MAC deposition, but may accelerate the deposition process of MAC in later stages of infarction.

Key words Myocardial infarction · Complement · Membrane attack complex · Homologous restriction factor of complement · CD59

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Introduction

Myocardial infarction is defined as ischaemic myocardial necrosis due to an inability of the vascular supply to meet the metabolic demand of the tissue [25]. Several studies have demonstrated deposition of the complement membrane attack complex (MAC) on dead cardiomyocytes in areas of myocardial infarction, suggesting an involvement of the complement system in the pathogenesis of myocardial tissue injuries evolving after coronary occlusion [6, 9, 14, 15, 23, 27, 28, 34, 37–39, 41]. However, the exact mechanism of complement deposition on infarcted cardiomyocytes remains to be elucidated.

Recently, our group [36] and Väkevä et al. [37, 38] demonstrated the expression of the 20 kDa homologous restriction factor of complement (HRF20; CD59) [4, 21, 35] on myocardial cells of human hearts immunohistochemically, and indicated its importance in defence against complement-mediated injury to infarcted myocardium. The latter investigators [37, 38] and Zimmermann et al. [42] pointed out that loss of HRF20 and the decay-accelerating factor (DAF; CD55) [2, 18, 33] may play an initiating role in MAC deposition. However, cases of early infarction were few in these studies and they were performed using frozen sections only. Recently Väkevä et al. [39], using a rat analogue of HRF20 [8], has demonstrated that deposition of C9 occured earlier than the loss of HRF20 in the acute myocardial infarction of rats. This report prompted us to analyse more precisely the relationship between loss of HRF20 and deposition of MAC in acute myocardial infarction of human hearts.

In the present study, the sequence of complement-mediated injury in human myocardial infarction was studied in detail by the method using acetone-fixed and paraffin-embedded tissues which has distinct advantages for the preservation of histological architecture [36]. We provide evidence that the deposition of MAC precedes loss of HRF20 in myocardial infarction in human hearts. The pathological significance of this factor in myocardial infarction is re-evaluated in this light.

Materials and methods

Cardiac tissues were obtained at autopsy within 3 h of death from nine individuals (seven males and two females, aged 47–84 years) who died of acute myocardial infarction. The ages of the infarcts, estimated from the first clinical presentation (chest pain), ranged from 3.5 h to 12 days; 3.5 h in patient 1, 4 h in patient 2, 20 h in patient 3, 30 h in patient 4, 4 days in patient 5, 5 days in patient 6, 8 days in patient 7, 11 days in patient 8 and 12 days in patient 9.

Short-axis (transverse) cuts were made through the ventricles one-third to two-fifths of the way down from the apex of the left ventricle to obtain slices of fresh myocardium with a thickness of 4–6 mm. After fixation in acetone and trimming to a suitable size for standard 1- by 3-inch glass slides, the pieces of trimmed tissue were embedded in paraffin following the AMeX method [30] (briefly, tissues were fixed in acetone at 4°C for 3 days, then cleared in methyl benzoate and xylene, consecutively, and embedded in paraffin). In each case, another adjacent slice was fixed in 10% neutral buffered formalin (pH 7.2) and embedded in paraffin by standard procedures.

Haematoxylin and eosin (H&E)-stained sections were made routinely from all tissue blocks. For comparative study and establishment of the precise distributions of infarcted areas, serial sections from acetone-fixed tissues were used for H&E as well as immunohistochemical stainings.

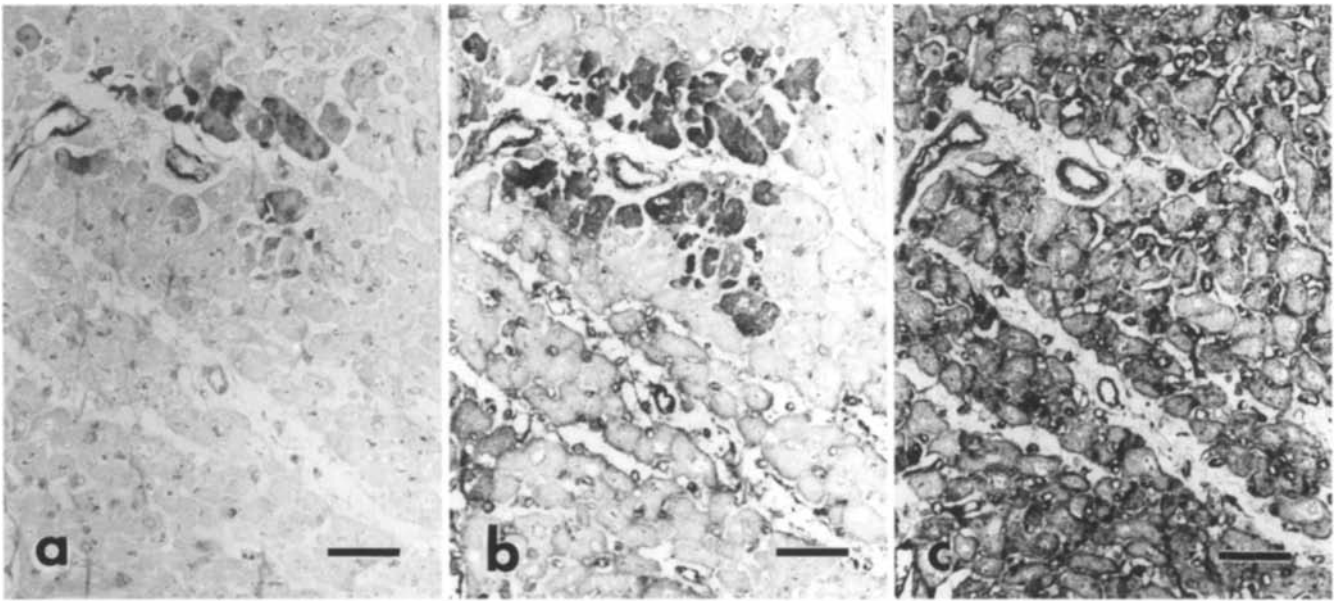
The anti-HRF20 mouse monoclonal antibody, 1F5 (mouse IgG1), was prepared from hybridoma clone 1F5 by the method described previously [20]. The specificity of the antibody has been documented [21]. The anti-DAF mouse monoclonal antibody, 1C6 (mouse IgG1), was obtained from Wako Pure Chemical Industries (Osaka, Japan). For immunohistochemical detection of MAC, anti-human C5b-9 neoantigen mouse monoclonal antibody (aE11, mouse IgG2a) was purchased from Dakopatts (Copenhagen, Denmark). Rabbit antisera to human C1q and C3d, and rabbit antibodies (F(ab′)2) to human IgG (gamma-chains) and IgM (mu-chains) were also obtained from Dakopatts.

Acetone-fixed, paraffin-embedded tissues were employed for immunohistochemical studies. Immunoreactive HRF20, DAF, complement components and immunoglobulins were localized using the avidin-biotin-peroxidase (ABC) method [7]. Biotinylated anti-mouse and -rabbit IgG antibodies and ABC standard kits were obtained from Vector Laboratories (Burlingame, Calif., USA). Paraffin sections were serially cut at 3 μm and deparaffinized with xylene. After washing with phosphate-buffered saline (PBS), the sections for HRF20 were treated with 0.3% (v/v) hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase. In the sections for complement components, this procedure was avoided, and proteolytic digestion was performed: incubation for 10 min at 37°C in 0.02% type III trypsin (Sigma, St. Louis, Mo., USA) solution [40]. Sections for human immunoglobulins were treated to inactivate endogenous peroxidase as well as digested by the proteolytic enzyme. All sections were then immersed in 1:20 dilution of nonimmune goat serum for 20 min to block nonspecific immunoglobulin binding sites in the tissues, and after blotting of excess serum, incubated with the primary antibodies described above for 1 h at room temperature. Control sections were treated with either non-immune mouse immunoglobulins (IgG1, IgG2a) (Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA) or non-immune rabbit immunoglobulins (MBL, Nagoya, Japan). The sections were rinsed and incubated sequentially with secondary antibody (goat biotinylated anti-mouse IgG antibody, or goat biotinylated anti-rabbit IgG antibody) for 30 min, and with avidin-biotin-peroxidase complex for 50 min. After the sections were washed with PBS, the peroxidase reaction was developed by incubation of the sections in 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma) solution containing 0.003% (v/v) hydrogen peroxide and 10 mM sodium azide. Methyl green was used as a counterstain.

Results

In patients 1 and 2 (myocardial infarctions of less than 4 h of duration), the infarcted areas were difficult to identify in H&E stained sections because of the shortness of time between onset of the disease and death. However, small clusters of cardiomyocytes bearing immunoglobulins (IgG, M), C1q, C3d and MAC were distributed focally in the left ventricular wall and interven-

Fig. 1a–c Complements and HRF20 in infarcted myocardium (patient 2, infarction of 4 h). The proteins were localized on adjacent sections. The immunostaining of membrane attack complex (MAC; **a**) was weaker than that of C3d (**b**), and no MAC deposition was found in C3d-negative cells. MAC-immunoreactivity was present on some but not all of the C3d-positive cells. However, HRF20 (**c**) was demonstrated on the surface of these cardiac muscle cells as well as cells with no complement deposition. (**a–c** ×180; *bars*=50 μm)



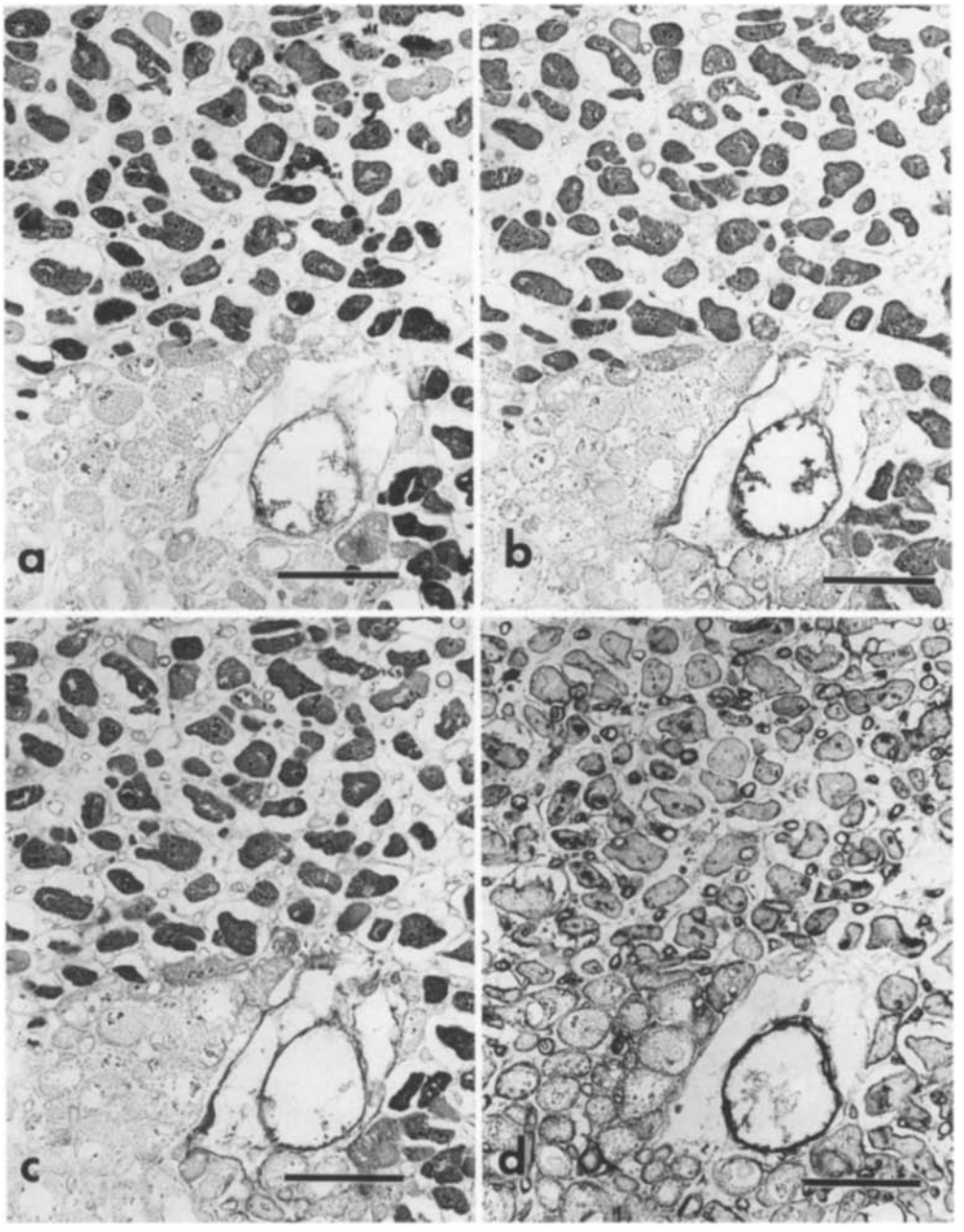


Fig. 2a–d Complement, IgM and HRF20 in infarcted myocardium (patient 3, infarction of 20 h). The proteins were localized on adjacent sections. Note that, although MAC (**a**), C3d (**b**) and IgM (**c**) are distributed within the cardiomyocytes in the same area of myocardial infarction, no loss of HRF20 (**d**) from the surface of these infarcted cardiomyocytes is apparent. (**a–d** ×360; *bars*=50 μm)

tricular septum (Fig. 1a, b). Immunoglobulins, C1q and C3d were found in parallel in the same cells. These proteins were diffusely stained in the cytoplasm, with no linear deposition on the cell membrane. MAC-immunoreactivity was present on some but not all of the C3d-positive cells (also positive for immunoglobulins and C1q). The immunostaining of MAC was much weaker than the immunoglobulins, C1q and C3d, and no MAC-deposition was found in C3d-negative cells (Fig. 1a, b). However, HRF20 was clearly demonstrated on the surface and intercalated disks of these cardiac muscle cells as well as cells with no complement or MAC deposition (Fig. 1c). C3d-positive cardiomyocytes were slightly shrunken, but did not show loss of HRF20 from their cell surface. Besides supposedly injured cardiomyocytes, MAC was strongly positive on the blood vessels, including arteries and veins, and some connective tissues as previously described [31].

In patient 3 (myocardial infarction aged 20 h), H&E-stained sections showed easily recognizable microscopic changes such as oedema, wavy fibres, shrunken eosinophilic cytoplasm, contraction band necrosis and mild infiltration of neutrophils into the lesions. In such areas of infarction, MAC, C3d, C1q and immunoglobulins were intensely stained in the cytoplasm of infarcted cardiomyocytes (Fig. 2a–c). While HRF20 was lacking in some cells, it was preserved in most cells of the infarcted area (Fig. 2d) and was clearly expressed on the surface and intercalated disks of cardiomyocytes in the non-infarcted areas where deposition of immunoglobu-

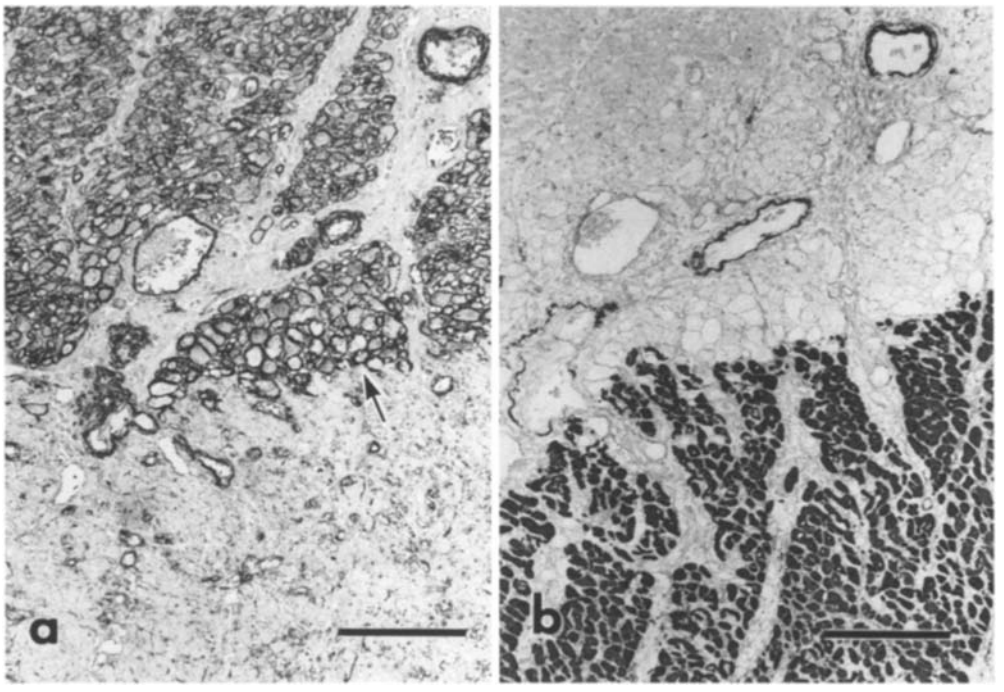


Fig. 3a, b HRF20 and MAC in infarcted myocardium (patient 5, infarction of 4 days of age) localized on adjacent sections. Note complete loss of HRF20 (**a**) from the infarcted cardiomyocytes in the area of myocardial infarction showing intense deposition of MAC (**b**). Cardiomyocytes in the narrow zone adjacent to the infarcted area which are swollen with no deposition of MAC demonstrate stronger expression of HRF20 (*arrow*) than normal cardiomyocytes outside this zone. (**a, b** ×90; *bars*=200 μm)

lins, MAC or other complement components was lacking (Fig. 2a–d).

In patients 5–9 (myocardial infarction aged 4–12 days), typical features of myocardial infarction were evident in the H&E sections. Cardiomyocytes in these lesions showed marked deposition of MAC and complete loss of HRF20 (Fig. 3a, b). MAC in dead cardiomyocytes was strongly stained in the cytoplasm. C1q, C3d and immunoglobulins were also positively stained. In the non-infarcted area where deposition of MAC or other complement components was lacking, HRF20 was clearly expressed (Fig. 3a, b) as in the other cases above. The cardiomyocytes in the narrow zone adjacent to the infarcted area, which were swollen with no deposition of complement components and IgM, demonstrated stronger expression of HRF20 than normal cardiomyocytes outside this zone (Fig. 3a).

DAF was demonstrated moderately to weakly on the endothelial cells of blood vessels in all the specimens, but not on sarcolemmal membranes of the cardiomyocytes in both normal and infarcted areas except for only very weak linear staining on cells in some non-infarcted regions.

Discussion

Complement activation on cell membranes is controlled by membrane inhibitors such as HRF20 [4, 21, 35], DAF [2, 18, 33] and the C8 binding protein [32]. HRF20 is a 20,000 Da glycosyl phosphatidylinositol anchored membrane glycoprotein which interferes with the terminal stage of complement action [4, 17, 21, 35]. Together with other homologous restriction factors, such as DAF which inhibits C3 activation [2, 18, 33], it has been found in various tissues and is considered to play an important role in protecting host cells from MAC-mediated damage [13, 19, 26].

Deposition of complement components including MAC in infarcted areas of the heart has been reported in humans [9, 15, 29, 31, 37, 38, 42] and experimental animals [23, 38], suggesting involvement of the complement system in the pathogenesis of myocardial tissue injuries following coronary occlusion. This is in line with the significant roles well known for complement in the initiation and control of inflammation [5]. Loss of membrane inhibitors of homologous complement activation from the myocardial cells under infarcted conditions has been proposed as a possible explanation for MAC deposition [37, 38]. However, whether the involvement of the membrane inhibitors is essential and at what stage it might act have not been established. The present study provided evidence that such loss of inhibitors is not the initiating step in human myocardial infarction. This is supported by a recent report using rat homologue of HRF20 [8], which demonstrated that deposition of C9 preceded the loss of HRF20 in experimental myocardial infarction of rats [38].

The present demonstration of MAC deposition in infarcted cardiomyocytes before loss of HRF20 indicates that the latter cannot be the primary causal event in human hearts. In the very early stage of infarction C3d and immunoglobulins were stained in the cytoplasm (but not on the surface) more strongly than MAC. If HRF20 loss was responsible, early deposition of MAC would be expected on the plasma membrane, but no such staining was found at any site, suggesting that the process begins within the cells.

The sequence of morphological and biochemical changes following acute hypoxic injury of cardiac muscle has been studied previously [25]. The multiple events caused by ischaemia include loss of selective membrane permeability, detachment of ribosomes, decreased synthesis of protein, accumulation of lipid breakdown products, cytoskeletal changes and resultant cell membrane damage which is irreversible. It has been demonstrated that ischaemic injury to cardiomyocytes increases their permeability, permitting the influx of plasma proteins and efflux of cellular constituents[3, 10, 11, 16]. As a result, initial MAC deposition in dead cardiomyocytes may occur independently of complement-mediated injury to the plasma membrane. Thus, in the very early stages of myocardial infarction, complement could passively penetrate the cell membrane in the same manner as other plasma proteins such as immunoglobulins and fibrinogen, independently of and prior to the cell-surface MAC formation which causes cell-membrane damage. Since the molecular weight of complement is less than 460,000 Da [24], which is smaller than that of IgM, the membrane might not be an effective barrier.

While the initial trigger to activate the complement system in infarcted cells is not yet fully understood, non-immune activation of C1 by direct interaction of C1q with mitochondria may be important for MAC formation [12, 22, 27]. Damage to the mitochondrial membrane which is easily caused by ischaemia, could strongly activate C1, because the cardiolipin rich in this membrane is a potent activator of C1 [12, 27]. Non-immune activators of C1 from sources other than mitochondria have also been described, including components located within cells such as DNA and RNA [1]. Since C1q deposition was actually present in infarcted cells in the present study, activation of the classic pathway leading to MAC formation might have occurred in the cytoplasm. However, the existence of alternate pathways suggests that complement activation in infarcted areas might be more complicated. In a rat model of myocardial infarction, Väkevä et al. [39] suggested complement activation to be initiated by spontaneous activation of C3 and of the alternative pathway[38]. It should also be taken into account that HRF20 synthesis may be downregulated in this process of cell injury and its function compromised even when immunohistochemically present [39].

If the membranes lacking HRF20 in dead cardiomyocytes are an additional site of MAC deposition, the pathological significance of this deposition may be evident at later stages, since complement components such as C3a, C4a and C5a, which are generated during MAC formation, play a significant role in the progression of stromal tissue inflammation in areas of myocardial infarction [5]. It is possible that other defence mechanisms against MAC may actually be more important for cell protection. The immunohistochemical expression of DAF on the surface of normal cardiomyocytes and its loss from the infarcted cardiomyocytes were previously demonstrated using frozen sections [42]. Nevertheless, other authors, also using frozen sections, reported that no or only very weak linear staining of sarcolemmal membranes of the cardiomyocytes of both normal and infarcted myocardial infarction could be detected [38]. In the present study using acetone-fixed and paraffin-embeddded sections, the monoclonal antibody did not work sufficiently well to demonstrate DAF on the cardiomyocytes, even in normal hearts. The issue of the role of DAF remains to be clarified.

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