Complement inhibition reduces injury in the type 2 diabetic heart following ischemia and reperfusion

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Am J Physiol Heart Circ Physiol 294: H1282–H1290, 2008. First published January 4, 2008; doi:10.1152/ajpheart.00843.2007.—Chronic inflammation exacerbates the cardiovascular complications of diabetes. Complement activation plays an important role in the inflammatory response and is known to be involved in ischemia-reperfusion (I/R) injury in the nondiabetic heart. The purpose of this study was to determine if increased complement deposition explains, in part, the increased severity of neutrophil-mediated I/R injury in the type 2 diabetic heart. Nondiabetic Zucker lean control (ZLC) and Zucker diabetic fatty (ZDF) rats underwent 30 min of coronary artery occlusion followed by 120 min of reperfusion. Another group of ZDF rats was treated with the complement inhibitor FUT-175 before reperfusion. Left ventricular (LV) tissue samples were stained for complement deposition and neutrophil accumulation following reperfusion. We found significantly more complement deposition in the ZDF LV compared with the ZLC (P < 0.05), and complement deposition was associated with significantly greater neutrophil accumulation. In whole blood samples taken preischemia and at 120 min reperfusion, neutrophils exhibited significantly more CD11b expression in the ZDF group compared with the ZLC group (P < 0.05). Furthermore, intracellular adhesion molecule (ICAM)-1 expression following I/R was increased significantly in ZDF hearts compared with ZLC hearts (P < 0.001). These results indicate that, in the ZDF heart, increased ICAM-1 and polymorphonuclear neutrophil (PMN) CD11b expression play a role in increasing PMN accumulation following I/R. The infarct size of the ZDF was significantly greater than ZLC (P < 0.05), and treatment with FUT-175 significantly decreased infarct size, complement deposition, and PMN accumulation in the diabetic heart. These findings indicate an exacerbated inflammatory response in the type 2 diabetic heart that contributes to the increased tissue injury observed following ischemia and reperfusion.

myocardial ischemia-reperfusion; FUT-175; inflammation; neutrophil accumulation; CD11b/CD18

ISCHEMIC HEART DISEASE is the leading cause of morbidity and mortality in the United States, and, in 1999, heart disease and stroke afflicted ~17 million people worldwide, accounting for 30% of all deaths (4). Among this population, diabetic patients have significantly more severe and fatal myocardial infarctions than nondiabetic patients. In fact, cardiovascular disease is the leading cause of death in diabetics, accounting for 60–75% of all diabetes-related deaths (4). Current findings indicate a relationship between chronic inflammation and the cardiovascular complications of diabetes (21, 56, 61). Chronic inflammation may contribute to the increased morbidity and mortality associated with cardiovascular heart disease in type 2 diabetic patients.

Restoring blood flow to the ischemic heart is clearly necessary for myocardial salvage. Paradoxically, reperfusion can further tissue damage due to oxidative injury (7, 19). Reactive oxygen species from activated, sequestered neutrophils are a prime source of oxidative injury during reperfusion (16). The neutrophil-mediated inflammatory response occurs early in myocardial reperfusion (43) and begins with neutrophil deposition and accumulation in the coronary microcirculation. Neutrophil activation includes surface expression of the CD11b/CD18 integrin, which aids in neutrophil adherence to the vascular endothelium. Hokama et al. (22) demonstrated increased CD11b expression on neutrophils from streptozotocin (STZ)-induced diabetic animals. Neutrophil activation contributes to endothelial dysfunction, neutrophil aggregation, and vascular obstruction. Neutrophil trapping and sequestration in the microcirculation is known to play a significant role in ischemia-reperfusion (I/R) injury (21, 42). Studies from our laboratory demonstrated that neutrophils rapidly accumulate in greater numbers in the coronary microcirculation of the diabetic heart (21). Thus neutrophil sequestration may be enhanced in the diabetic heart. However, the effects of neutrophil accumulation are not solely confined to mechanical plugging, the so-called “no-reflow” phenomenon. Once sequestered, neutrophils generate oxidants and proteases, exposing the myocardial vasculature and muscle to further injury (33, 34).

Complement activation constitutes another component of the inflammatory response involved in I/R injury. The complement system is part of the innate defense system responsible for the elimination of invading foreign cells and initiation of inflammation. There are three independent pathways that can initiate the complement cascade: the classical, alternative, and more recently described lectin pathway. Complement activation contributes to myocardial ischemic injury in humans (59) and animals (26, 57). Components C3a, C5a, and C5b-9 of the complement system have significant proinflammatory activity. Postmortem examination of human hearts identified deposition of the membrane attack complex (MAC, C5b-9) on damaged muscle fibers (49). Yasojima et al. (59) provided evidence that the mRNAs and proteins for all of the components of the classical complement pathway are expressed in the human
heart and that this expression is upregulated in areas of myocardial infarction.

There is an important relationship between complement and neutrophil accumulation in the setting of I/R injury. Hill and Ward (20) demonstrated that, when treated with reagents that deplete serum complement, rats fail to recruit leukocytes to regions of myocardial infarction. These studies suggest a nonimmunologic role for the complement system in response to acute inflammatory tissue injury. Other studies observed protective effects of complement inhibition independent of neutrophils in ischemic injury (23, 15). FUT-175 strongly inhibits, in an intense, specific, and reversible way, the expression of C1r and C1s of the classical pathway (5) and binds specifically to the Bb fragment of Factor B in the alternative pathway (24). However, the role of the classical and alternative pathways in diabetic ischemic heart disease is not known. Treatment with FUT-175 may elucidate the role of complement in I/R injury in the diabetic heart.

The present study was performed to test the hypothesis that complement deposition is enhanced in the type 2 diabetic heart and is associated with increased infarct size following ischemia. The specific goals of this study were to 1) examine and compare complement deposition; 2) determine if neutrophil accumulation correlates to complement deposition; and 3) examine the relationships between complement deposition, neutrophil accumulation, and infarct size. We found that complement deposition was increased in the Zucker diabetic fatty (ZDF) heart following I/R and that this deposition correlated with increased neutrophil accumulation as well as infarct size. Blocking complement with FUT-175 resulted in significantly decreased complement and neutrophil-mediated myocardial cell death. Our results indicate that complement activation plays a significant role in the severity of myocardial I/R injury in the type 2 diabetic heart.

MATERIALS AND METHODS

Animals. All procedures were reviewed and approved by the Institute for Laboratory Animal Research and were in accordance with the Guide for Care and Use of Laboratory Animals. Male ZDF (fa/–) rats and their aged-matched lean littersmates [Zucker lean control (ZLC) fa/–] were obtained from Charles River GMI Laboratories at 10 wk of age. Housing was under controlled conditions of light (12:12-h light-dark) and temperature (22–24°C). Rats were fed Purina 5008, a 6% fat rodent diet, ad libitum. This model for non-insulin-dependent diabetes mellitus begins to develop hyperglycemia and insulin resistance at ~7 wk of age, and glucose levels typically reach 500 mg/dl by 10–11 wk of age (11). Overt diabetes develops at ~12 wk with elevated insulin levels and elevated blood glucose levels. Myocardial I/R protocol. At 12–16 wk of age, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and placed on a heating pad to maintain normal body temperature. A polyethylene catheter (PE-10) was inserted and secured in the right femoral artery for blood sampling and blood pressure monitoring. The rats were intubated with a PE-200 tube, and a window thoracotomy was performed. After opening the chest wall, rats were ventilated with supplemental oxygen using a small animal respirator (model 683; Harvard Apparatus). Periodic measurements of pH, PO2, and PCO2 were made to ensure adequate ventilation using a Radiometer ABL5 Blood Gas Analyzer. After respiratory stabilization, the ribs were gently spread to expose the left side of the heart and visualize the left anterior descending coronary artery (LAD). A silk suture was placed around the LAD, and the ends of the suture were tightened and clamped to induce ischemia to the left ventricle distal to the ligated artery. Blanching of the myocardial tissue ensured proper ligation of the LAD. After 30 min of ischemia, the ligature was unclamped, and the ischemic myocardium was reperfused for 120 min.

Complement inhibition. FUT-175 (Futhan, nafamostat mesilate; BIOMOL International) is a serine protease inhibitor with potent inhibitory activity against the C1r and C1s subunits of the classical pathway of complement system activation, as well as factors B and D of the alternative pathway (18, 24). FUT-175 was found to prevent complement activation in several in vitro and in vivo models (38, 23, 50). For use, FUT-175 was dissolved in sterile saline and was dosed 5 min before reperfusion (1 mg/kg body wt) via intravenous bolus (50).

Myocardial infarct size determination. Following reperfusion, the LAD was reocluded, and Trypan blue dye was injected to delineate the area at risk (AAR) (10, 26, 57). A 2-mm heart section was taken ~3 mm distal to the suture. This coronal section of myocardial tissue was scanned with a high-resolution computer scanner (model 5370C; Hewlett-Packard). After scanning, the coronal section was placed in a 1.5% triphenyl tetrazolium chloride (TTC) solution and incubated for 30 min at 37°C (57). Following the TTC incubation, the section was placed in a 10% buffered formalin solution for 24 h and rescaned. The AAR was computed by dividing the area unstained by the Trypan by the total left ventricular (LV) area. The infarcted fraction was determined following Formalin incubation by measuring the area of necrosis divided by the total LV area. Infarct size is expressed as the percentage of the AAR (AAR/AAR × 100, where A1 is the area of the infarct).

Flow cytometry. Blood was drawn before ischemia from the femoral arterial catheter, anticoagulated with sodium citrate (Sigma), and diluted with Pharmingen Staining Buffer (BD Biosciences). Diluted whole blood was aliquot into two samples. Both samples were incubated for 25 min with phycoerythrin-Cy5-conjugated anti-CD45 (BD Pharmingen), a pan-leukocyte marker. For detection of activated neutrophils, one sample was treated with an IgG isotype control, and the other was treated with a fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (BD Pharmingen) for detection of activated neutrophils, as previously described (21). Blood samples were fixed with cold 1% paraformaldehyde before analysis. A FACSCaliber flow cytometer (Becton Dickenson) was used to measure the mean channel of fluorescence for CD11b in the CD45-positive neutrophil population. CellQuest software was used for analysis, and the total fluorescence intensity (TFI) was calculated as the product of the values given for the “percent gated” and “geometric mean” of the M2 region (35).

Histology. Following I/R, ZLC and ZDF hearts were excised and cut into 4-mm coronal sections. Sections were embedded and frozen in optimal cutting temperature compound-embedding medium, sectioned at 6 μm using a cryostat, and mounted on slides for histological staining. To examine complement deposition, tissue slides were first incubated in 3% BSA for 30 min at 37°C to reduce nonspecific binding before incubation with a horseradish peroxidase (HRP)-conjugated goat antirat C3 antibody (MP Biomedicals) at 1:500 dilution for 30 min. The C3 antibody was developed with diaminobenzidine (DAB) chromogen for 5 min at room temperature. Sections were then rinsed with PBS, mounted, and coverslipped. To control for nonspecific binding/staining, incubations were done by processing with DAB chromogen alone. Analysis of C3 immunostaining was performed blinded, in duplicate, and data were expressed as the percentage of C3 positive area to total LV area (%C3/LV).

A separate set of slides was stained for neutrophil sequestration using naphthol AS-D chloroacetate esterase (NCE; Sigma; see Ref. 29). Naphthol AS-D chloroacetate is enzymatically hydrolyzed by a specific esterase, liberating a free naphthol compound. Free naphthol then couples with a diazonium compound, forming highly colored (pink to fuschia) deposits at sites of enzyme activity. This enzyme is specific for cells of granulocytic lineage (neutrophils), and activity is weak or absent in monocytes and lymphocytes. Neutrophil counts...
were performed blinded, in duplicate, and data are expressed as the number of neutrophils per five fields at ×40 magnification.

Finally, intracellular adhesion molecule-1 (ICAM-1, CD54) expression was examined in ZLC and ZDF frozen cardiac tissue sections (62). Following 60 min in 3% BSA, slides were incubated in anti-ICAM-1 (BD Pharmingen) overnight at room temperature. The slides were rinsed and developed using an antimouse Ig-HRP detection kit (BD Pharmingen) and counterstained with hematoxylin and eosin Y. Slides were examined using an Olympus IMT2 microscope with a ×20 objective lens and a 1.5 optivar. Digital images were captured using a Hamamatsu ORCA 100 charge-coupled device camera using Simple PCI software (version 5.2; Compix, Sewickley, PA). An analysis macro was created to threshold on the HRP-stained pixels and discarded very small pixel features. The area that fell within the HRP threshold was measured using a precalibrated conversion to change pixels into square micrometers. The camera acquisition settings remained the same for both groups, and 100 nonoverlapping semirandom fields were examined for each group. Controls consisted of slides incubated in the absence of the primary antibody and resulted in no nonspecific endothelial staining.

Statistical analysis. All values are expressed as means ± SE of n independent experiments. Comparisons between nondiabetic and diabetic groups were made using a two-tailed independent t-test. For the CD11b data, a repeated-measures ANOVA was used for comparisons between time points within each experimental group. Differences were considered significant at P ≤ 0.05. SigmaStat 3.0 software (Jandel Scientific) was used for statistical analysis.

RESULTS

Characteristics. Table 1 presents the baseline characteristics of the ZLC and ZDF rats used in this study. There were no significant differences in age, mean arterial blood pressure, or heart rate between the lean controls and the diabetic animals. The ZDF animals were indeed diabetic, with significantly increased body weight compared with the lean controls. The ZDF animals had significantly increased blood glucose concentrations, measured with an AccuCheck Active blood glucose monitor, as well as significantly increased body weight compared with the lean controls.

LV infarct size. Myocardial injury following 30 min ischemia and 120 min reperfusion was assessed by examining the area of the infarct as a percentage of the AAR (%AI/AAR). The AAR did not differ between ZLC, ZDF, or ZDF + FUT rats (52.8 ± 3.7, 45.8 ± 4.9, and 41.4 ± 6.9% AAR/LV, respectively), indicating a comparable degree of ischemic insult between groups. However, infarct size was significantly greater in the ZDF rat hearts compared with their lean controls (ZLC: 27.4 ± 5.6% AI/AAR and ZDF: 56.5 ± 6.4% AI/AAR; P < 0.005; Fig. 1). Treatment with the complement inhibitor FUT-175 significantly decreased infarct size in ZDF rats, indicating a role for complement in the excessive I/R injury (ZDF + FUT: 32.8 ± 4.5% AI/AAR; P < 0.05; Fig. 1). Similar results were observed when the infarct size was evaluated as the percentage of infarcted area to the total LV area. Thus, under similar conditions of ischemia and reperfusion, diabetic rats had significantly greater myocardial injury than their lean controls, and this excessive injury could be attenuated by inhibiting complement with FUT-175.

Myocardial complement deposition. Complement activation and deposition plays a significant role in the manifestation of reperfusion injury (26, 62). To analyze complement activity, we immunohistologically stained LV cardiac tissue sections for complement component C3 (Fig. 2). C3 deposition appeared increased in the type 2 diabetic heart. Diabetic Zucker diabetic fatty (ZDF) hearts (filled bar; n = 8) demonstrated significantly greater infarct sizes compared with the nondiabetic Zucker lean control (ZLC; open bar; n = 9). *P < 0.05. Treatment with FUT-175 decreased infarct size in the ZDF (hatched bar; n = 6). #P < 0.05.

Table 1. Physical and hemodynamic characteristics for ZLC and ZDF animals

<table>
<thead>
<tr>
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<th>ZLC</th>
<th>ZDF</th>
<th>ZDF + FUT-175</th>
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</thead>
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<td>Age, wk</td>
<td>14.1±0.7</td>
<td>13.8±0.6</td>
<td>12.6±0.4</td>
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<td>Weight, g</td>
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<td>355.2±9.9*</td>
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<td>Glucose, mg/dl</td>
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<td>362.8±22.8*</td>
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</tr>
<tr>
<td>HR, beats/min</td>
<td>332.1±8.8</td>
<td>317.3±13.4</td>
<td>316.4±12.9</td>
</tr>
</tbody>
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Values are means ± SE. ZLC, Zucker lean control; ZDF, Zucker diabetic fatty; MAP, mean arterial pressure; HR, heart rate. *P < 0.05.
3.2% C3/LV; ZDF: 41.5 ± 3.4% C3/LV; \( P < 0.05 \). FUT-175 treatment significantly decreased C3 deposition in diabetic rat hearts (23.0 ± 3.9% C3/LV; \( P < 0.005 \); Fig. 2). No C3 deposition was observed in nonischemic heart tissue or in slides stained only with DAB chromogen (data not shown), indicating that these findings were not due to basal complement deposition or endogenous peroxidase generation.

**Neutrophil accumulation.** Neutrophil sequestration and infiltration has been found to play a significant role in reperfusion injury and endothelial dysfunction (21, 31, 33). Studies in nondiabetic animals found that tissue myeloperoxidase activity, a measure of neutrophil accumulation, is decreased following complement inhibition (57). Therefore, we examined the relationship between complement deposition and neutrophil accumulation in the type 2 diabetic heart. NCE histochemical staining was used to assess neutrophil accumulation in the hearts of ZLC and ZDF rats following I/R. We observed significantly greater neutrophil accumulation in the left ventricle of the ZDF compared with the ZLC [97 ± 16 vs. 53 ± 9 polymorphonuclear neutrophils (PMNs)/5 fields, respectively; \( P < 0.05 \)]. Similar results were found in a subset of slides where staining for complement C3 and neutrophils was overlapped. For these slides, neutrophils were counted only in the LV area containing positive C3 staining (ZLC: 45 ± 7 PMNs/5 fields; ZDF: 90 ± 5 PMNs/5 fields; \( P < 0.001 \); Fig. 3). By examining only regions where PMNs and C3 localized, we found a positive relationship with infarct size (ZLC: \( r = 0.75 \), ZDF: \( r = 0.53 \); Fig. 4). FUT-175 treatment resulted in significantly decreased PMN accumulation in the ZDF heart (Fig. 3; 41 ± 9 PMNs/5 fields; \( P < 0.001 \)), indicating that complement inhibition plays a role in PMN sequestration in the heart following I/R.

Differential blood counts were taken preischemia and after 15 min (R15) and 120 min (R120) reperfusion. We found that blood taken preischemia from ZDF rats had significantly elevated granulocyte percentages compared with ZLC blood (ZLC: 18.0 ± 1.5%; ZDF: 30.4 ± 3.0%; ZDF + FUT: 30.4 ± 4.7; \( P < 0.001 \); Fig. 5). There were no differences in granulocyte percentages in whole blood taken from ZLC, ZDF, or ZDF + FUT rats at R15 or R120. The increased granulocyte percentage observed in the preischemic ZDF blood may be due to chronic inflammation resulting from the metabolic abnormalities that characterize type 2 diabetes.

**Circulating neutrophil CD11b.** Upon activation, neutrophils increase their surface expression of the CD11b integrin. Using an FITC-conjugated anti-CD11b antibody, we measured the total FITC-CD11b fluorescence intensity (TFI) in each ZLC and ZDF preischemic whole blood sample, as well as after R15 and R120. We observed a significant increase in neutrophil CD11b expression in preischemic ZDF blood compared with the ZLC (ZLC: 10.1 ± 1.1 TFI; ZDF: 17.9 ± 1.5 TFI; \( P < 0.05 \); Fig. 6), suggesting that, under basal conditions, ZDF neutrophils are chronically activated, indicative of a low-grade inflammation present in these animals. Neutrophils from ZDF blood also significantly increased CD11b expression following 120 min of reperfusion compared with lean controls (ZLC: 65.9 ± 6.8 TFI; ZDF: 114.4 ± 19.0 TFI; \( P < 0.05 \); Fig. 6).

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**Fig. 3.** Leukocyte accumulation is increased in the type 2 diabetic heart. Naphthol AS-D choloroacetate esterase (NCE) staining for polymorphonuclear neutrophils (PMNs)/5 fields at 40X (ZLC: 45 ± 7 PMNs/5 fields; ZDF: 90 ± 5 PMNs/5 fields; \( P < 0.001 \)) compared with the nondiabetic ZLC (open bar; \( n = 7 \)). FUT-175 treatment decreases PMN accumulation in the ZDF heart (hashed bar; \( n = 4 \)). *\( P < 0.05 \), #\( P < 0.05 \).

**Fig. 4.** C3 deposition and PMN accumulation are associated with increased infarct size. PMNs were only counted in regions where C3 components were also present. These values correlated strongly with infarct size measurements (ZLC: \( r = 0.75 \); ZDF: \( r = 0.53 \)).

**Fig. 5.** Granulocyte percentages are increased in type 2 diabetic blood. Differential counts revealed a significant increase in granulocytes from whole blood samples taken preischemia from ZDF (closed circles) and ZDF + FUT (closed triangles) rats compared with ZLC (open circles) rats. *\( P < 0.001 \).
ICAM-1 was attenuated significantly (ZDF + FUT: 1,364 ± 1,029; P < 0.001; Fig. 7). These results suggest that there is enhanced endothelial cell activation following I/R injury in the diabetic heart. The decreased ICAM-1 expression observed following FUT-175 treatment may result from decreased complement-mediated vascular endothelial cell activation during reperfusion.

**DISCUSSION**

Diabetes is now considered a major risk factor for cardiovascular disease. The relative risk of ischemic heart disease in people with type 2 diabetes is more than two times that in the nondiabetic population. The ZDF rat model of type 2 diabetes demonstrates many of the characteristics inherent in human diabetes, including hyperglycemia, dyslipidemia, obesity, impaired wound healing, chronic inflammation, neuropathy, and nephropathy, making it a useful model for the study of I/R injury in type 2 diabetes.

In this study, we found that activation of the complement system is increased in the type 2 diabetic heart following I/R. These findings expand on previous work from our laboratory indicating that neutrophil accumulation is increased in the coronary microcirculation of the diabetic heart following I/R injury (21). Here we found increased complement deposition, infarct size, and neutrophil accumulation in the type 2 diabetic heart following LAD occlusion and reperfusion. Treatment with the complement inhibitor FUT-175 decreased complement deposition, infarct size, and PMN accumulation in the diabetic heart. We found that neutrophil CD11b expression is increased in whole blood from diabetic animals both preischemia and after 120 min of reperfusion injury, revealing a heightened immune response compared with control. Taken together, these results suggest that increased complement activation plays a role in increasing neutrophil-mediated I/R injury in the diabetic heart.

Our results support earlier studies that diabetic rats sustain significantly greater injury following acute myocardial ischemia compared with lean control rats. Previous investigations
demonstrated increased infarct size in the db/db mouse model of diabetes (25), as well as in the ZDF rat (60). Our study suggests that this injury is mediated by enhanced complement deposition and neutrophil accumulation. Because there were no differences in blood pressure or heart rate between the ZLC and ZDF animals, infarct size measurements between these groups following I/R injury cannot be attributed to a reduction in myocardial oxygen demand. In addition, our results parallel clinical observations where human diabetic patients sustain more severe and fatal heart attacks than their nondiabetic peers (1, 40). Type 2 diabetes is associated with chronic inflammation, thus we chose to examine the possible mechanism(s) within the immune response that may be responsible for increasing reperfusion injury in the ZDF model of type 2 diabetes.

**Complement deposition.** Previous studies established that complement deposition occurs in human (49) and animal models of I/R injury (26, 57, 62). Here, we present the finding that, in the setting of type 2 diabetes, there is enhanced complement C3 deposition in ischemic regions of the left ventricle following 120 min of reperfusion. Because of the lytic and proinflammatory consequences of complement system activation, enhanced complement deposition appears to mediate the observed increase in infarct size in ZDF animals.

Ross and Medof (44) found that i3b is an important ligand for CD11b-mediated neutrophil adherence. Additionally, complement component C5a is the principal chemotactic factor for circulating neutrophils and increases neutrophil adhesion to the endothelium by mobilizing internal stores of neutrophil complement receptor-1, CD11b/CD18, and CD11c (55). Sacks et al. (45) demonstrated that C5a is also capable of stimulating neutrophils to produce and release reactive oxygen species, proteolytic enzymes, and leukotrienes that influence platelet and endothelial function, inducing vasoconstriction and platelet aggregation. Indeed, we found a positive correlation between neutrophil accumulation and infarct size, especially in regions of complement deposition (Fig. 4). Following myocardial I/R, type 2 diabetic animals had significantly greater neutrophil accumulation within C3-deposited regions than nondiabetic animals, as indicated by the pronounced rightward shift in the correlation line in Fig. 4. Because FUT-175 treatment decreased PMN accumulation, we speculate that, in the setting of type 2 diabetes, increased complement deposition in response to I/R injury mediates neutrophil accumulation and plays a significant role in the observed increase in infarct size in the diabetic heart.

Reactive oxygen species stimulate gene transcription via nuclear factor-κB and extracellular signal-regulated kinase 1/2 (2), activate complement (13), and induce complement production in the isolated myocardium (54). However, no studies have examined complement activation and gene expression in the setting of a chronic proinflammatory state, one characteristic of type 2 diabetes. The finding that C5a indirectly induces the production of chemokines, cytokines, and other proinflammatory mediators (27, 46) provides yet another link between complement activation, inflammation, and enhanced tissue injury in the setting of I/R. Indeed, Collard et al. (13, 14) and Jordan et al. (26) demonstrated a significant role for complement activation in the setting of I/R injury in the nondiabetic heart and vasculature.

Adipocytes increase C3 production in response to insulin (48), and serum C3 concentration correlates strongly with fasting insulin levels (37). Adding to these findings, plasma C3 appears more closely associated with the diabetic state (or associated abnormalities) than with obesity (58). Engstrom et al. (17) reported a significant association between complement component C3 and diabetes, concluding that the risk of developing type 2 diabetes is related to levels of complement C3. Epidemiological evidence clearly links many of the chronic complications of diabetes to the prolonged and uncontrolled hyperglycemic state of these patients. One consequence of hyperglycemia is that it causes protein glycation and the formation of advanced glycation end-products. Indeed, studies have demonstrated that glycation inactivates the complement regulatory protein CD59, leading to increased MAC deposition and MAC-induced growth factor release (3, 39). However, these studies did not examine cardiovascular risk or disease. Thus, to our knowledge, our study is the first to report that elevated complement deposition is associated with increased tissue injury following I/R in the type 2 diabetic heart.

C3 deposition can result from activation of any of the three independent complement pathways (classical, alternative, or lectin). This study did not attempt to address which pathway was responsible for our finding of increased C3 deposition in the ZDF heart. Buerke et al. (9) described the importance of the classical pathway using C1-esterase inhibitors to attenuate myocardial injury in a feline model of I/R. The lectin pathway has been studied extensively in I/R injury by Stahl and colleagues. They found that the lectin pathway plays a particularly important proinflammatory role in myocardial I/R (26). Our finding that treatment with FUT-175 reduced infarct size in the ZDF heart to a similar size as the ZLC suggests that the classical and alternative pathways mediate the excessive complement and neutrophil-mediated injury observed in the ZDF heart.

**Neutrophil and ICAM-1 activation.** We found that neutrophil accumulation is increased significantly in the left ventricle of ZDF rats compared with nondiabetic rats following 120 min of reperfusion. Neutrophil accumulation in postischemic tissues is known to contribute to myocardial (35, 51), cerebral (52), and renal (41) reperfusion injury. Using intravital fluorescence microscopy, Hokama et al. (21) observed increased neutrophil accumulation during reperfusion in both the coronary capillaries and post-capillary venules in the STZ-induced diabetic heart. Our results indicate that, in the setting of type 2 diabetes, increased neutrophil accumulation is associated with greater infarct size, as well as increased PMN CD11b and ICAM-1 expression. Previous studies from our laboratory found that diabetic neutrophils produce increased amounts of reactive oxygen species and contribute to mechanical plugging in ischemia (22, 35). Our results indicate that inhibition of complement activation effectively decreases neutrophil accumulation in the type 2 diabetic heart, decreasing neutrophil-mediated I/R injury.

Oxidative stress in the type 2 diabetic rat heart may have contributed to our findings of increased complement deposition and neutrophil accumulation. Impairment in nitric oxide (NO)-mediated pathways has been implicated in the pathogenesis of cardiovascular disease in diabetes (53). Bitar and colleagues (8) found a marked reduction in aortic NO bioavailability in a genetic model of type 2 diabetes, resulting from increased...
oxidative stress. They concluded that the development of endothelial dysfunction in the aortic tissue of diabetic rats was linked to an exaggerated production of superoxide anion, resulting in inactivation of NO and impaired NO-dependent vasorelaxation. They also found increased activity of the NADH/NAD(P)H oxidase system and uncoupling of endothelial nitric oxide synthase. In addition to the deleterious effects of decreased NO availability on impaired vasodilatation, Kubes et al. (28) reported that inhibition of NO production resulted in a 15-fold increase in neutrophil adherence to feline mesenteric venules. Lefer et al. (32) reported that the presence of NO reduced neutrophil accumulation in an acute model of canine myocardial I/R injury. Free radicals upregulate complement expression in the isolated rat heart (54). Collard et al. (12) demonstrated that intracellular oxidative stress is necessary for complement activation and deposition on human endothelial cells.

In our study, the increase in sequestered neutrophils may be partly a consequence of differences in granulocyte numbers in diabetic blood compared with nondiabetic blood. We found a significant increase in the granulocyte percentage at baseline in whole blood samples taken from the ZDF compared with the ZLC (Fig. 5). However, there were no differences in granulocyte percentages during reperfusion. FUT-175 treatment did not attenuate the increase in circulating granulocytes throughout reperfusion, suggesting that FUT-175 has little effect on granulocyte recruitment during periods of acute inflammation. Indeed, FUT-175 treatment resulted in no change in PMN CD11b expression. Using flow cytometry, we did not find a difference between the number of CD11b-positive cells between the ZLC, ZDF, or ZDF + FUT groups.

The increase in neutrophil accumulation observed in the diabetic rat hearts following ischemia may also be due, in part, to increased expression of neutrophil-endothelial cell adhesion molecule expression. Neutrophil accumulation depends on CD11b/CD18 receptor adhesion to endothelial cells via ICAM-1 (CD54). CD11b is a β2-integrin constitutively present on the surface on neutrophils but is transformed to an active conformation and quantitatively upregulated upon neutrophil activation (6). Neutrophil β2-integrins play a vital role for the full expression of complement-dependent and oxygen radical-mediated injury of the lung and dermal vasculature (36). These results indicate that not only do diabetic neutrophils have increased CD11b expression preischemia but that this increased expression remains significantly greater than nondiabetic neutrophils during the early hours of reperfusion. These data indicate that diabetic neutrophils are chronically activated under basal conditions and that, following an acute inflammatory insult, such as ischemia, they have the capacity to increase their CD11b expression above and beyond nondiabetic neutrophils. Additionally, we found significantly greater ICAM-1 expression within the diabetic LV following I/R injury. Conflicting studies have reported an increase in ICAM-1 in diabetic retinal microvessels (30), but when the number of microvessels was considered no difference in ICAM-1 in the intestinal microcirculation of diabetic and nondiabetic animals was observed (47). In our study, the combined effect of increased PMN CD11b and ICAM-1 expression is likely responsible for the enhanced neutrophil accumulation that we observed in the diabetic heart and earlier in the diabetic coronary microcirculation (21). These data indicate that the diabetic heart experiences a hyperreactive inflammatory response to I/R injury.

FUT-175 is a synthetic protease-inhibiting agent that is highly effective against the enzyme activities of Clr, Cls, Factor B, and Factor D of the classical and alternative complement activation pathways. Several studies found that FUT-175 strongly inhibits complement-mediated hemolysis (5, 18). In the isolated rabbit heart, supplementing the perfusate with FUT-175 prevented the activation of complement and subsequent decline in cardiac functional parameters (23). This study indicated that the complement system, specifically the MAC, can contribute directly to the induction of myocardial tissue injury, even in the absence of other immunological factors and cellular elements normally present in whole blood. We found that FUT-175 significantly decreased vascular ICAM-1 expression in the diabetic heart, possibly because of decreased formation of the MAC on posts ischemic endothelial cells. In addition, Schwertz et al. (50) reported that FUT-175 treatment decreased creatine kinase release and leukocyte accumulation in a rabbit model of ischemia and reperfusion.

In the present study, we found that complement deposition and neutrophil accumulation are increased in the type 2 diabetic rat heart following I/R injury and that the localization of these immune system components is associated with increased infarct size. Inhibiting complement with FUT-175 decreased complement- and neutrophil-mediated reperfusion injury in the diabetic heart. Thus, complement appears to play an important role in diabetic I/R injury. Elucidating the role of complement in type 2 diabetic I/R injury will aid in the development of improved pharmacological interventions and treatments for ischemic heart disease in this growing patient population.

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