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Pleiotropic Effects of Neutrophils on Myocyte Apoptosis and Left Ventricular Remodeling During Early Volume Overload

Mikhail A Kolpakov^{1,*}, Rachid Seqgat^{1,*}, Khadija Rafiq¹, Hang Xi¹, Kennneth B Margulies², Joseph R Libonati¹, Pamela Powel³, Steven R Houser¹, Louis J Dell'italia³, and Abdelkarim Sabri^{1,4}

¹Cardiovascular Research Center and Department of Physiology, Temple University School of Medicine, PA

²University of Pennsylvania School of Medicine, PA

³University of Alabama at Birmingham, AL

Abstract

Most of the available evidence on the role of neutrophils on pathological cardiac remodeling has been pertained after acute myocardial infarction. However, whether neutrophils directly contribute to the pathogenesis of cardiac remodeling after events other than acute myocardial infarction remains unknown. Here we show that acute eccentric hypertrophy induced by aorto-caval fistula (ACF) in the rats induced an increase in the inflammatory response characterized by activation of the STAT pathway and increased infiltration of neutrophils in the myocardium. This early inflammation was associated with a decrease in interstitial collagen accumulation and an increase in myocyte apoptosis. Neutrophil infiltration blockade attenuated MMP activation, ECM degradation, and myocyte apoptosis induced by ACF at 24hrs and attenuated the development of eccentric hypertrophy induced by ACF at 2- and 3-weeks, suggesting a causal relationship between neutrophils and the ACF-induced cardiac remodeling. In contrast, sustained neutrophil depletion over 4-weeks resulted in adverse cardiac remodeling with further increase in cardiac dilatation and macrophage infiltration, but with no change in myocyte apoptosis level. These data support a functional role for neutrophils in MMP activation, ECM degradation, and myocyte apoptosis during eccentric cardiac hypertrophy and underscore the adverse effects of chronic anti-neutrophil therapy on cardiac remodeling induced by early VO.

Keywords

Neutrophils; volume overload; apoptosis; remodeling

Introduction

Pressure and volume overload (VO) induced an alteration in myocardial structure to accommodate chronic changes in myocardial demand. This cardiac remodeling often becomes

⁴Address correspondence to: Abdelkarim Sabri, Ph.D., Cardiovascular Research Center, Temple University, MRB 801, 3420 N. Broad Street, Philadelphia, PA 19140, Tel. 215-707-4915; Fax. 215-707-5737, sabri@temple.edu.

*Both authors contributed equally to this work.

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maladaptive, with ventricular dilatation and pump dysfunction that both culminate to heart failure (HF).[1] One principal feature characterizing HF is the loss of contractile myocytes that contributes to chamber dilation, fibroblast proliferation, myocardial scarring, and depressed ventricular function.[1,2] The mediators of myocyte death are important topics for study and previous work suggested that inflammation may play a role in pathological cardiac remodeling. [3] Although beneficial at early stages after myocardial injury, inflammatory cells release free radicals and proteolytic enzymes within the myocardium that may contribute to myocyte death, extracellular matrix (ECM) remodeling, and subsequent myocardial dysfunction. Pathophysiologically relevant concentrations of these inflammatory cell products mimic many aspects of pathological remodeling, including left ventricle (LV) dysfunction, activation of fetal gene expression, myocyte hypertrophy, and myocyte apoptosis.[3] However, it is still debated whether inflammation is a cause or a consequence of myocyte loss *in-vivo* and whether myocyte loss due to inflammation can have direct long term consequences on cardiac remodeling and function.

Polymorphonuclear neutrophils are the most abundant leukocytes in the body and play a fundamental role in host defense by phagocytosing invading microorganisms. Based on studies showing that depletion of neutrophils from the circulation reduces myocardial injury after ischemia-reperfusion, neutrophils have been implicated as having a direct role in causing myocardial injury.[4,5] Part of the neutrophil damaging properties is associated with their release of cytotoxic factors such as oxygen free radicals and arachidonic acid metabolites that extend myocardial injury after ischemia-reperfusion.[6-8] However, neutrophils may also produce high levels of proteases in response to inflammatory mediators, including serine proteases, collagenases, and gelatinases.[9] These enzymes are involved in ECM protein degradation and play a crucial role in the alteration of both the geometry and mechanical properties of the myocardium.[9,10]

The functional role of neutrophils in cardiac remodeling has mainly been examined in settings of acute myocardial infarction, models that have been associated with significant neutrophil infiltration and myocyte loss.[4,5] However, the role of neutrophils following cardiac events other than acute myocardial infarction has never been studied. The current study explores the role of neutrophils in response to acute hemodynamic stress of volume overload (VO). We utilized a neutrophil depletion strategy to examine the functional contribution of these cells on myocardial structural and molecular adaptations during early VO. We showed that neutrophil activation regulates MMP activation and ECM degradation, and promotes myocyte apoptosis during early stimulus of VO.

Materials and Methods

Animal Preparation

All animal protocols have been approved by the Institutional Animal Care Committee of Temple University University. Abdominal aorto-caval fistula (ACF) was performed in male Sprague-Dawley rats (250-300g) as previously described.[11] Age-matched sham- and ACF-operated rats were generated for echocardiographic and hemodynamic study at 12-hrs, 24-hrs, 2-days, 5-days, and 4-weeks. After each time, animals were sacrificed and tissues were collected for immunohistochemistry or enzyme activity assays analysis. In a third group of animals, 0.5 mg/kg anti-rat neutrophil (anti-RP-3 monoclonal antibody (mAb), generously provided by Dr. Sendo F, Yamagata University, Japan) or anti-IgG mAbs (Sigma Aldrich) were injected subcutaneously 2-days before the start of the surgery and sham or ACF animals were sacrificed after 24-hrs or 4-weeks. Another subset of rats was injected subcutaneously with anti-RP-3 or anti-IgG mAbs 2-days before the start of the surgery and each 5-days until the animals were sacrificed after 4-weeks.

Histology and Immunohistochemistry

Details of procedures for collagen quantification and immunolabeling of paraffin sections are provided in the Supplementary methods.

Western blotting

Western blotting was performed using standard techniques as described in the Supplemental section.

Assessment of Apoptosis

Details of procedures for assessing apoptosis were described in details in the Supplementary methods.

Statistical Analyses

Data reported are mean $\pm$ SEM. Statistical significance was evaluated using ANOVA post-hoc test. A *P* value less than 0.05 was considered significant.

Results

Morphometric and hemodynamic data (Table 1)

There was no significant difference in body weight (BW) between the control and fistula groups. However, there were small but significant increases in total heart weight (HW)/BW ratio at 2-days after ACF which increased further to 24% at 5-days post-ACF ($p < 0.05$ vs. shams). This increase in HW resulted from an increase in both left and right ventricular weights (data not shown). There was a significant decrease in mean arterial pressure due to ACF at 12-hrs and waned towards shams at 5-days ($p < 0.05$ vs. sham). In response to the acute decrease in mean arterial pressure, mean heart rate increased at 12-hrs after induction of ACF and remained elevated throughout the time course. LV end systolic pressure (LVESP) was similar between sham and ACF animals. In contrast LV end-diastolic pressure (LVEDP) was increased markedly at 2 and 5-days ACF ($p < 0.05$ vs. sham), while no significant differences were noted at 12, and 24-hrs.

In Vivo diastolic and systolic function (Figure 1)

There were no significant changes in LV end-systolic dimension (LVESD) in ACF groups compared to shams (Figure 1A). In contrast, LV end-diastolic dimension (LVEDD) was increased as early as 12-hrs post-ACF and reached significance at 24-hrs post ACF (Figure 1B), while the ratio of LVEDD to wall thickness (LVEDD/wt) was first increased significantly at 24-hrs ($p < 0.05$) and remained high at 2- and 5-days after ACF (Figure 1C). The increase in LVEDD/wt was also driven by a decrease in wall thickness at 24-hrs ($p < 0.05$ vs. sham, data not shown). LV ejection fraction started to increase at 24-hrs after ACF and reach statistical significance at 2 and 5-days after ACF compared to sham groups (Figure 1D).

ACF decreases interstitial collagen

Degradation of myocardial collagen typically results in ventricular dilatation and a decrease in ventricular stiffness and constitutes a key feature of early VO-induced cardiac hypertrophy. [11] We next assessed interstitial collagen content by morphometric analysis of picro-sirius red-stained LV sections. We found a significant decrease in mean collagen volume percent 12-hrs after induction of the ACF ($3.9 \pm 0.2\%$ vs. $1.1 \pm 0.1\%$, $p < 0.001$) that persisted at day 2 ($2.1 \pm 0.1\%$, $p = 0.002$) (Figures 1E, 1F). Collagen volume percent reached a value above the sham control group by 5-days ACF, but this change did not achieve statistical significance compared to shams ($4.7 \pm 0.6\%$, $p = 0.06$).

Early ACF induces neutrophil recruitment into LV myocardium

Although contributing sources of collagenolytic activity in the VO remain to be determined, primary candidates are endogenous matrix metalloproteases (MMPs) released by both cardiac non myocyte and myocyte cells and proteases released by inflammatory cells. To test whether collagen degradation induced after early ACF was associated with the infiltration of neutrophils, the first infiltrating cells during the inflammatory response,[12] we performed immunostaining using antibodies that specifically recognize rat neutrophils (anti-RP-1 mAb). [13] Induction of ACF caused an increase in neutrophil infiltration within the LV myocardium as early as 12-hrs, reaching maximum by 24-hrs and returning to sham's values by 2-days after ACF (Figures 2A and 2B). The same trend of increase in neutrophil infiltration was confirmed using immunostaining with anti-myeloperoxidase and anti-Cat.G antibodies and Leder's staining (Figure 2A).

To further quantify the extent of neutrophil infiltration, we measured the activity of Cat.G, a specific serine protease released by activated neutrophils. Cat.G activity was increased within the LV after 12 and 24-hrs after ACF (Figure 2C). This time course was well correlated with the increased infiltration of neutrophils. Taken together, these data showed that early induction of VO increases neutrophil infiltration and activation in the LV myocardium.

Early ACF activates the STAT pathway

The signal transducer and activator of transcription (STAT) family of proteins are activated in response to various physiological insults and inflammatory cytokines, leading to their dimerization and translocation to the nucleus.[14] We next assessed whether ACF-induced neutrophil infiltration in the LV was associated with an increase in STAT-1 and -3 activation. Figure 3A showed an increase in Y⁷⁰¹-STAT-1 and Y⁷⁰⁵-STAT3 phosphorylation, which is required for their transcriptional activation, by Western blot in response to ACF but with different kinetics. Stat-3 phosphorylation occurred at 12-hrs and remained high 5 days after ACF induction. In contrast, STAT-1 phosphorylation was rapid with maximum phosphorylation observed at 12-24-hrs after ACF. Along with this increase in STAT1 and STAT-3 phosphorylation, we examined p38 MAPK, ERK_{1/2}, and JNK_{1/2} phosphorylation after ACF by immunoblot analysis. These MAPKs have been shown to regulate STAT activity in response to many stimuli.[14] Control animals presented basal phosphorylation of these kinases and induction of ACF led to a transient increase in JNK1, but not JNK2, phosphorylation with maximal response occurred at 12-24 hrs after ACF (Figure 3B). In contrast, phosphorylation of ERK_{1/2} was significantly increased early after ACF and its phosphorylation was sustained until 5 days after ACF. P38 MAP kinase phosphorylation induced by ACF was transient and modest in magnitude compared to sham samples. These data together suggest that ACF differentially regulates the activation of these MAP kinases with transient activation of stress kinases, JNK1 and p38 MAP kinase, pathway and sustained activation of the survival ERK_{1/2} pathway.

Early induction of ACF is associated with cell death by apoptosis

Loss of ECM, increase in inflammatory proteases, and increase in STAT-1 activity, all have been shown to promote myocyte apoptosis.[5,14,15] To evaluate the frequency of myocyte death after early induction of VO, TUNEL staining was performed on LV sections. Significantly more TUNEL-positive myocytes were observed in LV myocardium at 12-, 24-hrs, and 2-days after ACF compared to shams (Figure 4A). The TUNEL-positive cells were identified as cardiomyocytes by anti-tropomyosin immunostaining. The increase in cell apoptosis was further confirmed by measurement of nucleosomal fragments by ELISA (Figures 4B) and caspase-3 activity using caspase-3 fluorogenic substrate (Figure 4C). It is noteworthy that a significant correlation between caspase-3 activity and Cat.G activity was observed during

the early induction of ACF (Figure 4D) implicating a causal relationship between neutrophil activation and myocyte apoptosis.

We next examined molecular mechanisms responsible for ACF-mediated myocyte apoptosis. Relative to sham controls, ACF heart samples showed rapid and transient increases in the levels of pro-apoptotic molecules such as PARP cleavage and Bim compared to sham controls (Figure 4E). This increase was transient and the maximum accumulation was observed at 12-24-hrs after ACF. Interestingly, ACF also induced an increase in anti-apoptotic pathways as evidenced by an increase in Bcl2 expression and a decrease in Ser¹¹²-Bad phosphorylation. The activation of these anti-apoptotic pathways was slow in kinetics and was sustained until 5-d after ACF (Figure 4E). Thus, ACF induced significant and transient increases in pro-apoptotic signaling molecules, whereas the activation of survival signaling molecules was slow in kinetic and sustained overtime.

Neutrophil depletion prevents ECM degradation and myocyte apoptosis induced after acute ACF

To evaluate the contribution of neutrophils in the early changes following ACF, neutrophils were depleted by administration of anti-RP-3 monoclonal antibodies (mAbs). Sham or ACF animals received anti-RP-3 mAbs (0.5 mg/kg) two days prior to surgery. In every experiment, control animals received equivalent amounts of anti-IgG isotype mAbs. As described previously,[16] we observed a selective depletion of circulating neutrophils between 24- and 96-hrs (~85%) after a single administration of anti-RP-3 mAbs, with rebound neutrophilia observed at 120-hrs (data not shown). Concomitant with this depletion of circulating neutrophils, neutrophil infiltration in the LV myocardium and the subsequent increase in Cat.G activity were markedly prevented in 1-day ACF compared to ACF animals treated with IgG (Figures 5A and 5B). No significant effects on neutrophil infiltration and Cat.G activity were observed in LV myocardium from shams treated with anti-RP-3 mAbs. Interestingly, neutrophil depletion was sufficient to prevent collagen loss induced by ACF at 24-hrs. Figure 5C shows decreased collagen volume percent in ACF animals treated with IgG for 24-hrs and neutrophil depletion significantly abrogated this loss of collagen suggesting that neutrophils play a role in ECM processing.

Given the role played by neutrophils in MMP activation,[9] we next assessed whether neutrophil recruitment to the LV was required for ACF-induced MMP activation. In-gel zymography showed an increase in MMP-2 activity at 24-hrs after ACF compared to shams (Figure 5D). Neutrophil depletion completely prevented MMP-2 activation induced by ACF, without having a significant effect on basal MMP-2 activity in treated shams. We also measured general MMP activity using fluorogenic substrate as another independent method to measure MMP activity and found that ACF-induced increase in total MMP activity was prevented after neutrophil depletion (Figure 6E). These data provide evidence that neutrophil infiltration contributes to MMP activation during early induction of ACF.

We next examined whether myocardial neutrophil infiltration was functionally linked to myocyte apoptosis. Though neutrophil depletion had no effect on basal myocyte apoptosis in shams, it completely prevented ACF-induced increases in caspase-3 activation, TUNEL positive myocytes, and DNA fragmentation (Figures 5F-5H). Neutrophil depletion also attenuated PARP cleavage, STAT-1/3, and MAP kinases activation induced by 1d ACF (Supplemental Figure S1). These data together indicate the role of infiltrating neutrophils on downstream signaling induced by early hemodynamic stress of VO that lead to ECM degradation and myocyte apoptosis.

Effects of transient and sustained neutrophil depletion on cardiac remodeling induced by ACF

We next sought to investigate whether neutrophil depletion would affect long term myocardial remodeling after induction of ACF for 4-weeks. Examination of cardiac dimensions by echocardiography at 4-weeks post ACF showed no differences between shams injected with Ig G or anti-RP-3 mAbs (Figure 6 and data not shown). However, the induction of ACF for 4-weeks increased cardiac hypertrophy as assessed by HW/BW ratio (Table 2). ACF also increased cardiac dilatation as determined by an increase in LVEDD, LVESD, and LVEDD/wt ratio (Figures 6A-6C). Interestingly, despite these structural changes, cardiac function as assessed by LV ejection fraction was slightly elevated in 4-weeks ACF compared to shams (Figure 6D). Neutrophil infiltration, as determined by anti-RP-1 immunostaining, and Cat.G activity in the LV were similar between sham and 4-weeks ACF (data not shown). The single injection of anti-RP-3 mAbs (which induced neutropenia for 96-hrs) significantly attenuated the increase in LVEDD, LVESD, and LVEDD/wall thickness ratio induced by ACF at 1- and 2-weeks. However, this beneficial effect of neutrophil depletion was lost over time and LVEDD, LVESD, and LVEDD/wt ratio reached ACF operated values at 4-weeks after surgery (Figures 6A-6C). Concomitant with LV remodeling, HW/BW ratio was not significantly different between ACF controls and ACF animals treated with anti-RP-3 mAbs at 4-weeks (Table 2). These data suggest that the pathophysiological consequences of neutrophil infiltration are either resumed after few days following ACF or that the *de novo* infiltration of neutrophils masked the beneficial effect of transient neutrophil depletion therapy on long term cardiac remodeling induced by ACF. To test this hypothesis, ACF operated animals were injected with anti-RP-3 mAbs 2-days prior to surgery followed by repeated antibody injection each 5 days for 4-weeks. Following this protocol, we maintained low levels of circulating neutrophils during the 4-week period of the experiment. Interestingly, prolonged neutrophil depletion for 4-weeks actually exacerbated ACF-induced cardiac hypertrophy (+29% in HW/BW vs. 4-weeks ACF, $P<0.05$) and the LV dilatation (+10% in LVEDD; +14% in LVESD; +15% in LVEDD/wall thickness ratio vs. 4-weeks ACF, $P<0.05$). These data together show that while transient depletion of neutrophils delayed LV hypertrophy and dilatation induced by ACF, continuous neutrophil depletion resulted in adverse ACF-induced LV hypertrophy and dilatation.

To further characterize the phenotype of ACF rats at 4-weeks, histological and mRNA analyses of hypertrophic markers were performed. Microscopic analysis of picro-sirius red-stained histological sections revealed a decrease in LV collagen volume present in 4-weeks ACF compared to shams (Supplemental Figure S2). However, neither transient nor prolonged neutrophil depletion therapy significantly affected ACF-induced collagen loss at 4-weeks. Consistent with these data, ACF increased both MMP-2 expression and activity at 4-weeks compared to shams and neutrophil depletion did not significantly affect this MMP-2 activation induced by ACF (Supplemental Figure S3). We also examined cardiac atrial natriuretic factor (ANF), α -skeletal actin (α -SK actin), and β -myosin heavy chain (MHC) mRNA levels by RT-PCR as markers of cardiac hypertrophy. We found that cardiac ANF and α -SK actin, but not β -MHC, mRNA levels were significantly increased in 4-weeks ACF heart samples, indicating that the molecular program for cardiac hypertrophy was initiated (Supplemental Figure S4). Neither single nor repeated injections of anti-neutrophil antibodies significantly affected ANF and α -SK actin expression induced by 4-weeks ACF. It is noteworthy that ACF induction for 4-weeks was not associated with myocyte apoptosis as determined by TUNEL staining and caspase-3 activity assay and administration of either a single or multiple injections of anti-RP3 antibodies have no effect on the level of myocyte apoptosis (Supplemental Figure S5).

We next assessed whether adverse cardiac remodeling observed in ACF animals treated with prolonged neutrophil depletion was related to changes in the inflammatory response. Therefore

we measured macrophage accumulation by immunostaining using an antibody directed against monocyte/macrophage antigen CD68. ACF significantly increased CD68-positive macrophages accumulation in the LV myocardium at 4 weeks compared to shams (Figures 6E and 6F). Transient neutrophil depletion attenuated ACF-induced CD-68 positive cell infiltration. In contrast, prolonged neutrophil depletion significantly enhanced these cells accumulation compared to 4-weeks ACF rats. This accumulation of CD68-positive macrophages was characteristic for these cells as accumulation of CD11a-positive cells, a surface glycoprotein found in the majority of leukocytes, did not significantly change in 4-weeks ACF hearts or after transient or prolonged neutrophil depletion (Figure 6G). Interestingly, the trend in CD-68 positive macrophage infiltration after ACF was also observed for monocyte chemoattractant protein-1 (MCP-1) expression, a chemokine involved in monocyte recruitment and migration,[17] (Figure 6H). ACF induced a significant increase in MCP-1 mRNA expression compared to shams that was attenuated by transient neutrophil depletion and exacerbated by prolonged depletion of neutrophils. However, this latter increase did not reach statistical significance. We also found that ACF induced expression of inflammatory cytokines interleukin (IL)-1 β , IL-6, granulocyte colony-stimulating factor (G-CSF), and tumor necrosis factor (TNF)- α mRNA compared to shams. Transient neutrophil depletion significantly attenuated IL-1 β , G-CSF, and TNF- α expression induced by 4-weeks ACF and has no effect on IL-6 expression. In contrast, prolonged neutrophil depletion significantly exacerbated the increase in IL-1 β expression and the decrease in G-CSF expression induced by 4-weeks ACF. No effect of prolonged neutrophil depletion on TNF- α or IL-6 expression induced by 4 weeks ACF was observed. These data together showed that prolonged neutrophil depletion enhances infiltration of macrophages in the LV and exacerbates the expression of cytokines and chemokines involved in adverse cardiac remodeling, IL-1 β [18] and MCP-1[17], respectively, along with attenuation of a cytokine involved in cardioprotection, G-CSF.[19]

Discussion

The current study provides new insights into the mechanisms of myocyte apoptosis during early eccentric remodeling in response to hemodynamic stress of VO. We found that induction of ACF leads to an early inflammatory response characterized by activation of STAT-1 and STAT-3 along with increased infiltration of neutrophils in the LV myocardium. Moreover, we found that transient blockade of neutrophil infiltration was sufficient to attenuate MMP activation/collagen degradation, myocyte apoptosis, and early cardiac dilatation induced by acute ACF. Interestingly, sustained blockade of neutrophil infiltration resulted in adverse cardiac remodeling characterized by exacerbation of macrophage infiltration. These novel findings showed the pleiotropic effects associated with neutrophil infiltration during early LV remodeling in a pure VO of ACF.

Early geometrical changes in LV chamber and cardiomyocyte remodeling with ACF are associated with an acute inflammatory response characterized by an increase in neutrophil infiltration along with an increase in neutrophil protease activity. Moreover, we found that cardiac Cat.G activation, MMP activation, and ECM degradation are less evident in neutrophil depleted rats with early ACF, which supports the contribution of neutrophils to early VO-induced MMP activation and ECM degradation. Several studies showed increased infiltration of neutrophils in human failing myocardium and after cardiac injury in animal models of HF. [4,5] However, in all these studies infiltration of neutrophils was pronounced and was associated with a marked increase in cardiac myocyte loss and ECM accumulation. This is in stark contrast with acute ACF-induced VO, where neutrophil infiltration and myocyte loss were relatively modest and ECM degradation was rather observed. While our study did not delineate the mechanisms by which neutrophil activation affected ECM and cardiac remodeling after ACF, previous studies highlighted the capacity of neutrophils to produce high levels of

collagenases (i.e. MMP-8, neutrophil collagenase), gelatinases (i.e. MMP-9, gelatinase B), and serine proteases (i.e. Cat.G, elastase) in response to inflammatory mediators that can process ECM thereby promoting cardiac remodeling.[20,21] Moreover, neutrophils also release free radicals during different models of cardiac diseases, in which they are involved in pathological cardiac remodeling.[6] Thus neutrophil activation may regulate several downstream targets to modulate ECM and cardiac remodeling.

One interesting finding in this study is that acute VO was associated with an increase in myocyte apoptosis as evidenced by an increase in TUNEL positive myocytes, DNA fragmentation, and caspase-3 activity. Again this increase occurred early (12-48-hrs) and the rate of myocyte apoptosis returned toward sham values by 5-days after ACF. It is noteworthy that the level of myocyte apoptosis observed in ACF hearts was modest and occurred throughout the LV myocardium. Similar levels of myocyte loss have been shown to promote cardiac failure with appearance of dilated cardiomyopathy.[22] In addition, these low levels of myocyte apoptosis are significantly more deleterious to ventricular function when they occur dispersed throughout the myocardium, as in certain cardiomyopathies, than when they are restricted to a specific segment, as occurs in a myocardial infarction.[23] Considering apoptosis as a progressive event, accumulation of cell loss over time in a non proliferating organ like the heart may result in adverse consequences. Consistent with its effect on myocyte apoptosis, acute ACF also induced a transient increase in cleaved PARP and Bim expression, two known pro-apoptotic molecules.[24] However, ACF also increased the expression and activation of anti-apoptotic molecules Bcl2 and Bad, respectively, suggesting that complex regulations between pro- and anti-apoptotic pathways govern cardiomyocyte cell death in response to acute ACF. Interestingly, we found that myocyte apoptosis was completely absent in neutrophil depleted animals at 24-hrs after ACF suggesting that neutrophils either directly or through release of cytotoxic factors lead to myocyte apoptosis. Neutrophils have been shown to promote myocyte apoptosis in-vitro and in various cardiac diseases.[25] This seems to be mediated through neutrophil-endothelial cell interaction and rapid production of reactive oxygen species.[26, 27] However, emigrated neutrophils also release serine proteases that lead to MMP activation and ECM degradation therefore leading to cardiomyocyte apoptosis.[28] The fact that neutrophil depletion prevented both MMP activation and ECM degradation induced by ACF and that the kinetics of ECM degradation coincide with that of myocyte apoptosis after ACF strongly suggests that one of the earliest events leading to myocyte apoptosis after ACF may involve a loss of survival signaling emanating from ECM receptors, integrins. Further studies are needed to delineate the role of ECM/integrin signaling pathways in neutrophil response during acute VO.

The elucidation of the molecular signaling pathways associated with ACF showed early activation of the STAT pathway. The activation of this pathway has been shown to occur in response to inflammatory cytokines therefore providing additional evidence that inflammatory pathways were activated in response to acute ACF. We found that STAT-1 and STAT-3 phosphorylation was increased in response to ACF but with different kinetics. STAT-1 phosphorylation induced by ACF was transient and correlated with the transient increase in myocyte apoptosis. In contrast, STAT-3 phosphorylation was slow and sustained overtime. Knowing the opposing effects of STAT-1 and -3 activation in modulating cardiomyocyte growth in response to inflammatory cytokines, it is tempting to speculate that the early activation of STAT-1 induced by ACF may be involved in myocyte apoptosis, whereas the sustained activation of STAT-3 may play a role in myocyte hypertrophy and cardiac remodeling. These findings are concomitant with the role of STAT-1 activation in reducing the basal activity of anti-apoptotic gene promoters, Bcl2 and Bcl-x, and the implication of STAT-3 activation in myocyte protection and hypertrophy.[14] Associated with these changes in the STAT-1 and -3 phosphorylation induced by ACF, we investigated potential targets within the MAP kinases pathway. We found a rapid and transient increase in JNK-1 phosphorylation

in ACF hearts compared to sham that correlated with myocyte apoptosis. In contrast, activation of ERK_{1/2} and p38 MAP kinase was modest and sustained overtime. While the role of these MAP kinases in myocyte growth is still debated as both pro- and anti-apoptotic effects were associated with their activation,[29] it is generally admitted that JNK activation mediate myocyte apoptosis while activation of ERK_{1/2} leads to myocyte growth and survival.[29] Collectively, these data emphasize that ACF activates both pro- and anti-apoptotic signaling pathways with rapid and transient activation of pro-apoptotic pathways, STAT-1 and JNK-1, that correlated with myocyte apoptosis.

In addition to the acute alterations in ECM remodeling and myocyte growth, we showed in this study that early neutrophil infiltration have long term cardiac remodeling and functional consequences. ACF induction for 4-weeks produced time-dependent cardiac hypertrophy characterized by an increase in LV chamber dimension and a decrease in LV wall thickness. However, LV ejection fraction was slightly higher compared to sham group, suggesting compensated cardiac function. It is of interest that acute neutrophil depletion exhibited reduced LV dilatation and increased LV wall thickness at 1-, 2-, and 3-weeks after ACF that returned towards untreated ACF values by 4-weeks, suggesting that the early appearance of eccentric cardiac hypertrophy induced by ACF may be initiated by neutrophil infiltration. These observations extend previous studies showing that administration of either a mast cell stabilizer or a broad-spectrum MMP inhibitor reduced adverse cardiac remodeling in response to VO. [30,31] Surprisingly, repetitive neutrophil depletion over 4-weeks after ACF led to further increases in cardiac hypertrophy and LV dilatation compared with ACF alone. This enhanced increase in cardiac dilatation did not significantly affect LV ejection fraction, ECM accumulation, myocyte apoptosis, or ANF and α -SK actin gene expression compared to ACF untreated group. However, this represents an early phase in the ACF and increases in LVEDD (Figure 6A) and, in particular, LVESD (Figure 6B) would suggest a poor prognosis for prolonged treatment. Interestingly, we found that prolonged anti-neutrophil therapy enhanced the expression of MCP-1, the main chemotactic factor for the migration of monocytes/macrophages,[17] along with an exacerbation in macrophage accumulation in the LV compared to 4-weeks ACF animals. Although the role of MCP-1 in mediating myocyte apoptosis and pathological cardiac remodeling have been documented,[17] the role of macrophages in cardiac remodeling is complex as both beneficial (early phase) and detrimental (late phase) outcomes have been linked to macrophage activation/secretion.[32] Along with macrophage accumulation, prolonged neutrophil depletion also enhanced the expression of IL-1 β , a cytokine known to induce myocyte apoptosis and play a role in adverse cardiac remodeling,[18] while decreasing the expression of G-CSF, a cytokine that plays a role in cardioprotection.[19] However, how prolonged neutrophil depletion modulates macrophage accumulation and cytokine/chemokine expression and how these inflammatory mediators affect cardiac remodeling in response to ACF needs further investigation.

Limitations

The effects of transient and continuous depletion of neutrophils on cardiac remodeling were studied only at 4-weeks after ACF, a time where cardiac function is well compensated. It would be of interest to determine whether this transient treatment is beneficial to animals undergoing HF (approximately at 21-weeks after ACF).[11] However, we believe that other factors may influence the course of cardiac remodeling long-term as the hemodynamic stress emanating from VO persists. Thus, as we move from compensatory cardiac hypertrophy to HF phase in response to ACF, differential stirring changes in the factors involved in cardiac remodeling would occur.

In conclusion, the study showed that early neutrophil infiltration is likely to exert a direct effect on MMP activation/ECM degradation and myocyte apoptosis thereby triggering LV

remodeling in response to acute VO. The study also emphasizes the need to better control the activity of neutrophils for effective cardiac remodeling that may constitute a reasonable therapeutic target for treatment of VO-induced cardiac dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Echocardiographic and histological analysis of rats following ACF

(A-D) LV remodeling in animals subjected to ACF compared with age-matched shams. LV end-systolic dimension (LVESD) (A), LV end diastolic dimension (LVEDD) (B), LVEDD/wall thickness ratio (C), and LV ejection fraction (D). (E) Representative picrosirius red staining (original magnification, $\times 40$) was used to quantitate collagen in sham and ACF groups. (F) Interstitial collagen accumulation as determined by morphometric analysis of picrosirius red stained LV sections. Values are mean $\pm$ SEM. $^* = p < 0.05$ vs. sham

B **C**

Figure 2. Acute ACF increases neutrophil infiltration and activation in the LV myocardium (A) LV sections from sham (a, c, e, g) and rat subjected to ACF for 1-day (b, d, f, h) were immunolabeled with anti-rat neutrophil RP-1 (a, b), anti-myeloperoxidase (MPO) (c, d), or anti-Cat.G antibodies (e, f), or stained with chloroacetate esterase (Leder) stain (g, h) to localize neutrophils. Bar=50µm. (B) Number of RP-1 positive cells in the LV myocardium of shams and rats subjected to ACF. (C) LV homogenates from sham or ACF rats were assessed for Cat.G activity assay using specific fluorogenic substrate. Results are expressed as relative fluorescence unit (RFU)/min/mg protein. Values are mean ±SEM. * $P<0.05$ vs. sham.

Figure 3.

Acute ACF induces differential activation of STAT-1/3 and MAP kinases. Representative immunoblots showing accumulation of Y⁷⁰¹-STAT1 and Y⁷⁰⁵-STAT3 (A) or phospho-p38 MAPK, -ERK_{1/2}, and -JNK (B) in LV extracts from sham and ACF operated rats. Blots were stripped and blotted with anti-STAT-1 or STAT-3 antibodies (A) or with anti-p38 MAPK, -ERK_{1/2}, or -JNK antibodies (B). Top, Representative autoradiogram (with each lane from a single gel exposed for the same duration). Bottom, Fold induction (n=6 each group). * *P*<0.05, ACF vs. shams.

C **D**

Figure 4. Acute ACF induces left ventricular myocyte apoptosis

(A) *Up*, LV tissue sections from sham or 1-day ACF were assessed for apoptosis using TUNEL assay (green), tropomyosin (red), and DAPI (4',6-diamidino-2-phenylindole) (blue) staining. Bar=40μm. *Bottom*, The number of TUNEL-positive myocytes was expressed as a percentage of total nuclei detected by DAPI staining. (B-C) LV homogenates from shams or animals subjected to ACF were assayed for DNA fragmentation assay using anti-histone antibody ELISA (B) or caspase-3 activity using specific fluorogenic substrate (C). Results are expressed as relative OD₄₁₀-OD₅₀₀/mg protein (B) or as relative fluorescence unit (RFU)/min/mg protein (C). Values are mean ±SEM. **P*<0.05; ***P*<0.01 vs. sham. (D) Correlation of caspase-3 activity and Cat.G activity. Pearson correlation *r*=0.66, *P*<0.001. (E) Representative

immunoblot of PARP cleavage, Bim, Bcl-2, Ser¹¹²-Bad, and Bad levels in sham and ACF operated animals. Actin was used as a loading control.

A **B**

C **D**

Figure 5. Neutrophil depletion prevents ACF-induced MMP activation, collagen loss, and myocyte apoptosis

Animals were injected with either anti-IgG or anti-RP-3 mAbs (0.5 mg/kg) 48-hrs prior to ACF induction for 1-day and compared to shams. (A) Quantification of RP-1 positive cells in LV sections as assessed by immunohistochemistry using anti-RP1 mAb. (B) Cat.G activity was assayed using fluorogenic substrate. (C) Interstitial collagen accumulation as determined by morphometric analysis of picrosirius red stained LV sections. (D-F) LV homogenates from sham or ACF animals treated with anti-IgG or anti-RP-3 mAbs were assayed for MMP-2 activity using gelatin zymography (D), total MMP activity (E), or caspase-3 activity (F) using fluorogenic substrates specific for MMP or caspase-3, respectively. Results are expressed as

relative fluorescence unit (RFU)/min/mg protein. (G) Number of TUNEL-positive myocytes expressed as a percentage of total nuclei detected by DAPI staining. (H) DNA fragmentation as assessed by anti-histone antibody ELISA. Results are expressed as relative OD₄₁₀-OD₅₀₀/mg protein. Values are mean $\pm$ SEM. * P <0.05 vs. sham+IgG, † P <0.05 vs. ACF+IgG.

E

F **G**

Figure 6. Effect of transient versus sustained neutrophil depletion on cardiac remodeling and hypertrophy induced by ACF

(A-D) Indices of LV remodeling in sham+IgG, ACF+IgG, ACF+1xRP-3 (ACF rats injected one time with anti-RP-3 mAb), and ACF+6xRP-3 (ACF rats injected six times with anti-RP-3 mAb) for 4-weeks. LVEDD (A), LVESD (B), LVEDD/wall thickness ratio (C), and LV ejection fraction (D). (E) LV sections were immunolabeled with anti-rat CD-68 to localize macrophages and counterstained with hematoxylin. Bar=50 μ m. (F-G) Number of CD-68 (F) or CD-11a (G) positive cells in the LV myocardium of shams and rats subjected to ACF treated with IgG or anti-RP-3 antibodies. (H) Relative expression of mRNA levels of MCP-1, IL-6, IL-1 β , G-CSF, and TNF- α in different groups. The value of each hybridized probe was normalized to that of β -actin in each template set as an internal control and expressed as the ratio to sham+IgG. Values are mean $\pm$ S.E.M. ($n=8$ for sham, $n=5$ for each experimental group). * $P<0.05$ vs. sham+IgG, and # $P<0.01$ vs. ACF+IgG.

Table 1
Hemodynamic measurements in age matched sham and ACF.

	6-hrs		12-hrs		24-hrs		2-d		5-d	
	Sham (n=8)	ACF (n=8)	Sham (n=7)	ACF (n=8)	Sham (n=7)	ACF (n=8)	Sham (n=6)	ACF (n=8)	Sham (n=7)	ACF (n=8)
BW (g)	291±10	302±5	271±6	267±4	281±11	290±12	282±16	291±6	284±4	281±4
HW/BW (mg/g)	3.3±0.06	3.3±0.04	3.4±0.08	3.5±0.04	3.3±0.06	3.4±0.08	3.3±0.04	3.7±0.05*	3.4±0.09	4.2±0.11*
MAP (mm Hg)	103±6	85±3*	90±5	70±2*	83±3	77±4	76±2	71±2	85±3	74±2
Heart Rate (bpm)	285±12	336±14*	285±15	348±11*	260±14	320±10*	259±6	296±7*	240±10	289±5*
LVEDP (mm Hg)	5±2	7±2	2±1	5±2	3±2	6±1	2±1	6±1*	2±1	7±2*
LVESP (mm Hg)	58±3	72±4	62±2	61±2	64±3	60±2	66±3	56±3	58±2	71±5

Values are mean ±SEM. HW, heart weight; BW, body weight; MAP, mean arterial pressure; LVEDP, LV end-diastolic pressure; LVESP, LV end-systolic pressure.

* $P < 0.05$ vs. age-matched sham.

Table 2

LV hemodynamics in 4-weeks sham and ACF treated with anti-IgG or anti-RP-3 antibodies.

	4W-Sham+IgG (n=6)	4W-ACF+IgG (n=6)	4W-ACF+1xRP3 (n=6)	4W-ACF+ 6xRP3 (n=6)
Heart rate (bpm)	280±8	282±5	277±16	283±13
MAP (mmHg)	113±10	98±10	105±6	111±9
HW (g)	1.1±0.09	1.76±0.08 [*]	1.7±0.09 [*]	1.94±0.09 ^{*†}
BW (g)	415±9	373±6	361±5	367±5
HW/BW (mg/g)	2.8±0.01	4.7±0.02 [*]	4.9±0.04 [*]	5.5±0.03 ^{*†}

Values are mean ±SEM. MAP, mean arterial pressure; HW, heart weight; BW, body weight.

^{*} $P < 0.05$ vs. 4W-sham+IgG;[†] $P < 0.05$ vs. 4W-ACF+IgG