Pathophysiology of right ventricular heart disease: the role of structure, apoptosis and inflammation
Campian, M.E.

Citation for published version (APA):
Campian, M. E. (2012). Pathophysiology of right ventricular heart disease: the role of structure, apoptosis and inflammation
CHAPTER V

Early inflammatory response during development of right ventricular heart failure in rat

Maria E. Campian; Maxim Hardziyenka; Kora de Bruin; Berthe L.F. van Eck-Smit; Jacques M.T. de Bakker; Hein J. Verberne; H. L. Tan


“*The function of wisdom is to discriminate between good and evil*”

George Bernard Shaw
Abstract

Introduction: Inflammatory activation plays an important role in the pathogenesis and progression of left ventricular (LV) heart failure. In right ventricular (RV) heart failure development and progression, the role of inflammatory activation is little known. We aimed to study the role of inflammatory activation in RV heart failure by serial monitoring during disease progression.

Methods: RV heart failure following pulmonary hypertension was induced by monocrotaline (MCT) injection in rats. Two groups were studied: MCT-treated rats (MCT-rats), and age-matched controls (CON-rats). Serial echocardiography and in vivo 67-Gallium (67Ga) scintigraphy were performed. Local inflammation of RV was studied by (1) ex vivo semi-quantitative 67Ga-autoradiography, (2) immunohistochemistry of myeloperoxidase (MPO), a marker of neutrophiles activity, and (3) mRNA assays of tumor necrosis factor alpha (TNF-α).

Results: In MCT-rats, 67Ga-scintigraphy showed increased myocardial uptake which started during early RV disease stages. 67Ga autoradiography revealed that this increased 67Ga-uptake occurred in RV and interventricular septum, but not in LV. The stage-dependent increases of in vivo 67Ga RV myocardial uptake were paralleled by increases in mRNA gene expression for TNF-α in RV, and increased MPO staining in RV.

Conclusion: RV heart failure development and progression is associated with an early increase in RV inflammation. 67Ga-scintigraphy may be used for the serial assessment of inflammation and monitoring of disease progression in RV heart failure.
Introduction

Inflammatory activation plays an important role in the pathogenesis and progression of left ventricular (LV) heart failure [1,2]. Patients with LV heart failure have increased levels of circulating inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-1β, and various chemokines, e.g., monocyte chemoattractant peptide (MCP)-1 and macrophage inflammatory protein (MIP)-1α [3-5]. Furthermore, levels of activated circulatory cytokines correlate directly with the severity of LV disease [4,6,7]. Accordingly, studies in experimental models and preliminary clinical experience have suggested a possible therapeutic role for cytokine inhibition in heart failure [8-10]. However, specific TNF-α blockade in a trial of patients with stable heart failure from various causes did not yield the expected benefit [11]. To better understand the role of both systemic and local inflammatory responses in mediating ventricular dysfunction, further research is needed.

In contrast to LV heart failure, the role of inflammatory activation in right ventricular (RV) heart failure development and progression is little known. The current understanding of the molecular mechanisms of RV heart failure is small in comparison to that of LV. However, understanding how RV heart failure differs from LV heart failure might be essential for patient management. So far, only a limited number of studies have been performed to determine the role of inflammation in RV heart failure [12,13]. Therefore, the aim of this study was to determine the association between inflammatory activation and RV heart failure progression in the monocrotaline (MCT) rat model.
Methods

Study design

The study was performed according to the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996). In total forty-four male 8-weeks old Wistar rats, weighing 225-285g at the beginning of the experiment, were studied. RV disease was induced with a single intraperitoneal injection (60mg/kg body weight) of MCT (MCT-rats). MCT injection leads to severe pulmonary disease in the absence of intrinsic heart and lung disease. The ensuing pulmonary hypertension results in RV hypertrophy and RV failure. As a control group (CON-rats), we used age-matched rats that received an intraperitoneal saline injection. Previously, we found that RV disease progression in this model follows a specific time course composed of subsequent RV disease stages: RV hypertrophy, RV dilation and RV failure. We used serial echocardiography to identify the RV hypertrophy and RV dilation stages, and clinical assessment (presence of body weight loss, cyanotic ears, cold limbs, and dyspnea) to identify the RV failure stage (Table 1).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RV hypertrophy</th>
<th>RV dilation</th>
<th>RV failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days after MCT-injection</td>
<td>age-matched</td>
<td>19.6±2</td>
<td>23.5±0.6</td>
<td>38.3±5</td>
</tr>
<tr>
<td>RVFWT (mm)</td>
<td>0.62±0.03</td>
<td>0.96±0.04</td>
<td>1.2±0.02</td>
<td>0.9±0.03</td>
</tr>
<tr>
<td>RVEDD (mm)</td>
<td>4±0.2</td>
<td>4.4±0.4</td>
<td>6.4±0.7</td>
<td>6.8±0.9</td>
</tr>
<tr>
<td>Ascites</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Echocardiographic and postmortem findings. RV, right ventricle; RVFWT, right ventricle free wall thickness; RVEDD, right ventricle end-diastolic diameter. Data are expressed as mean±SEM.
Cardiac function and *in vivo* inflammation was assessed by serial echocardiographic measurements and *in vivo* $^{67}$Gallium ($^{67}$Ga) scintigraphy, respectively, in 10 rats of each group (MCT and CON). To establish whether the signal, which was obtained during *in vivo* $^{67}$Ga-scintigraphy, arose from RV or LV, we harvested the hearts of the MCT-rats at the completion of the time course study, i.e., when RV failure had occurred. In these hearts, we conducted *ex vivo* $^{67}$Ga autoradiography. Confirmation that the signal obtained by $^{67}$Ga-scintigraphy reflected inflammation was obtained by immunohistochemistry for the detection of myeloperoxidase (MPO) activity, and assessment of mRNA gene expression of tumor necrosis factor alpha (TNF-α). These studies were performed in the hearts of 12 MCT-rats (n=4 sacrificed during the RV hypertrophy; n=4 with RV dilation; and n=4 with RV failure) and 12 CON-rats.

**Echocardiography**

Transthoracic two-dimensional, M-mode and Doppler echocardiography was performed in accordance with the standards of the American Society of Echocardiography, using a 10MHz transducer in anesthetized, spontaneously breathing, rats (3% isoflurane). RV free wall thickness (RVFWT) was measured in the two-dimensional short-axis parasternal view below the tricuspid valve or in the long-axis parasternal view by M-mode, depending on the quality of visualization. RV end-diastolic diameter (RVEDD) was measured as the maximal distance from the RV free wall to the interventricular septum from the apical four-chamber view. Each parameter was averaged over 3 cardiac cycles. As reported before15, RV hypertrophy was defined by RVFWT>0.7mm (22.2±1.7days after MCT injection in MCT-rats), RV dilation by RVEDD>4.5mm (27.0±1.7 days in MCT-rats).
48 hours after injection of 40MBq $^{67}$Ga-citrate in a lateral tail vein, anterior planar scintigraphy was performed with a dedicated single pinhole system designed for SPECT [16]. Acquisition in the left anterior oblique position was not feasible due to overprojection of nonspecific bone marrow uptake. Rats were immobilized in a perspex cylinder, mounted on a stepping-motor driven system, and positioned above the up-facing pinhole collimator of the $\gamma$-camera. This enables anterior scintigraphy at a standardized orientation and distance from the pinhole aperture. Acquisitions were performed under the same anesthesia as used during echocardiography. We used a tungsten insert with a 3mm pinhole aperture. SPECT images could not reliably be reconstructed due to the limited $^{67}$Ga myocardial uptake, especially in controls and early stages of RV failure. SPECT was therefore not part of the current analysis. The $\gamma$-camera was connected to a HERMES acquisition and processing station (HERMES Medical Solutions). Static images of the thorax were obtained for 20min with a 15% energy window at the 93 and 185keV $^{67}$Ga-photon peaks in a 128x128 matrix. One standardized region of interest (ROI) was drawn over the myocardium (specific activity) and two over extra-thoracic soft tissue in the axilla (nonspecific activity). As there were no differences in the results for the two axillary ROIs, the data were combined to produce aggregate axillary uptake data for further analysis. Myocardial uptake ratio was calculated as the ratio of specific to nonspecific uptake (expressed as mean counts/pixel) as follows: (myocardium-nonspecific)/nonspecific. The images were analyzed blinded to the disease stage of the rats.

Tracer autoradiography
Tracer autoradiography using phosphor imaging was conducted in hearts which were quickly frozen after excision, and sliced into 50μm short axis slices. Every fifth slice was mounted on a glass plate and covered with a Saran wrap to prevent contamination of the phosphor plate. The short axis slices were exposed to a Fuji BAS-MS imaging plate for ~24h. The images were scanned at 50μm resolution with a 16-bit pixel depth using a Fuji FLA-3000 phosphor imager and analyzed using AIDA image software (Version 3.20.007). For $^{67}$Ga, this technique has a strong linear relation between activity and photo-stimulated luminescence, and a high resolution of 0.30±0.03mm, expressed as full width half-maximum [17].

Real-time quantitative RT-PCR

We extracted total RNA from the hearts using RNA NucleoSpin kit as described by the manufacturer's protocol with the aim of measuring mRNA levels of TNF-α. cDNA was synthesized on 2μg mRNA with an RNA PCR kit (Reverse Transcriptase Invitrogen) using the oligo-dT primer. Reaction mixtures were incubated for 30 min at 42°C, 5min at 99°C, and 5min at 5°C. SYBR Green quantitative PCR assays were performed using the MX3000P Multiplex Quantitative PCR System (Stratagene) and Brilliant SYBR Green QPCR Master Mix kit (Stratagene). Primer sequences used to amplify various cDNAs were: forward=gctcacaaggctgctgaag, reverse=gacagcctggtcaccaaat. A typical PCR protocol was performed under the following conditions: 10min at 95°C, followed by 50 three-temperature cycles (95°C denaturation for 30s, annealing temperature for 1min, and 72°C extension for 1min). The specificity of the SYBR Green assays was confirmed by melting point analysis and gel electrophoresis in the presence of ethidium bromide. Gene expression of the housekeeping gene GAPDH was used for normalization.
Immunohistochemistry

Tissue sections (7μm thick) were cut from formalin-fixed, paraffin-embedded hearts. Sections were deparaffinized and rehydrated by passage through a graded series of ethanol and distilled water. For MPO immunohistochemistry, the antigen was retrieved by heating the slides in a pressure cooker in Tris-buffered saline with 0.075% Tween-20 (pH 7.6) for 10 min. Endogenous peroxidase activity was quenched by incubation in 0.3% v/v H₂O₂ in methanol for 20 min at room temperature. Sections were incubated with primary antibody polyclonal rabbit anti-human MPO antibody diluted 1:500 at 25°C for 1 hour followed by a secondary antibody (Envision kit 1 hour at 25°C). Hematoxilin-eosin was used as counterstaining. Six paraffin sections (7μm thickness) from each tissue block were obtained at 210μm distances in the coronal plane. In each section, 5 random microscopic fields from RV were analyzed.

The number of MPO-positive infiltrates (inflammatory infiltrates index) was determined in 10 microscopic fields (light microscopy, 20 X magnifications) from RV and LV of each animal. We studied 12 rats in each study group, i.e., 4 rats at each of the 3 stages of RV disease progression (RV hypertrophy, RV dilation, and RV failure). The reader was blinded to the disease stages.

Statistical analysis

All data are expressed as mean±SEM. Means between the various groups were compared for differences with analysis of variance (ANOVA). In case of multiple comparisons, a post-hoc Bonferroni correction was applied (SPSS for Windows 15.1, SPSS Inc.). A p-value <0.05 was considered to indicate a statistically significant difference.
Results

*Serial in-vivo detection of inflammation with 67Ga-scintigraphy*

Figure 1A shows $^{67}$Ga uptake in a MCT-rat in the RV failure stage (right) and an age-matched CON-rat (left). MCT-rats exhibited a significant increase in $^{67}$Ga uptake during RV disease development (Figure 1B, squares). $^{67}$Ga uptake increased at early disease stages, becoming already significantly higher than baseline levels at the RV hypertrophy stage. Thereafter, $^{67}$Ga-uptake continued to rise and reached a maximum at the RV dilation stage. Although $^{67}$Ga-uptake slightly declined towards the end of the study (not statistically significantly different from the RV dilation stage), it remained elevated compared to baseline. In CON-rats, $^{67}$Ga-uptake did not change throughout the study (Figure 1B, circles).

*Ex vivo quantitative $^{67}$Ga autoradiography*

To establish whether the increased myocardial $^{67}$Ga-uptake, found during *in vivo* $^{67}$Ga-scintigraphy, occurred in RV, LV or inter-ventricular septum, we conducted *ex vivo* semi-quantitative $^{67}$Ga autoradiography in the hearts of MCT-rats in the RV failure stage. We found that $^{67}$Ga-uptake was increased in RV and inter-ventricular septum, but not in LV (Figure 2). In CON-rats, the autoradiography signals in RV and inter-ventricular septum did not differ from those of LV.
**Figura 1**

**A.** Representative planar pinhole $^{67}$Ga-scintigrams in a MCT-rat in the right ventricle (RV) dilation stage (right panel) and an age-matched control rat (left panel). Regions of interest were drawn around the myocardium (red line) and in the axillary region (yellow, blue) to determine the myocardial uptake ratio. The MCT-rat shows increased $^{67}$Ga myocardial uptake.

**B.** Myocardial uptake ratios at various times after MCT injection, n=10 per group. P<0.05 from day 21 (RV hypertrophy) until day 35 in MCT-rats (squares) vs. age-matched control rats (circles). Data are expresses as mean±SEM. * p=0.004 vs. age-matched control; # p<0.001 vs. MCT-rat on day 14; ^ p=0.002 vs. MCT-rat on day 21.

**Gene expression profiles**

Myocardial TNF-α expression levels in RV of MCT-rats were significantly higher compared to CON-rats (where they were undetectable) at all disease stages (p<0.001, Figure 3). The expression levels of myocardial TNF-α during the development of the different disease stages showed a significant increase from RV hypertrophy to RV dilation and RV failure. However, there was no significant difference in TNF-α myocardial expression between
RV dilation and RV failure. We did not detect TNF-α transcript activity in LV tissue preparations.

**Figure 2**

**67Ga autoradiography:** A. Representative short axis myocardial slices from a monocrotaline (MCT)-injected rat in the RV failure stage (right) and an age-matched control (CON) rat (left). There is increased signal intensity in right ventricle (RV) and interventricular septum (IVS) compared to left ventricle (LV). B. Photo-stimulated luminescence (PSL) for RV, IVS, and LV (PSL/mm²). PSL values are corrected for background. n=10 in CON-rats and MCT-rats each. Data are expressed as mean±SEM. RV MCT vs. RV CON, p<0.001; LV MCT vs. LV CON, P=0.08; IVS MCT vs. IVS CON, p=0.002
**Figure 3**

*Time course of TNF-α gene expression during RV disease progression.* Expression of mRNA TNF-α in the right ventricle (RV) at the RV hypertrophy, RV dilation, and RV failure stages. Myocardial TNF-α mRNA expression was undetectable in hearts of age-matched control rats (CON). Data are expressed as mean±SEM.

*Immunohistochemistry*

To obtain histological confirmation that $^{67}$Ga-uptake reflected inflammation, we performed immunohistochemistry analysis to detect neutrophiles activity using MPO staining.

Figure 4A (arrows) shows MPO-positive infiltrates in MCT-rats at different disease stages. We observed disease stage-dependent changes in inflammatory infiltrates index, which started at the RV hypertrophy stage, and continued into the RV failure stage (Figure 4B). We did not detect inflammatory infiltrates in the LV of MCT-rats nor in age-matched CON-rats.
**Figure 4**

A. Representative examples of neutrophile activity expressed as myeloperoxidase antibody activity (arrow) in the right ventricle (RV) at the RV hypertrophy, RV dilation, and RV failure stages (20X magnification).

B. Inflammatory infiltrates index during RV disease progression, n=4 per disease stage in each group. Data are mean±SEM.

**Time course of myeloperoxidase activity during RV disease progression.** A. Representative examples of neutrophile activity expressed as myeloperoxidase antibody activity (arrow) in the right ventricle (RV) at the RV hypertrophy, RV dilation, and RV failure stages (20X magnification). B. Inflammatory infiltrates index during RV disease progression, n=4 per disease stage in each group. Data are mean±SEM.
Discussion

Our study provides evidence of an association between inflammatory activation and RV disease progression. The inflammatory activation exhibited a particular time course, becoming elevated at an early disease stage (RV hypertrophy), peaking at the stage of RV dilation, and remaining elevated compared to baseline throughout disease progression to RV failure. The inflammatory response was non-invasively assessed with $^{67}$Ga-scintigraphy and reflected local inflammation in RV, as confirmed by $^{67}$Ga autoradiography, immunohistochemistry, and gene expression profiles.

Inflammation and heart failure

Studies into the presence of an inflammatory response in patients with heart failure began more than four decades ago, when elevated C-reactive protein levels were found in the serum of patients with chronic heart failure and the severity of disease was correlated with high levels of C-reactive protein [18]. Levine et al. [19] observed elevated levels of circulating TNF-α in patient with heart failure. A “cytokine hypothesis” was since proposed as a basic mechanism in heart failure [20]. This hypothesis states that a systemic activation of pro-inflammatory cytokines is triggered by a cardiac event (e.g., myocardial infarction) and is associated with deleterious effects on LV function; this, in turn, accelerates the progression of heart failure. However, the results of the RENEWAL study failed to show a clinically relevant benefit of the specific TNF-α antagonist etanercept on the rate of death or hospitalization due to chronic heart failure [11]. Although one interpretation of those results is that specific anti-inflammatory mediators are not viable targets in heart failure, the prevailing point of view is that targeting a single component of the inflammatory cascade is not sufficient to counteract a disease as complex as heart failure. Whether broader-spectrum anti-
inflammatory strategies (e.g., statins, immunoadsorption, or immune modulation therapy) will have any added value in heart failure is currently being addressed in ongoing clinical trials.

Despite the wealth of data on the role of inflammation in LV failure, little is known on the role of inflammation in RV heart failure. So far, it has been demonstrated that acute RV heart failure following pulmonary embolism is associated with up-regulated chemokine expression and infiltration of both neutrophiles and monocyte/macrophages. Furthermore, treatment with anti-MPO antibody reduced RV MPO activity and prevented RV dysfunction [12,13]. In accordance with these studies [13], our results suggest the presence of early neutrophile activation in RV that might contribute to the development of the disease. Our results also demonstrate that noninvasive serial monitoring of myocardial inflammation during RV disease progression is feasible and therefore has potential clinical relevance.

**RV failure: inflammation and apoptosis**

With the use of the MCT-rat model, we previously demonstrated that development of RV heart failure is associated with cardiomyocyte death and collagen deposition [21]. Inflammation and cell death are mechanisms which are activated early during RV disease progression, while fibrosis occurs at later stages. When novel therapeutic strategies are considered, this might imply that combined inhibition of inflammation and apoptosis at early stages of RV disease must be targeted. However, these questions are subject of ongoing investigation.

**Serial non-invasive assessment of disease progression with $^{67}$Ga-scintigraphy**

In general, $^{67}$Ga is handled as ferric iron and thus it is bound to transferrin (and concentrates) in areas of inflammation, such as an infection site and
areas of rapid cell division. $^{67}$Ga binds to transferrin, leukocyte lactoferrin, bacterial siderophores, inflammatory proteins, and cell membranes in neutrophiles. This allows sites with tumor, inflammation, and both acute and chronic infection to be visualized with scintigraphic techniques [22-25]. $^{67}$Ga-scintigraphy for the detection of inflammation has been reported in cases of cardiac mycotic aneurysm [26], infective endocarditis [27], and infective complications of prosthetic valve surgery [28]. Controversial data were reported in cases of detection of inflammatory foci using $^{67}$Ga-scintigraphy in myocarditis [29]. Although $^{67}$Ga-scintigraphy has a high sensitivity, a major disadvantage of $^{67}$Ga-scintigraphy is its limited specificity.

In recent years, $^{18}$F-fluorodeoxyglucose positron emission tomography ($^{18}$F-FDG PET) has emerged as an alternative and superior method to assess inflammation. However, for the myocardial, one of the preferred substrate of metabolism is glucose. Therefore, although $^{67}$Ga-scintigraphy is limited by its limited specificity, the technique is most likely to be superior to $^{18}$F-FDG for the detection of myocardial inflammation. Thus, it is anticipated that the ability to detect inflammation serially in vivo using $^{67}$Ga-scintigraphy, as demonstrated in the present study, will not only facilitate mechanistic studies related to inflammation, but will also allow for monitoring the time course of the disease or the response to various treatments aimed at counteracting RV heart failure.

**MCT model of right ventricular failure**

The MCT-rat model of RV failure is based upon a single MCT injection that leads to severe pulmonary disease in the absence of intrinsic heart and lung disease30. The resulting pulmonary hypertension in turn induces RV hypertrophy and RV failure. We have previously demonstrated in this model
that distinct disease stages occur during the progression of RV disease, and can be precisely identified by echocardiography and clinical assessment [15]. Using serial echocardiography allowed us to pinpoint inflammation to specific RV disease stages. Defining the disease stages precisely is of great value to conduct such studies. Thus, in this model, the remodeling events that culminate to RV failure can be accurately linked to the specific disease stages.

Conclusion
Inflammation is an early mechanism that may contribute to the development of RV failure. In vivo $^{67}$Ga-scintigraphy may be used for the serial assessment of inflammation to monitor disease progression in RV failure.

Acknowledgments
Dr. H.L.Tan was supported by the Royal Netherlands Academy of Arts and Sciences (KNAW), the Netherlands Organization for Scientific Research (NWO, ZonMW-Vici 918.86.616), and Actelion Pharmaceuticals Netherlands Co.
References


