

**GEORGE EMIL PALADE UNIVERSITY OF MEDICINE, PHARMACY, SCIENCE AND TECHNOLOGY OF TÂRGU
MUREȘ**

SCHOOL OF DOCTORAL STUDIES

- SUMMARY OF PHD THESIS -

Title: Antifibrotic and Angiogenic Effects of S100A8/A9 Blockade in Acute Myocardial Infarction

Principal PhD coordinator: Assoc. Prof. Dr. Alexandru Șchiopu

Cotutelle PhD coordinator: Prof. Dr. Ovidiu S. Cotoi

PhD student: Răzvan-Gheorghiță Mares

Introduction:

Acute myocardial infarction (AMI) is a disease associated with high morbidity and mortality. Although advances in critical care, pharmacologic interventions, and emergency management of AMI by percutaneous coronary revascularization have significantly reduced acute mortality, survivors remain at increased risk of developing heart failure. Thus, a better understanding of the pathogenesis of cardiomyocyte necrosis, myocardial dysfunction, and ventricular remodeling is urgently needed to develop new therapies for AMI patients. Therapies targeting the innate immune response may represent a viable solution to reduce myocardial damage, improve cardiac function and prognosis in AMI patients. However, immunosuppressive approaches used in previous clinical trials have failed, and to date there are no clinically implemented therapeutic strategies that adequately modulate the inflammation/myocardial repair balance in the acute phase after AMI.

Objectives:

The objectives of this study were the following:

- To implement and validate an experimental platform for the study of myocardial infarction within the George Emil Palade University of Medicine, Pharmacy, Sciences and Technology in Târgu Mureș, which will allow local, national or international research teams to effectively test new molecules and pharmaceutical compounds for the treatment of myocardial infarction;
- To integrate within the platform of a new and minimally invasive AMI model in mice, which at the moment is not implemented in any other research center in Romania, as well as of associated techniques that will allow the characterization of local and systemic changes caused by myocardial ischemia;
- To validate the ability of the platform to measure the effects of treatments on cellular processes and histological changes post-AMI;
- To evaluate the effects of ABR-238901 treatment, a blocker of S100A8/A9 protein, on infarct size and post-AMI myocardial fibrosis;
- To evaluate the effects of S100A8/A9 blockade on neovascularization after AMI.

General methodology:

Wild-type (C57BL/6) female mice, 8-12 weeks of age, 20-25 g body weight, underwent permanent left coronary artery (LAD) ligation using a novel and minimally invasive surgical approach. All surgeries were performed at the Experimental Station of "George Emil Palade" University of Medicine, Pharmacy, Science, and Technology of Targu-Mures according to local protocols approved by the Scientific Research Ethics Commission of the University. Mice were randomly assigned into 3 groups: sham-operated mice (Sham group), AMI mice receiving PBS (AMI group) and AMI mice treated with 30mg/kg of the small-molecule S100A8/A9 and S100A9 blocker ABR-238901 (ABR) diluted in PBS (AMI+ABR group). We used electrocardiographic measurements to monitor the induction of acute myocardial ischemia and to immediately confirm the success rate of the procedure. AMI was histologically confirmed by Hematoxylin and Eosin (H&E) and Trichrome-Masson stains. Additionally, the size of

the myocardial damage was determined by Trichrome-Masson staining and by the triphenyl tetrazolium chloride (TTC) assay to distinguish between necrotic and viable myocardium in the early period after AMI. Histological analyses of heart sections were performed at 1, 3 and 7 days after AMI induction. To collect the heart, the mouse was anesthetized by intraperitoneal injection of a ketamine-xylazine solution. We analyzed myocardial tissue sections collected from 5-6 different levels along the transversal axis of the heart, starting from the apex and up to the level of the coronary suture. H&E staining was used to visualize the inflammatory infiltrate and cardiomyocyte loss secondary to acute ischemia. Trichrome-Masson staining was used to stain collagen fibers, in order to evaluate the extension of fibrosis in the infarcted myocardium. We performed immunohistochemical staining for S100A9, a pro-inflammatory alarmin that increases rapidly in the infarcted myocardium, and for the lymphocyte antigen 6G (Ly6G), a marker specifically expressed on the surface of mouse neutrophils, to evaluate histological changes in the early acute inflammatory period after AMI. Further, we used immunohistochemical staining of the endothelial cell marker CD31 to visualize capillaries in the infarcted and remote myocardium. In separate experiments at the Institute for Cellular Biology and Pathology "N. Simionescu" Bucharest, we performed a proteomic analysis of left ventricle extracts by qualitative mass spectrometry on day 7 post-MI to evaluate the angiogenic mediators and processes modulated by S100A9 inhibition. We also analyzed by flow cytometry the dynamics of innate immune cells (neutrophils, monocytes and macrophages) in blood and heart at 1, 3 and 7 days after AMI.

Results:

The total surgical procedure time of AMI induction was up to 3 minutes, with a heart exposure time for LAD ligation of up to 20 seconds. The time to full recovery of the animals after cessation of inhalatory anesthesia was up to 5 minutes. The short-term post-operative animal survival reached up to 80%, and the long-term survival rate (from 1 hour after surgery until harvest) was up to 95%. The overall survival rates (including surgery-related death) were about 65-75%. After AMI, the inflammatory infiltrate mainly consists of neutrophils that peaked on day 1 after infarction. The myocardial destruction and the inflammatory infiltrate were much more pronounced on day 3, but the neutrophil presence was highly reduced compared to day 1. The ability of S100A9 to reflect the presence of neutrophils in the infarcted myocardium was confirmed by significant associations between the extent of the S100A9 and Ly6G staining, expressed as average percentage of left ventricular area, on both day 1 and day 3 post-AMI. The S100A9 blockade lowered the presence of inflammatory cells and the infiltration of Ly6G-positive neutrophils and on the S100A9 presence in the myocardium on days 1 and 3. The extent of myocardial fibrosis was significantly reduced in ABR-238901-treated mice both on 3 and 7 days after AMI. In ABR-treated mice, a significant decrease of myocardial fibrosis was also observed in the remote myocardium at 7 days post-AMI. Furthermore, we demonstrated that infarct size was significantly reduced both at 3 and 7 days after AMI by blocking S100A9 for 3 days. S100A9 inhibition did not significantly influence myocardial vasculature on days 1 and 3 post-AMI. However, ABR treatment determined a 2.5-fold increase in vascular area from day 1 to day 7 compared with only a 1.6-fold increase in the control PBS-treated mice. Among the 25 proteins significantly modulated by treatment, 10 proteins showed at least 4-fold differences between ABR-treated AMI mice and control mice treated with PBS. The ABR treatment counteracted the increase of 8 of the 10 proteins compared to the PBS-treated MI group, inducing a significantly lower abundance of Anx1 (~16-fold), AnxA2 (~5-fold), Fgf1 (~10-fold), Emc10 (~5.5 fold), RhoA (~4.7-fold), Cdc42 (~4.7-fold), Camp (~4.4-fold), and Ngp (~4-fold). In contrast, Flna and Rtn4 were increased by S100A9 blockade by ~10-fold and ~5-fold, respectively.

General conclusions:

We described and implemented in our University a novel and minimally invasive and experimental model of AMI induction in mice. Furthermore, we have successfully implemented histological and immunohistochemical as well as flow cytometry techniques that allow extensive tissue and cellular analysis of local and systemic changes caused by acute myocardial ischemia. Our results promote S100A9 as a potential biomarker of neutrophil involvement in AMI and a promising therapeutic target to modulate neutrophil recruitment and function, and thereby reduce excessive inflammation after AMI. Furthermore, we demonstrated that S100A9 blockade during the acute inflammatory phase after AMI reduces infarct size both at 3 and 7 days after coronary occlusion and significantly decreases post-ischemic cardiac fibrosis. Our data revealed that the treatment promotes neovascularization in both the infarcted and the remote myocardium. The underlying mechanisms for the pro-angiogenic effects involve upregulation of Flna and Rtn4, and downregulation of RhoA, Ngp, and Camp.

Originality of the thesis:

In this project we have established a minimally invasive and highly effective experimental model of AMI induction in mice by permanent LAD ligation. According to our knowledge, at the moment this surgical intervention is successfully performed in Romania only in the "George Emil Palade" University of Medicine, Pharmacy, Sciences and Technology in Târgu Mureş. In addition, we integrated and synchronized for the first time in our University peripheral research modules with the surgical procedure, such as small animal electrocardiography, animal treatment, tissue harvesting, histological processing and analysis and cellular extraction and flow-cytometrical analysis, which allow the study of the local and systemic changes caused by acute myocardial ischemia in mice. Moreover, the entire experimental platform currently represents an important tool that supports the understanding of the role of the innate immune system and inflammation in the pathogenesis of AMI, that can be used by biotech and pharmaceutical companies and research organizations for developing and testing of novel therapies for myocardial infarction. The results of the present work indicate for the first time the pro-inflammatory alarmin S100A9 as a potential biomarker of neutrophil involvement in AMI and a promising therapeutic target to reduce excessive inflammation after AMI. Another novelty is showing that S100A9 blockade in the first 3 days after AMI attenuates post-ischemic myocardial fibrosis and significantly reduces myocardial infarct size. Importantly, we show for the first time that S100A9 blockade promotes post-AMI myocardial neovascularization by increasing Flna and Rtn4 expression and decreasing RhoA, Ngp, and Camp expression. These findings promote S100A9 blockade as a novel and promising immunomodulatory therapy in AMI and identify novel proteins with therapeutic potential.