Secretome of apoptotic cells causes cardioprotection and inhibits ventricular remodeling after acute myocardial infarction

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"Hic locus est ubi mors gaudet succurrere vitae"
(This is the place where death delights to help the living)

Giovanni Battista Morgagni (1682 – 1771)

Inscription at the Institute of Anatomy, Medical University Vienna

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Zusammenfassung

Der akute Myokardinfarkt gefolgt von linksventrikulärem Remodelling ist eine der Hauptursachen für die chronische Herzinsuffizienz in der westlichen Welt. Vorangegangene Untersuchungen zeigten, dass die Injektion apoptotischer Zellen im Tiermodell des Herzinfarkts die Infarktgröße signifikant reduzieren, die linksventrikuläre Funktion erhalten und das Einwandern regenerativer Zellpopulationen in die Ischämiezone fördern konnte. Ziel dieser Studie war, die diesen Effekten zugrundeliegenden Mechanismen zu entschlüsseln. Ein besonderes Augenmerk wurde auf die Rolle parakriner Faktoren, die von apoptotischen Zellen sezerniert werden, gelegt.

Suspensionen apoptotischer Zellen wurden in einem Rattenmodell des akuten Herzinfarkts einerseits intravenös als auch intramyokardial injiziert. Morphologische und funktionelle Analysen wurden mittels Histologie, Planimetrie und Echokardiographie durchgeführt.
Die kardioprotektiven Eigenschaften von Zellkulturüberständen apoptotischer Zellen wurden in einem weiteren Rattenmodell des akuten Herzinfarkts mittels permanenter Gefäßligatur und ebenfalls in einem Großtiermodell mit anschließender Reperfusion evaluiert.

Planimetrische und echokardiografische Analysen zeigten eine signifikante Reduktion der Infarktgröße mit geringeren Anzeichen von linksventrikulärer Dilatation und eine verbesserte linksventrikuläre Funktion in Tieren, denen Suspensionen apoptotischer Zeller injiziert wurden. In einer histologischen Auswertung zeigte sich, dass das kardiale Narbengewebe von therapierten Tieren eine weit höhere Akkumulation von elastischen Fasern aufwies.
Durch die ausschließliche Verabreichung von Zellkulturüberständen apoptischer Zellen konnte ebenfalls sowohl im Kleintier- als auch im Großtiermodell eine Reduktion der Infarktgröße und eine Verbesserung kardialer Funktionsparameter erzielt werden. *In vitro* Assays zeigten, dass humane Kardiomyozyten, die mit Zellkulturüberständen apoptotischer Zellen inkubiert wurden, eine Hochregulierung von anti-apoptotischen Mediatoren (Bcl-2, BAG1) und eine Aktivierung von kardioprotektiven Signalkaskaden (u.a. Akt, Erk1/2, CREB) zeigten.

Diese Daten zeigen, dass die Verabreichung von apoptotischen Zellen beziehungsweise deren Zellkulturüberständen, zytoprotektive Signalwege in Kardiomyozyten induzieren und so den Infarktschaden reduzieren kann.

Abstract

Heart failure developing after acute myocardial ischaemia is a major cause of morbidity and mortality in the western world. In our previous study we showed that intravenous injection of apoptotic peripheral blood mononuclear cell (PBMC) suspensions preserved cardiac function in a rat acute myocardial infarction (AMI) model. Based on these results, we sought to investigate other ways of cell administration and analysed the composition of the fibrotic scar tissue. Moreover, we sought to study the effect of soluble factors secreted by apoptotic PBMC on ventricular remodelling after AMI.
Cell suspensions of apoptotic PBMC were injected intravenously (IV) or intramyocardially (IM) after experimental AMI in rats. The administration of cell culture medium or viable PBMC served as controls. To study the effects of paracrine factors secreted by apoptotic cells, supernatants of irradiated PBMC were collected and injected intravenously after myocardial infarction in an experimental AMI rat model and in a porcine closed chest reperfused AMI model.

Rats injected with suspensions of apoptotic PBMC (either intravenously or intramyocardially) evidenced a significant reduction of infarct dimensions and preservation of cardiac function. Histology showed that the ratio of elastic and collagenous fibres within the scar tissue was altered in a favourable fashion in rats injected with apoptotic cells compared to controls.
The administration of supernatants of apoptotic PBMC resulted in a reduction of myocardial scar tissue formation in both the rat and the porcine model. In the large animal reperfused AMI model higher values of ejection fraction (57.0% vs. 40.5%, $p<0.01$), a better cardiac output (4.0 vs. 2.4 l/min., $p<0.001$) and a reduced extent of infarction size (12.6% vs. 6.9%, $p<0.02$) were found. *In vitro* experiments showed that exposure of primary human cardiac myocytes with paracrine factors secreted by apoptotic PBMC induced the activation of pro-survival signalling-cascades (AKT, Erk1/2, CREB) and increased anti-apoptotic gene products (Bcl-2, BAG1).

Intravenous and intramyocardial injection of apoptotic cell suspensions preserved left ventricular function and altered the composition of cardiac scar tissue with increased expression of elastic fibres. Intravenous infusion of cell culture supernatants of

apoptotic PBMC attenuated myocardial remodelling in both experimental AMI animal models.

Introduction

Stem Cell Therapies for Acute Myocardial Infarction

New treatment strategies and early reperfusion within a narrow time window has significantly reduced the mortality following acute myocardial infarction (AMI). However, ischaemic heart failure secondary to the initial event still remains widely prevalent and represents an increasing economic burden in the western world [1].

Great expectations emerged in the scientific world when Orlic *et al.* discovered in 2001 that the injection of bone marrow stem cells expressing the marker c-kit lead to the regeneration of myocardial tissue and reduced ventricular remodelling after AMI [2]. These findings were supported by an abundance of experimental studies published shortly after [3-5]. Over the following years many randomized controlled clinical trials were implemented in order to investigate whether injection of autologous stem cells supports healing processes after AMI or can even regenerate viable myocardium.

One of the largest clinical trials (the REPAIR-AMI trial) evaluating regenerative effects of bone marrow cells in patients after AMI was published in 2006 [6]. The REPAIR-AMI investigators showed that in patients being injected with autologous bone marrow derived progenitor cells, the global left ventricular ejection fraction (EF) was improved significantly after four months and mortality was reduced within the first year.

However, the ASTAMI trial failed to show or prove any beneficial effects of stem administration after AMI [7]. The authors speculated that the way of cell administration (i.e. by intracoronary delivery) has many limitations, only a small percentage of the injected cells might remain in the ischaemic heart and a further large proportion of transplanted cells might die within the next few days [8, 9].
The BOOST study was the clinical trial investigating cardiac cell therapy after AMI with the longest follow-up period. Even though initial results were very convincing showing a significant improvement of EF after six months of follow-up, results failed to show significance after 18 months and to an even lesser extent after 61 months [10-12].

In order to further evaluate these controversial results meta-analysis were conducted. Some suggested that cell therapy after AMI might improve EF but it does not prevent ventricular remodelling whereas others stated that there is not enough evidence to support the clinical application of stem cell therapy after AMI [13, 14]. However, the mechanistic principles of cardiac stem cell therapy still remain unclear as many other interactions such as pro-angiogenic effects, immunomodulation or paracrine signalling might be involved.

The Dying Stem Cell Hypothesis

Inflammation due to myocardial necrosis after ischaemia is an integral part in the pathophysiology of cellular responses after AMI. These inflammatory reactions in the ischaemic myocardium contribute to the detrimental processes after AMI subsequently leading to loss of further cardiomyocytes and to ventricular remodelling. Thum *et al.* stated in their hypothesis published in 2005 that immunomodulatory signals induced by transplanted apoptotic stem cells might be responsible for the (mildly) beneficial effects seen in clinical trials [15]. In these trials, the proportion of cells already undergoing apoptosis ranged around 5 to 25 percent [10, 16-18]. The authors assumed that necrosis of cardiomyocytes within the ischaemic myocardium plays the predominant part in triggering pro-inflammatory signals in the cellular microenvironment. They proposed that the improvement in cardiac function seen after stem cells administration might be explained by the modulatory interactions of local immune cells in response to transplanted cells undergoing apoptosis. It was shown that in contrast to necrotic cells, apoptotic cells can inhibit inflammatory reactions. This phenomenon was extensively covered by the work of Fadok *et al.* [19, 20].

It was shown previously that the recognition of apoptotic particles by phagocytic cells, such as macrophages is mediated via interaction of phosphatidylserine receptors. This interaction requires exposure of phosphatidylserine, which usually is only found on the inner leaflet of the plasma membrane of every cell, to flip to the outer surface of the apoptotic cell. They further argued that the ingestion of apoptotic cells by macrophages leads to the expression

of the anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-beta).

In the pro-inflammatory microenvironment of necrotic tissues the increased expression of anti-inflammatory cytokines counteracting the signaling of pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-alpha), interleukin-1 beta (IL-1beta) and interleukin-6 (IL-6) might result in an accelerated resolution of detrimental inflammatory processes after AMI and might improve reparative effects.

The group of Fadok furthermore showed conclusively that phagocytes that have ingested apoptotic but not necrotic cells respond by a change of the secretion pattern of many pro-survival growth factors such as vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF). These factors play a major role in conferring cytoprotective signals to neighboring cells by up-regulation of the anti-apoptotic protein Bcl-2, via phosphatidylinositol 3-kinase (PI3K) and triggering of the AKT signal transduction pathway [21].

Apoptotic Cell Therapy for AMI

In our previous study we tried to verify the hypothesis by Thum *et al.* [15] using *in vitro* and *in vivo* experiments. In order to implement the findings of Fadok *et al.* [19-21] into Thum's hypothesis, we tested the immunosuppressive or immunomodulatory effects of apoptotic cells in *in vitro* assays and their cardioprotective potential in a rat model of AMI.

We could show that the addition of irradiated apoptotic peripheral blood mononuclear cells (PBMC) reduced the secretion of the pro-inflammatory cytokines Interleukin-1beta (IL-1beta) and Interleukin-6 (IL-6) in an *in vitro* assay of LPS (bacterial lipopolysaccharide) stimulated cell cultures of monocytes and PBMC. Similar effects were also observed in mixed-lymphocyte reaction assays [22].

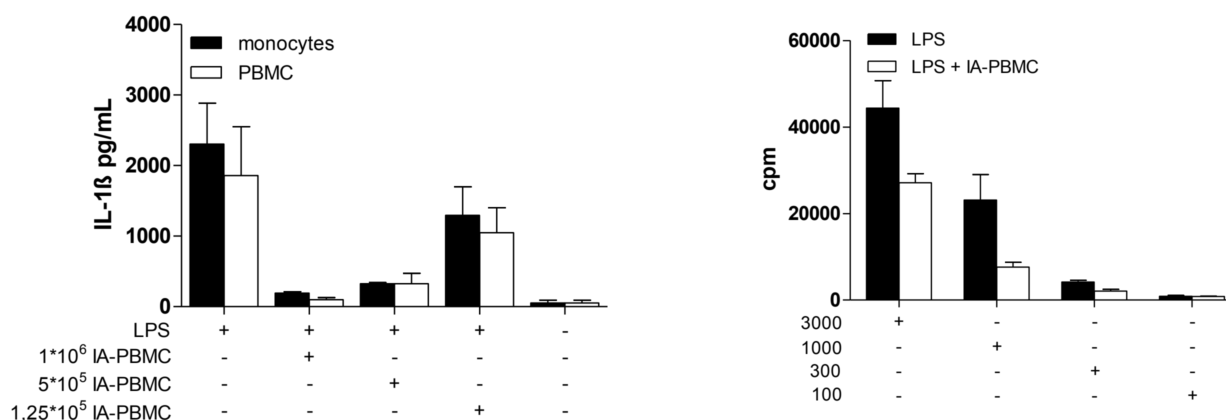

Figure 1 Co-incubation of LPS stimulated monocytes and PBMC with irradiated apoptotic PBMC suspensions reduced the secretory capacity of IL-1beta. Concomitantly, the addition of irradiated apoptotic PBMC to mixed-lymphocyte reactions decreased cell proliferation as indicated by less counts per minute (cpm)[22].

In the *in vivo* part of our previous study we injected syngeneic irradiated apoptotic PBMC suspensions in a rat model of AMI induced by ligation of the left anterior descending artery (LAD) via an intravenous route. We could show that the intravenous administration of irradiated apoptotic PBMC suspensions reduced infarct dimensions and scar formation six weeks after induction of AMI and also preserved ventricular function as evidenced by significantly improved values of ejection fraction (EF) and shortening fraction (SF). Moreover, higher numbers of cells staining positively for endothelial progenitor cell markers such as c-kit and Vascular endothelial growth factor receptor 2 (VEGF-R2) [23-25] were found within the cellular infiltrate in the ischaemic myocardium in animals injected with apoptotic cells. When evaluating cardiac tissue specimens, we also found higher numbers of macrophages in the ischaemic myocardium of treated animals compared to controls.

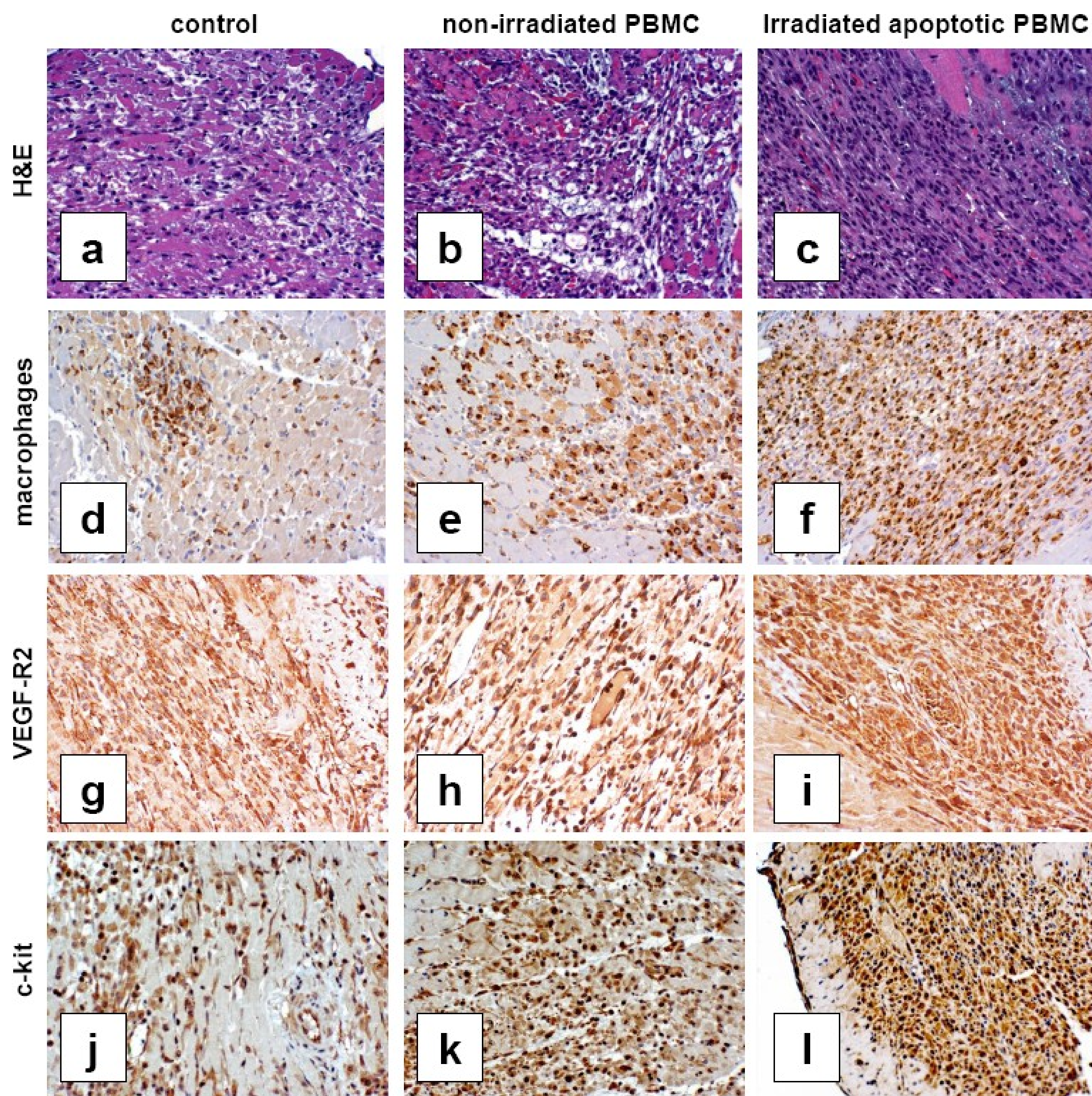

Figure 2 Tissue specimens stained with haematoxylin and eosin (H&E staining) evidenced that the cellular infiltrate was much denser in rats injected with apoptotic PBMC (c) compared to controls (a) and animals injected with non-irradiated viable cells (b). Moreover, higher numbers of macrophages and cells staining positively for VEGF-R2 and c-kit were found after injection of apoptotic PBMC following LAD ligation (f,i,l) compared to control groups (d,e,g,h,j,k)[22].

Six weeks after induction of AMI, hearts were explanted and infarct dimensions were calculated by means of planimetry. A significant reduction in infarct dimension was apparent in animals injected with irradiated apoptotic PBMC over controls and also over rats injected with non-irradiated viable cells.

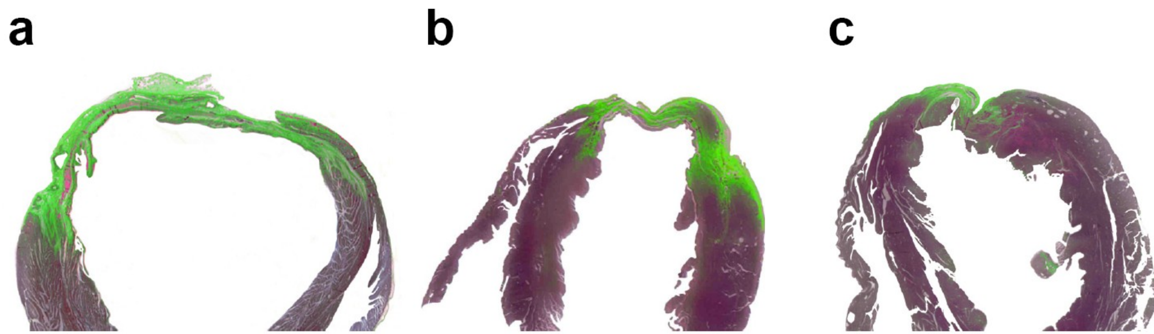

Figure 3 shows mid-ventricular sections of hearts explanted six weeks after induction of AMI. Large infarcts were common in the control group (a), a slight improvement was found in animals injected with viable cells (b) and the best outcome was observed in rats injected with irradiated apoptotic cells (c)[22].

Parameters of cardiac function were evaluated by means of echocardiography six weeks after AMI. Whereas both in controls as in animals injected with non-irradiated cells a significant loss of cardiac function was apparent compared to healthy rats (sham operated), functional parameters were almost completely preserved in animals with intravenous injection of irradiated apoptotic cells.

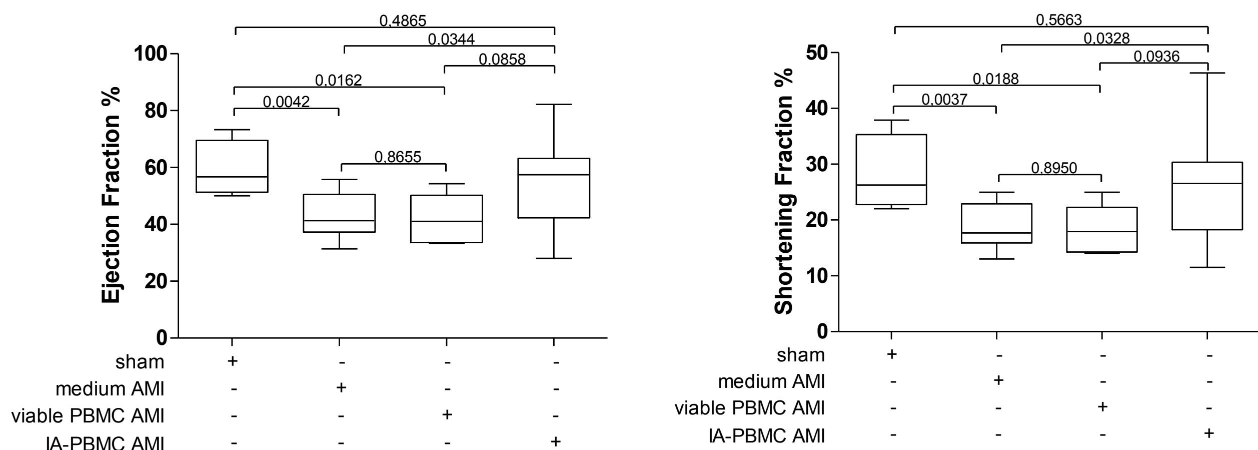

Figure 4 shows parameters of cardiac function (ejection fraction and shortening fraction) obtained six weeks after AMI by means of echocardiography. Rats injected with irradiated apoptotic cells presented a significant improvement of both ejection fraction and shortening fraction[22].

However, the exact mechanism how apoptotic cells reduced myocardial damage following AMI and preserved cardiac function still remained to be elucidated.

The Paracrine Paradigm

Over the last few years a new concept was developed in the field of regenerative therapies for AMI, namely that the main therapeutic effect seen in studies investigating stem cell therapy are conferred by paracrine factors that are secreted by the injected cells.

The first reports on this relatively new field of investigation date back to the year 2005, when Gnecchi *et al.* reported that the conditioned medium of mesenchymal stem cells stressed by incubation under hypoxic conditions significantly reduced the rate of cell death of adult rat ventricular cardiomyocytes caused by hypoxia [26]. The authors also tested their hypothesis in an *in vivo* model of AMI by permanent LAD ligation in a rat model and showed that the conditioned medium reduced the rate of apoptosis in the ischaemic myocardium. Moreover, infarct dimensions were reduced as well. The group of Gnecchi further proved their hypothesis in following studies and discussed their findings in many review articles [27-30].

In a sub-study conducted by the BOOST investigators, the regenerative effect of paracrine factors in human cardiac stem cell therapy was discussed for the first time [31]. In this study, the secretome of bone marrow derived cells and of peripheral blood cells was tested for its regenerative or cytoprotective effects in *in vitro* experiments, investigating coronary artery endothelial cell proliferation, migration, endothelial tube formation and aortic cell sprouting. Moreover, these cell culture supernatants protected rat cardiomyocytes from simulated ischaemia/reperfusion induced cell death in an *in vitro* model. Interestingly, both the supernatants of bone marrow cells and also of peripheral blood cells showed cytoprotective and regenerative effects in these assays with only marginal differences. The authors also compared the secretion pattern of the two cell types and found that bone marrow cells only produced slightly higher amounts of cytokines, chemokines and growth factors related to regenerative processes compared to peripheral blood cells.

These findings were supported by reports from the research group of Kalka *et al.*, they investigated the protective effects of endothelial progenitor cell (EPC) derived secretomes *in vitro* and in a rat model of hind limb ischaemia [32, 33]. These EPC derived secretomes reduced oxidative stress and the rate of apoptosis in cell

cultures of human umbilical cord vascular endothelial cells (HUVEC) stressed with H_2O_2. Moreover, they increased the expression of the anti-apoptotic factor Bcl-2 in HUVECs. In an *in vivo* study the authors investigated injection of stressed EPC derived secretomes in a rodent model of hind limb ischaemia. This therapy increased hind limb blood flow, capillary density and improved muscle viability and functional performance. Additionally, injection of EPC secretomes induced the mobilization of bone marrow derived EPC and their homing to sites of ischaemia.

Based on these recent reports we sought to further investigate our hypothesis of cardioprotection induced by apoptotic cells particularly with regard to paracrine factors and their effects on cytoprotective, anti-apoptotic and regenerative processes.

Aims of the study

In the first part of the study, the mechanisms how apoptotic cells might modulate the remodelling process following AMI were investigated in a rodent model of LAD ligation. Based on our previous results [22], another way of cell administration, i.e. via intramyocardial injection was investigated. Moreover, a major goal of the study was to analyse the composition of the extracellular matrix of the myocardial scar tissue. Interestingly, a strong accumulation of elastic fibres was found in the cardiac scar tissue of animals injected with apoptotic cell suspensions. In order to further investigate this issue, RT-PCR analysis and immunohistology for growth factors inducing elastin expression was performed.

The goal of the second part of the study was to define signalling mechanisms how apoptotic cells can confer cardioprotective effects to the ischaemic myocardium. As paracrine effects were more and more in the focus of research on protection against myocardial ischaemia, cell culture supernatants of irradiated apoptotic PBMC were harvested, lyophilised in order to improve practicability (this compound was termed *APOSEC*, i.e. **apo**ptotic cell **sec**retome) and were injected in a rat model of AMI. Moreover, the mechanisms of cardioprotection mediated by apoptotic cell supernatants were investigated in various *in vitro* assays using human cardiac myocytes. As the administration of apoptotic cell derived supernatants showed convincing results in the small animal model and in *in vitro* assays, a porcine model

of reperfused AMI was implemented. Using this type of animal model, it was possible to test the administration of apoptotic cell derived supernatants in an experimental setting that is much more comparable to the clinical scenario of AMI.

Materials and Methods

Descriptions of proceedings investigating the cardioprotective properties of apoptotic PBMC [34]

Acquisition of syngeneic rat PBMC suspensions for *in vivo* experiments

Experiments including animals were approved by the committee for animal research of the Medical University of Vienna and the Federal Ministry of Science and Research (ethics vote: BMBWK-66.009/0278-BrGT/2005). All animal experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 85-23). To obtain syngeneic rat PBMC for *in vivo* experiments, animals were anaesthetized and heparin was injected intravenously. Venous whole blood was aspirated by direct punctuation of the heart. Approximately 10-12 millilitres (ml) of blood were collected from each animal. The aspirated blood specimens were processed immediately and were diluted 1:2 in Hank's balanced salt solution HBSS (Lonza, Switzerland). The diluted blood suspensions were shifted carefully in a 50 ml tube containing Ficoll-Paque solution (GE Healthcare Bio-Sciences AB, Sweden). Tubes were centrifuged for 15 minutes at 800g at room temperature without brake. This cell separation technique generates a layer (buffy coat) of lymphocytes and monocytes in a very high degree of purity. Buffy coats of mononuclear cells were aspirated, washed once in HBSS and resuspended in 1 ml of fresh UltraCulture serum-free cell culture medium (Lonza, Switzerland). Cell concentrations were determined on a Sysmex automated cell counter (Sysmex Inc., USA).

The separated PBMC suspensions were subjected to Caesium-137 irradiation (Department of Transfusion Medicine, General Hospital Vienna) with 45 Gray (Gy) in order to induce apoptotic cell death. Non-irradiated cells served as controls. The obtained cells were cultured in a humidified atmosphere for 18 hours. Induction of apoptosis was measured by using a co-staining for Annexin-V/Propidium iodine (Becton Dickinson, USA) on a flow cytometer. Annexin-positivity of PBMC was

determined to be >80% and these cells were consequently classified as apoptotic PBMC (termed irradiated apoptotic PBMC, IA-PBMC).

Induction of acute myocardial infarction in male Sprague-Dawley rats

Acute myocardial infarction was induced in adult male Sprague-Dawley rats (weight 300 - 350g) by ligating the left anterior descending artery (LAD) as previously described [35, 36]. In short, animals were anaesthetized intraperitoneally with a mixture of xylazine (1mg per 100g bodyweight) and ketamine (10mg per 100g bodyweight), intubated using a venous catheter and ventilated mechanically. Rats were placed in a dorsal position and a left intercostal thoracotomy was performed and a ligature beneath the left atrium was placed around the LAD using 6-0 Prolene polypropylene suture. Immediately after the onset of ischaemia, cultured cell suspensions of $8.5*10^6$ viable or apoptotic PBMC suspended in 0.3 ml UltraCulture medium were injected into the femoral vein. In a second treatment group, apoptotic cells ($8.5*10^6$ cells) were also injected directly into the myocardium at five different sites of the peri-infarct zone. Injection of cell culture medium alone and sham operation served as controls in this experimental setting. The mortality rate in these experiments was between 20% and 30%.

Histological and immunohistological evaluations

Tissue specimens were collected from animals that were sacrificed either 72 hours or 6 weeks after experimental infarction. A thoracotomy was performed, hearts were explanted and fixed in 10% neutral buffered formalin. Heart specimens were then sliced at three layers at the level of the largest extension of infarcted area (n=6 for 72 hours analyses, n=10-12 for 6 weeks analyses) and embedded in paraffin. The tissue samples were stained according to a haematoxylin-eosin (H&E) and Elastica van Gieson (EVG) staining protocol.

Immunohistological analysis of specimens obtained 72 hours after AMI was performed using antibodies directed to CD68 (MCA 341R, AbD Serotec, UK), c-kit

(sc-168, Santa Cruz Biotechnology, USA), FLK1 (sc-6251, Santa Cruz Biotechnology, USA), IGF-I (sc-9013, Santa Cruz Biotechnology, USA) and FGF-2 (sc-79, Santa Cruz Biotechnology, USA). Specimens were evaluated on an Olympus AX70 microscope (Olympus Optical Co. Ltd., Japan) at a 200x magnification. Images were captured digitally using Meta Morph v4.5 Software (Molecular Devices, USA). Image J planimetry software (Rasband, W.S., Image J, U.S. National Institutes of Health, USA) was used to determine the area of necrosis after 72 hours and the dimension of myocardial infarction after 6 weeks. The extent of infarcted myocardial tissue (expressed as % of left ventricle) was calculated by dividing the area of the circumference of the infarcted area by the total endocardial and epicardial circumferenced areas of the left ventricle. Planimetric evaluation after six weeks was carried out on tissue samples stained with EVG for better comparison of vital myocardium and fibrotic areas. Infarction size was expressed as a percentage of the total left ventricular area. Tissue specimens stained with EVG were furthermore analysed microscopically for the ratio of elastic and collagen fibres within the left ventricular scar tissue. ImageJ planimetry software was utilized to calculate the elastin to collagen ratio by dividing the area occupied by elastic fibres by the total area of collagenous scar tissue.

Evaluation of cardiac function by echocardiography six weeks after induction of myocardial infarction

Animals were anaesthetized six weeks after induction of myocardial infarction as described above. Echocardiographic examination was conducted on a Vivid 7 system (General Electric Medical Systems, USA). All analyses were performed by an experienced observer blinded to the treatment groups to which the animals were allocated. M-mode tracings were recorded from a parasternal short-axis view and functional systolic and diastolic parameters were obtained (ejection fraction, EF; shortening fraction, SF; left ventricular end-diastolic diameter, LVEDD; left ventricular end-systolic diameter, LVESD, left ventricular end-diastolic volume, LVEDV; left ventricular end-systolic volume, LVESV). Ejection fraction was calculated as follows:

EF(%)=((LVEDV-LVESV)/LVEDV)*100. Shortening fractional was calculated as follows: SF(%)=((LVEDD – LVESD)/ LVEDD)*100.

Separation of human PBMC for *in vitro* experiments

Experimental procedures were approved by the local ethics committee of the Medical University of Vienna (ethics committee vote: EK-Nr 2010/034) and were conducted in compliance with the principles of the Declaration of Helsinki. Human peripheral blood mononuclear cells (PBMC) were obtained from young healthy volunteers by venous blood withdrawal after informed consent. Blood specimens in EDTA tubes were processed immediately and PBMC were obtained by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation as described above.

Apoptosis of PBMC was induced by Caesium-137 irradiation with 60 Gray (Gy) for *in vitro* experiments. Cells were resuspended in serum-free UltraCulture medium and cultured in a humidified atmosphere at 37° Celsius for 24 hours at a density of $2.5*10^6$ cells/ml, n=5). The induction of apoptosis in PBMC was measured by Annexin-V/Propidium iodine co-staining (Becton Dickinson, USA) on a flow cytometer. In order to characterize apoptotic cells, the Annexin-V-positivity of PBMC was determined to be >80%. Non-irradiated PBMC served as controls in all *in vitro* experiments and were termed “viable PBMC”.

Moreover, supernatants of cell cultures of irradiated and non-irradiated cells were harvested after 24 hours and were stored at -80° Celsius until further analyses were conducted.

Cell culture of human fibroblasts exposed to supernatants of apoptotic PBMC, RNA isolation and cDNA preparation

Cell culture supernatants were obtained from viable PBMC, irradiated apoptotic PBMC and mixed co-cultures of viable and apoptotic cells after 24 hours (cell density $2.5*10^6$ resuspended in fresh UltraCulture medium) as described above. Human

primary fibroblasts (Cascade Inc., USA), seeded at a density of $1*10^5$ cell per ml were exposed to supernatants obtained from viable PBMC, apoptotic PBMC and mixed cultures of viable and apoptotic cells for 24 hours. Fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, USA) that was supplemented with 10% fetal bovine serum (FBS, PAA, Austria), 25mM L-glutamine (Gibco BRL, USA) and 1% penicillin/streptomycin (Gibco BRL, USA) and seeded in 12-well plates. After RNA extraction of fibroblasts (using RNeasy, QiIAGEN, Austria) following the manufacturer's instruction, cDNAs were transcribed using the iScript cDNA synthesis kit (BioRad, USA).

Quantitative real time polymerase chain reaction (RT-PCR)

RT-PCR was used to quantify mRNA transcription of elastin, collagen type I, collagen type III, collagen type V, Interleukin 8 (IL-8), Matrixmetalloproteinase 1 (MMP1), Matrixmetalloproteinase 3 (MMP3) and Matrixmetalloproteinase 9 (MMP9). The expression of mRNA was quantified by RT-PCR on a LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Germany) according to the manufacturer's protocol. The primers for elastin (forward: 5´-CCTACTTACGGGGTTGG-3´, reverse: 5´- GCCGAGCAGACAAGAA-3´), collagen type I (forward: 5´- GTGCTAAAGGTGCCAATGGT-3´, reverse: 5´-CTCCTCGCTTTCCTTCCTCT-3´), collagen type III (forward: 5´-GTCCATGGATGGTGGTTTTC-3´, reverse: 5´- CACCTTCATTTGACCCCATC-3´), collagen type V (forward: 5´- GTCCATACCCGCTGGAAA-3´, reverse: 5´-TCCATCAGGCAAGTTGTGAA-3´), IL-8 (forward: 5´-CTCTTGGCAGCCTTCCTGATT-3´, reverse: 5´-TATGCACTGACATCTAAGTTCTTTAGCA-3´), MMP1 (forward: 5´-GGTCTCTGAGGGTCAAGCAG-3´, reverse: 5´- CCGCAACACGATGTAAGTTG-3´), MMP3 (forward: 5´- TGCTTTGTCCTTTGATGCTG-3´, reverse: 5´-GGCCCAGAATTGATTTCCTT-3´), MMP9 (forward: 5´-GGGAAGATGCTGGTGTTCA-3´, reverse: 5´-CCTGGCAGAAATAGGCTTC-3´) and β-2-microglobulin (β2M) (β2M, forward: 5´-GATGAGTATGCCTGCCGTGTG-3´, reverse: 5´-CAATCCAAATGCGGCATCT-3´) were designed as described previously

[37]. The relative expression of the target genes was calculated by comparison to the house keeping gene β2M using a formula as previously described [38].

Semi-quantitative evaluation of cytokines and growth factors secreted by apoptotic PBMC by means of membrane arrays

Cell culture supernatants of irradiated and non-irradiated human PBMC (cultured at a density of $2.5*10^6$/ml) were obtained from 4 healthy volunteers after informed consent. Membrane arrays for the detection of cytokines and growth factors in pooled supernatants were performed to analyse factors that are secreted by apoptotic cells in comparison to viable cells. Supernatants were screened for the presence and relative levels of a total of 274 cytokines by using a commercially available human cytokine antibody membrane array (AAH-CYT-4000, Ray Biotech, USA). Array experiments were performed according to the manufacturer's protocol. The obtained results were analysed using Image J software (Rasband, W.S., Image J, U.S. National Institutes of Health, USA). The secretion levels of each factor were expressed as relative to the respective positive controls in column 1 and 2 (VIABLE SN, APO SN). The fold increase over non-irradiated cell culture supernatants was expressed in column 3 (APO SN divided by VIABLE SN), see Table 1.

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<u>**Descriptions of proceedings investigating the cardioprotective properties of cell culture supernatants of apoptotic PBMC (APOSEC) [39]**</u>

Production of $APOSEC^H$, $APOSEC^R$ and $APOSEC^P$– Supernatants derived from irradiated apoptotic PBMC

Human peripheral blood mononuclear cells (PBMC) were obtained from young healthy volunteers after informed consent as described above. Apoptosis of PBMC was induced by Caesium-137 irradiation with 60 Gray. Irradiated and non-irradiated cells were resuspended in fresh serum-free UltraCulture Medium (Lonza, Switzerland) and cultured for 24 hours in various cell densities ($1*10^6$, $2.5*10^6$ and $25*10^6$ cells/ml, n=5) in a humidified atmosphere at 37° Celsius. After 24 hours, supernatants were collected and were subjected to ELISA content analysis or were lyophilised for further experiments. For lyophilisation, cell culture supernatants were dialyzed against ammonium acetate (at a concentration of 50mM) over night at 4°C. The obtained liquid ($APOSEC^H$) was sterile filtered using a 0.2µm filter (Whatman Filter 0.2µm FP30/o,2 Ca-S, Germany), frozen and lyophilised overnight (Lyophilisator Christ alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Germany).

For *in vivo* rat experiments, syngeneic rat PBMC were separated by density gradient centrifugation from venous whole blood obtained from heparinized rats as described above. PBMC were irradiated by Caesium-137 (45 Gy) and cultured for 24 hours at a cell density of $25*10^6$ cells/ml (resuspended in UltraCulture medium, Lonza, Switzerland). APOSEC for *in vivo* rat experiments ($APOSEC^R$) was further processed as described for $APOSEC^H$. Supernatants of non-irradiated rat PBMC cell cultures and fresh UltraCulture medium served as controls.

For large animal experiments, blood was obtained from anaesthetized pigs by direct puncture of the heart. Three pigs were anaesthetized with an intravenous bolus injection of 10 mg/kg ketamine and 1.3 mg/kg azaperone. A left thoracic dermal incision was conducted and a direct puncture of the heart was then performed under sterile conditions using a hollow needle. Arterial blood was drawn using 50 ml syringes. Blood obtained in syringes during this procedure was immediately transferred into heparinised plastic bags (3000 ml) for blood products. PBMC were

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then obtained according to the same protocol as described above. CellGro serum-free medium (Cell Genix, Germany), a "*Good Manufacturing Practice*" certified culture medium, was utilized for porcine PBMC derived APOSEC production ($APOSEC^P$). APOSEC for porcine experiments ($APOSEC^P$) was processed as described for $APOSEC^H$.

In order to avoid possible cross-species detrimental immune reactions, we opted to utilize APOSEC preparations solely in a syngeneic fashion (human APOSEC, $APOSEC^H$; rat APOSEC, $APOSEC^R$; porcine APOSEC, $APOSEC^P$).

Membrane array and ELISA analysis of cytokines and growth factors in $APOSEC^H$

Human PBMC derived APOSEC ($APOSEC^H$) was screened for cytokines and angiogenic factors using two commercially available array systems (ARY005, ARY007, Proteome Profiler Arrays, R&D Systems, USA). Membrane array experiments were performed according the manufacturer's instructions. Moreover, supernatant levels of cytokines and growth factors secreted by irradiated and non-irradiated PBMC in various concentrations ($1*10^6$, $2.5*10^6$ and $25*10^6$ cells/ml, n=5) were measured by utilizing commercially available enzyme-linked immunosorbent assay systems (ELISA, Duoset, R&D Systems, USA) kits for the quantification of IL-8, GRO-α, ENA-78, VEGF, IL-16, IL-10, TGF-β, sICAM-1, RANTES, IL-1ra, MIF, PAI-1, IGF-I, HGF, FGF-2, MCP-1, MMP9, SDF-1, G-CSF, GM-CSF and HMGB1 (IBL International GmbH, Germany). In brief, ninety-six–well plates (Nunc Maxisorp plates, Nunc GmbH & Co. KG, Germany) were coated overnight with capture antibodies at room temperature. After blocking of plates, supernatant samples and standard proteins were added to the wells. After a two hour incubation period and a washing step, a biotin-labelled antibody was added to each well and incubated for an additional two hours. Plates were washed and streptavidin-horseradish peroxidase was added. Colour reaction was achieved using a tetramethylbenzidine substrate solution (TMB; Sigma Aldrich, USA) and was stopped by a 1N sulphuric acid stop solution (Merck, Germany). Immediately thereafter, optical density values were

measured at 450 nm on a plate reader (Victor3 Multilabel plate reader, PerkinElmer, USA).

Rat AMI model and APOSEC^R treatment

Small animal experiments investigating APOSEC^R administration in rats were approved by the committee for animal research, Medical University of Vienna and the Federal Ministry of Science and Research (vote: 66.009/0168-II/10b/2008). Acute myocardial infarction was induced in adult male Sprague-Dawley rats (weight 275-300g) by ligating the LAD as described previously. Immediately after the onset of myocardial ischaemia, lyophilised supernatants obtained from $8.5 * 10^6$ either irradiated apoptotic PBMC or non-irradiated viable cells resuspended in 0.3 ml fresh UltraCulture medium (Lonza, Switzerland) were injected into the femoral vein. The administration of fresh UltraCulture cell culture medium served as negative control. In sham operated animals a left lateral thoracotomy was performed but no ligation was placed around the LAD.

Histological and immunohistological analysis and determination of myocardial infarction size by planimetry

Rats were sacrificed either 72 hours or 6 weeks after experimental infarction was induced. Hearts were explanted, placed in formalin overnight and were then sliced in three layers at the level of the largest extension of infarcted area (n=6 for 72 hours analyses, n=9 for 6 weeks analyses). The obtained tissue samples were stained according to a haematoxylin-eosin (H&E) and Elastica van Gieson (EVG) protocol. Short-term immunohistological evaluation on specimens obtained 72 hours after induction of AMI was performed using antibodies directed to CD68 (MCA341R, AbD Serotec, UK) and c-kit (sc-168, Santa Cruz Biotechnology, USA). Image J planimetry software (Rasband, W.S., Image J, U.S. National Institutes of Health, USA) was utilized in order to measure the extent of myocardial infarction after 6 weeks.

Enzymatic digestion of infarcted myocardium and flow cytometric analysis

Hearts were explanted from 6 rats with LAD ligation three days after infarction was induced (n=3 APOSEC injected rats, n=3 medium controls). The infarcted areas of explanted hearts were excised, cut into small cubic pieces (1mm in diameter) and incubated with collagenase (2.4 U/ml, Sigma Aldrich, USA) for 12 hours at 4°C as previously described [40, 41]. After that digestion period, cell suspensions were washed and obtained cells were subsequently incubated with primary antibodies directed to CD68 (MCA341R, AbD Serotec, UK) and c-kit (sc-168, Santa Cruz Biotechnology, USA). After an incubation period with a secondary antibody, cell suspensions were analysed for total CD68+ and c-kit+ cell numbers by means of flow cytometry (FACS Calibur, Becton Dickinson, USA).

Assessment of cardiac function by means of echocardiography

Echocardiographic examinations were conducted on a Vivid 7 system (General Electric Medical Systems, USA) as previously described. All measurements were performed by an experienced evaluator blinded to treatment or control groups. Values for left ventricular ejection fraction (EF), shortening fraction (SF), left ventricular end-systolic diameters (LVESD), left ventricular end-diastolic diameters (LVEDD), left ventricular end-systolic volumes (LVESV) and left ventricular end-diastolic volumes (LVEDV) were assessed.

Large animal AMI model

In order to investigate the cardioprotective effects of APOSEC preparations, we opted for a large animal model of the clinically more relevant setting of ischaemia and reperfusion. For this setting, the porcine closed chest reperfused AMI infarction model was chosen [42-44]). In this model, conditions are more similar to those in human AMI and primary percutaneous coronary intervention (PCI) than in any other animal model, and they thus allow a translational research approach. These large

animal experiments were conducted at the Institute of Diagnostics and Oncoradiology, University of Kaposvar, Hungary and were approved by the University of Kaposvar (ethics vote: 246/002/SOM2006, MAB-28-2005).

In total, 32 adolescent pigs (female Large Whites weighing approximately 30kg) were sedated with 12 mg/kg ketamine hydrochloride, 1 mg/kg xylazine and 0.04 mg/kg atropine. An intratracheal intubation was then performed to maintain thorough anaesthesia with a mixture of isoflurane, O_2 and N_2O. During anaesthesia, O_2 saturation and electrocardiography were monitored continuously. Vascular access to the right femoral artery and the right femoral vein was performed and 6 Fr (French scale) and 7 Fr introduction sheaths were then inserted into artery and vein, respectively. Heparin (200 IU/kg) was administered and a 6 Fr guiding catheter (Medtronic Inc., USA) was introduced into the left coronary ostium and a coronary angiography was performed (using Ultravist contrast medium, Bayer Healthcare, Germany).

For the induction of AMI, a balloon catheter (diameter: 3 mm, length: 15 mm; Boston Scientific, USA) was inserted into the LAD right after the origin of the second major diagonal branch. The LAD was then subsequently occluded by inflating the balloon slowly at 4 – 6 standard atmospheres (atm), (n=11 animals in the control group, n=10 in the treatment high dose and n=7 in the treatment low dose group). The occlusion of the LAD was controlled with angiography. Forty minutes after start of the occlusion, the lyophilised supernatant obtained from $250 * 10^6$ (low dose group), $1 * 10^9$ (high dose group) irradiated apoptotic porcine PBMC or lyophilised serum-free cell culture medium (CellGro Medium, Cell Genix, Germany) was resuspended in 250 ml of 0.9% physiologic sodium chloride solution and was administered intravenously over the next 25 minutes. 90 minutes after LAD occlusion, the balloon was slowly deflated and reperfusion was established again. A control coronary angiography was performed to prove the patency of the infarct-related artery and to exclude arterial injury in all animals. Moreover, all animals received 75mg clopidogrel and 100mg acetylsalicylic acid.

In situ viability staining using tetrazolium chloride

Either 24 hours or 30 days after AMI, euthanasia was performed by the administration of saturated potassium chloride. In order to delineate infarcted (necrotic) areas and areas at risk for ischaemia *in situ* double-staining with 1% Evans blue dye and a 4% solution of 2,3,5-triphenyltetrazolium chloride (TTC) was performed after 24 hours. In short, after explantation of the heart, the LAD was occluded again at same position where the balloon was situated before and both coronary arteries were perfused with an Evans blue solution to delineate the area at risk and non-risk regions of the myocardium. The hearts were cut in into 7 mm thick slices starting from the apex towards the level of the occlusion (6-7 layers per heart). The slices were then incubated in 500 ml of TTC solution at 37 °C in a shaking water bath for 20 minutes. Subsequently, all slices underwent an overnight bleach cycle at room temperature in 4,5% formalin. After bleaching, slices were photographed using a digital camera (Panasonic HDC-HS700, Japan) mounted on a fixed stand. Planimetric analysis was performed using Image J software (Rasband, W.S., Image J, U.S. National Institutes of Health, USA).

Hearts of all animals in the 30 days follow-up groups were fixed in formalin and embedded in paraffin for histological staining (H&E, Movat's pentachrome staining).

In order to prove that the coronary circulation was comparable between all three treatment groups, the Bypass Angioplasty Revascularization Investigation Myocardial Jeopardy Index (BARI score) was calculated based on LAD and LCX pre-occlusion angiograms as previously described [45].

Measurement of Troponin I levels after AMI

Serum samples were obtained from venous blood at the start of the experimental procedure, at the start of reperfusion and after 24 hours. Levels of Troponin I were determined by ELISA (Uscn Life Science Inc., China) according to the manufacturer's instructions.

Determination of cardiac functional parameters after AMI by magnetic resonance imaging (MRI)

Cardiac MRI was performed using a 1.5-T clinical scanner (Avanto, Siemens, Germany) three and 30 days after LAD occlusion as previously described [46]. MR images were acquired using a retrospectively ECG-gated, steady-state free precession cine MRI technique in short-axis and long-axis views of the heart. Delayed enhancement images were obtained after injection of 0.05 mmol/kg of contrast medium, short-axis and long-axis images were obtained 10 to 15 minutes after injection. The images were analysed using Mass 6.1.6 software (Medis, The Netherlands). After segmentation of the left ventricular endocardial and epicardial borders, end-diastolic and end-systolic volumes and left ventricular ejection fractions were calculated. The left ventricular and infarcted myocardial mass was determined from the cine and delayed enhancement MR images. The extent of myocardial infarction was expressed relative to the left ventricular mass. Data analyses and interpretations were performed by an experienced observer blinded to all study results.

Cell culture of primary human cardiomyocytes and immunoblot analysis

Primary human ventricular cardiac myocytes were obtained (CellSystems Biotechnologie, Germany) and cultured in cardiac myocyte medium (CellSystems Biotechnologie, Germany) at 37°C. In order to investigate the cytoprotective activity of APOSEC preparations, $3*10^5$ human cardiomyocytes were seeded in 6-well plates and cultivated in either basal medium without serum and growth factors or in basal medium supplemented with $APOSEC^H$ (APOSEC derived from $0.25*10^6$, $2.5*10^6$ and $25*10^6$ PBMC) for 24 hours.

For Western Blot analysis, $3*10^5$ human cardiac myocytes were incubated with $APOSEC^H$ (PBMC cell density for APOSEC production, $2.5*10^6$ per ml) or with lyophilised UltraCulture medium for 5, 10, 30 and 60 minutes and for 24 hours. Immunodetection was performed with anti-phospho-c-Jun (1 µg/ml, New England Biolabs, USA), anti-phospho-CREB (1 µg/ml, New England Biolabs, USA), anti-

phospho-AKT (1 µg/ml, New England Biolabs, USA), anti-phospho-Erk1/2 (1 µg/ml, New England Biolabs, USA), anti-phospho-Hsp27 (Ser15) (1 µg/ml, New England Biolabs, USA), anti-phospho-Hsp27 (Ser85), anti-phospho-BAG1 (C-16) (1 µg/ml, Santa Cruz Biotechnology, Germany), anti-Bcl-2 (2µg/ml, Acris, Germany), followed by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antisera (dilution 1:10000; Amersham BioSciences, Germany). In parallel, identical blots were performed for the equivalent non-phosporylated factors as controls. Moreover, a membrane-array (R&D Systems) analysing apoptosis mediating factors was performed with lysates from cardiac myocytes treated with either medium or $APOSEC^{H}$ (PBMC cell density $2.5*10^{6}$) for 24 hours according to the manufacturer's instructions.

Statistical methods

All statistical analyses were performed using Graph Pad Prism software (Graph Pad Software, USA). Data are shown as mean ± standard error of the mean (SEM). The Wilcoxon-Mann-Whitney-test or Student's t-test were utilized to calculate significances between groups. In boxplot figures, whiskers indicate minimums and maximums, the upper edge of the box indicates the 75th percentile and the lower one indicates the 25th percentile. P-values <0.05 were considered statistically significant.

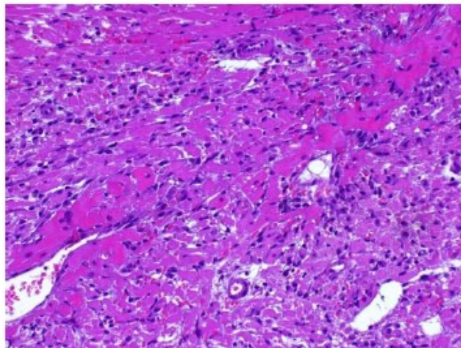
Results

Results of experiments investigating the cardioprotective properties of apoptotic PBMC [34]

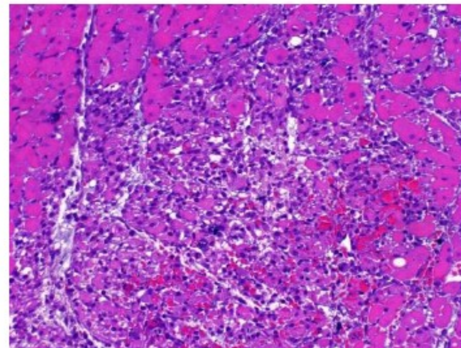
Histological analysis of cardiac specimens obtained three days after AMI

Three days after ligation of the LAD, hearts were explanted, fixed in formalin and stained histologically by means of H&E histology. In control animals and rats injected with non-irradiated viable PBMC, the cellular infiltrate in the infarcted myocardium evidenced a mixed pattern consisting of neutrophils, monocytes /macrophages and dystrophic or necrotic cardiac myocytes. In both treatment groups (intravenious injection of IA-PBMC, intramyocardial injection of IA-PBMC), the cellular infiltrate appeared more monomorphic and much denser compared to controls.
An analysis of cardiac sections performed three days after AMI by means of planimetry evidenced a significant reduction of the total infarcted area.

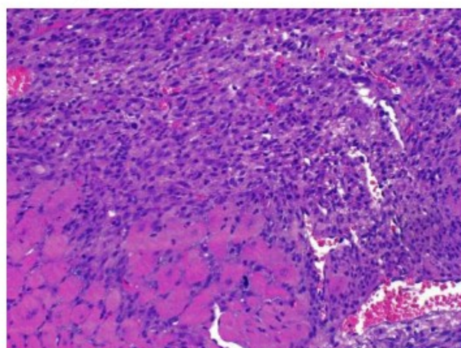
Medium Control



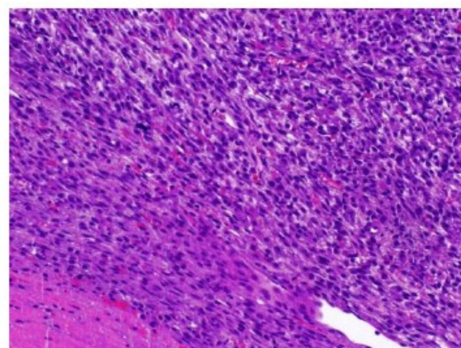
Viable PBMC IV



Apoptotic PBMC IV



Apoptotic PBMC IM



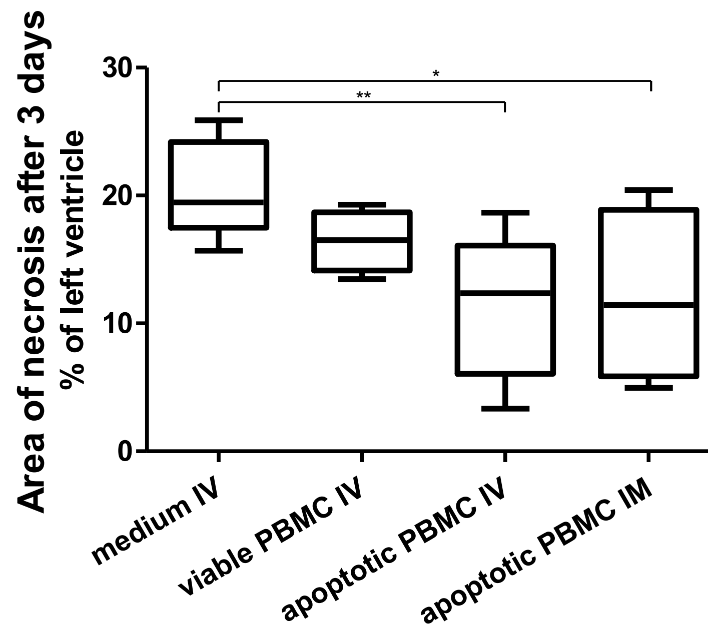


Figure 5 shows representative images of H&E stained specimens obtained three days after infarction and a quantification of the infarcted myocardium by planimetry.

Immunohistological staining for CD68, c-kit and VEGF receptor 2

In order to further characterize the cellular infiltrate we performed analyses by immunohistology for the markers CD68 (expressed on macrophages) and c-kit and VEGF receptor 2 (FLK1), both expressed on endothelial progenitor cells (EPC). Compared to the two control groups, much higher numbers of cells staining positive for CD68 were found in rats injected with irradiated apoptotic PBMC. The highest numbers were detected in animals that underwent direct intramyocardial injection of IA-PBMC. Moreover, the number of cells staining positive for the markers c-kit and FLK1 was increased in rats that were injected with IA-PBMC (either intravenously or intramyocardially), especially in the epicardial regions of the infarcted myocardium.

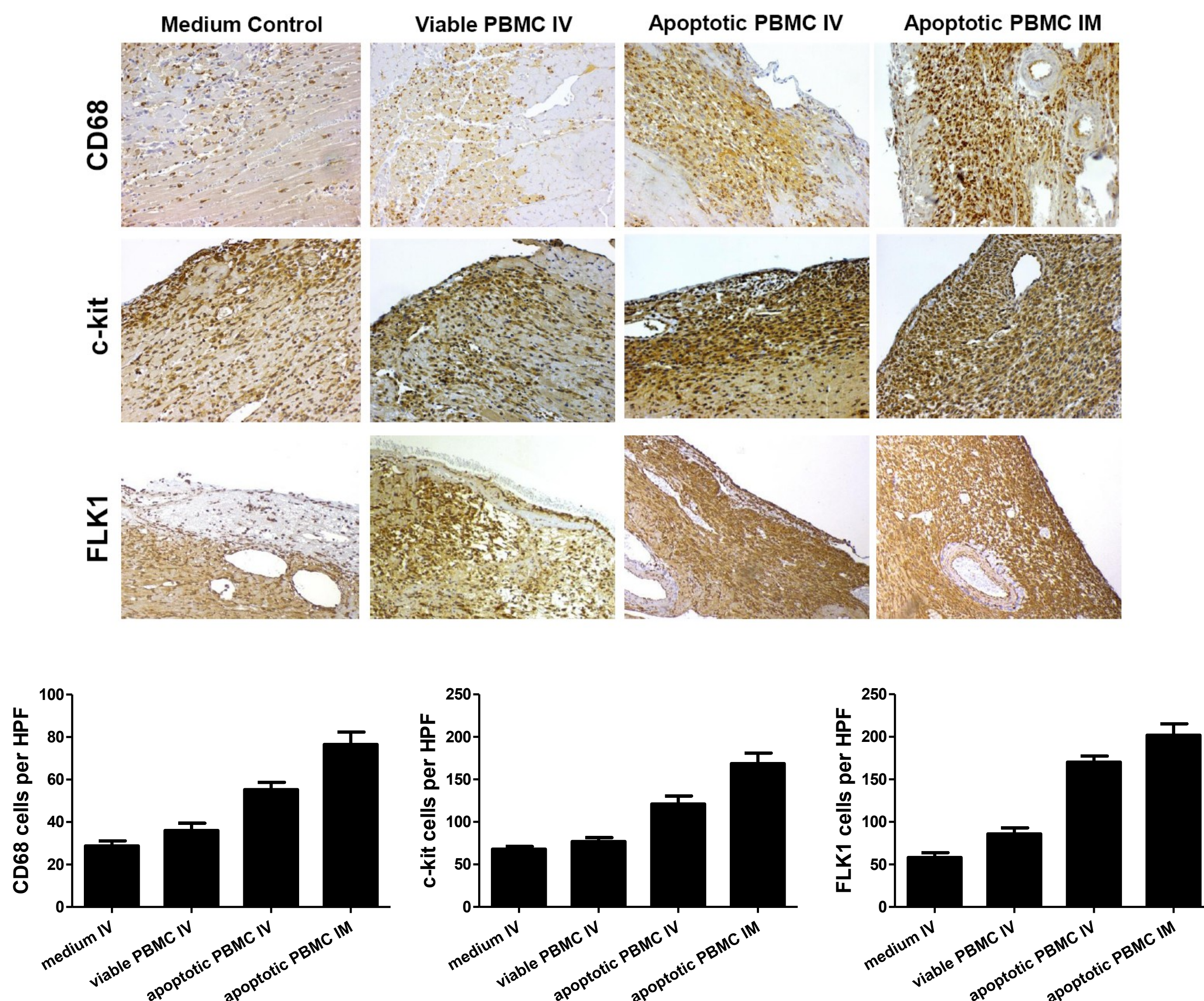


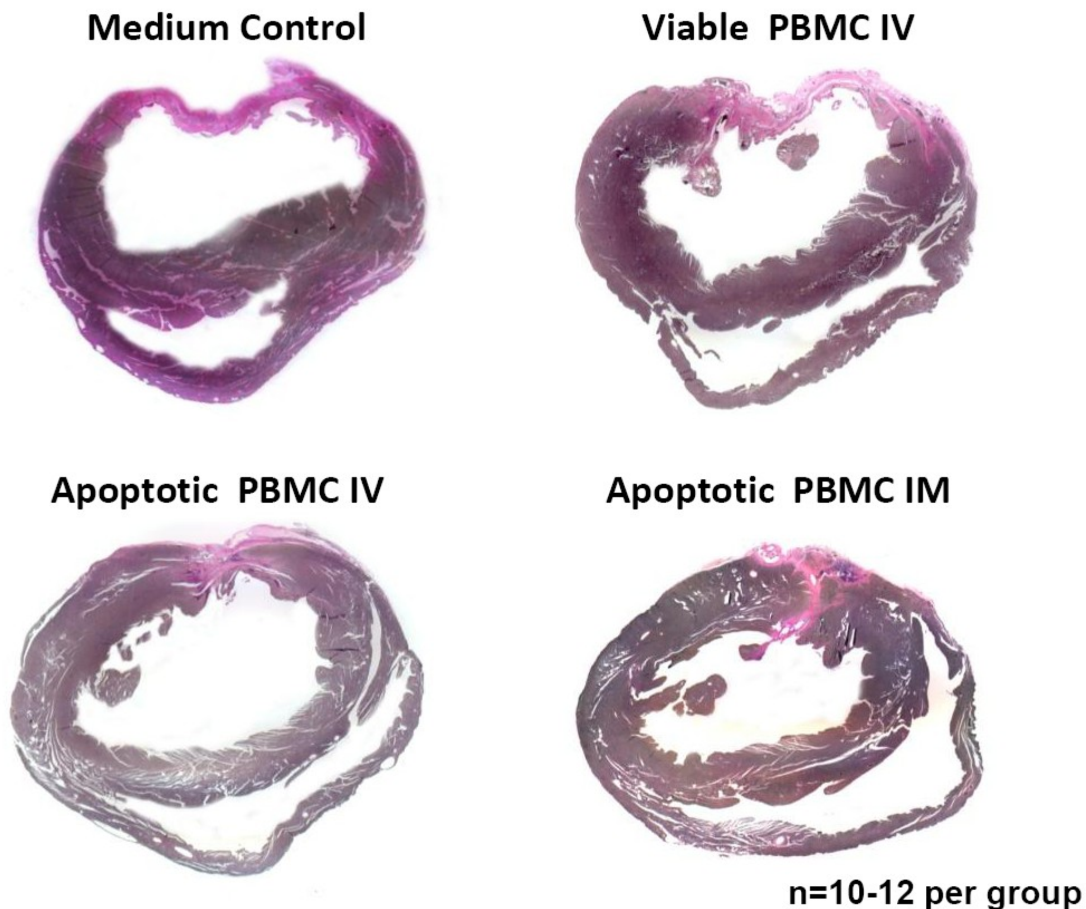
Figure 6 shows representative images of heart specimens stained for CD68, c-kit and FLK1 by means of immunohistology. Bar charts show the results obtained by quantification of high power fields (HPF).

The total number of cells quantified per high power-field (HPF) were 28.6±2.4 (±SEM) in control animals, 36.0±3.5 (±SEM) in animals injected with non-irradiated viable cells compared to 55.3±3.4 and 76.5±5.9 (±SEM) in rats injected with irradiated apoptotic cells (IV or IM, respectively). Within the infiltrate, most of the monocytic cells were identified to be highly positive for the marker c-kit and FLK1. HPF cell counts for c-kit were 68.0±3.1 (±SEM) in controls, 77.0±4.6 (±SEM) in rats that were injected with viable cell compared to 121.2±9.4 (±SEM) in intravenously (IV) and 168.6±12.4 (±SEM) in intramyocardially (IM) injected animals (see Figure 6). In specimens stained for FLK-1, these values were 58.3±5.6 (±SEM) in controls,

86.0±7.0 (±SEM) for viable cell injected rats, 170.3±7.1 (±SEM) for intravenously and 202.0±9.4 (±SEM) for intramyocardially injected animals (see Figure 6, n=5-6 per group).

Histological evaluation six weeks after LAD ligation

In order to determine the extent of fibrotic scar tissue within the left ventricular myocardium, cardiac specimens obtained six weeks after induction of AMI by LAD ligation were stained with Elastica van Gieson staining. A significant reduction in regards to scar dimension was found for animals injected with suspension of irradiated PBMC compared to controls. In both control groups, large infarct were common (between 14% and 25% of the left ventricle) whereas a significant reduction to values between 6% and 8% was evident in rats treated with apoptotic PBMC ($p<0.01$ vs. controls, $p<0.05$ vs. viable cell injected animals, n=10-12 per group). Moreover, the ventricular geometry was almost completely preserved in treated animals whereas signs of dilation could be found in animals injected with medium or non-irradiated cells.



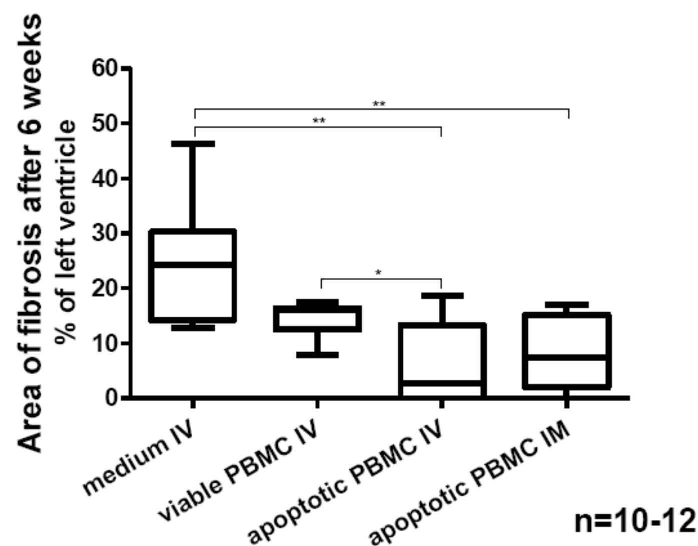


Figure 7 Hearts explanted from apoptotic cell injected rats six weeks after induction of AMI evidenced less myocardial damage compared to controls. Hearts obtained from medium as well as from viable cell injected rats appear more dilated and furthermore also show a greater extension of fibrotic tissue. A planimetric analysis performed on specimens collected six weeks after LAD ligation showed a mean scar extension of 25% in medium injected controls, 14% in viable cell injected animals compared to 6% (IV) and 8% (IM) in rats adminstered with apoptotic PBMC.

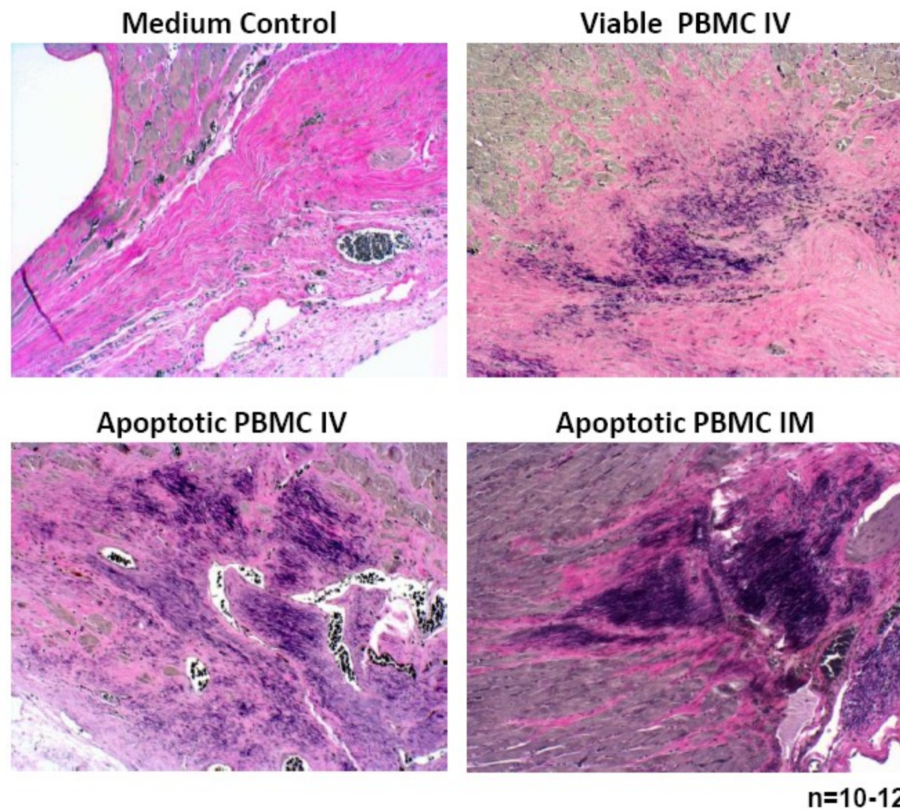
Assessment of cardiac function by echocardiography

Six weeks after induction of AMI by LAD ligation, all animals were sedated and parameters of cardiac function were assessed by means of echocardiography. In healthy rats without induction of AMI (i.e. sham operated animals) functional parameters were as follows: 60% ±4 (left ventricular ejection, EF), 29% ±2 (shortening fraction, SF), 9.2mm ±0.4 (left ventricular end-diastolic diameter, LVEDD), 6.5 mm ±0.3 (left ventricular end-systolic diameter, LVESD), 1.7 ml ±0.3 (left ventricular end-diastolic volume, LVEDV) and 0.7 ml ±0.1 (left ventricular end-systolic volume, LVESV). In control animals (injection of culture medium) mean EF, SF, LVEDD, LVESD, LVEDV and LVESV were impaired: 43% ±2 (EF), 19% ±1 (SF), 10.4 mm ±0.2 (LVEDD), 8.5 mm ±0.2 (LVESD), 2.3 ml ±0.1 (LVEDV) and 1.3 ml ±0.1 (LVESV). This was also true for animals that were injected with non-irradiated viable PBMC suspensions as evidenced by similar values: 42% ±3 (EF), 18% ±2 (SF), 11.0 mm ±0.4 (LVEDD), 9.0 mm ±0.5 (LVESD), 2.7 ml ±0.3 (LVEDV) and 1.6 ml ±0.2 (LVESV).
A significant improvement of regards to functional parameters were found in rats with intravenous injection of apoptotic cell suspensions: 53% ±4 (EF), 25% ±3 (SF), 8.9 mm ±0.3 (LVEDD), 6.8 mm ±0.4 (LVESD), 1.6 ml ±0.2 (LVEDV) and 0.8 ml ±0.1 (LVESV). Concomitantly, animals that underwent direct intramyocardial injection of apoptotic cell suspensions, these functional parameters were improved as well: 55% ±4 (EF), 26% ±2 (SF), 9.8 mm ±0.4 (LVEDD), 7.4 mm ±0.5 (LVESD), 2.1 ml ±0.2 (LVEDV) and 0.9 ml ±0.2 (LVESV).

Analysis of the composition of left ventricular scar tissue six weeks after AMI

By analysing Elastica van Gieson stained cardiac specimens obtained six weeks after AMI the composition of the fibrotic scar tissue was evaluated microscopically. Of special interest was the border zone between viable myocardium and scar tissue where a highly remarkably accumulation of elastic fibres was detected in animals injected with apoptotic cells in comparison to controls. To a lesser extent this phenomenon was also detectable in rats injected with viable cell suspensions. By

utilizing planimetry software, it was shown that the fibrotic scar in apoptotic cell (IV and IM) injected rats was composed by 5.5%±1.1 and 8.9%±2.2 of elastic fibres compared to 0.2%±0.1 in controls and 2.9%±0.2 in viable injected animals, ($p<0.001$ vs. control, n=10-12 animals per group).



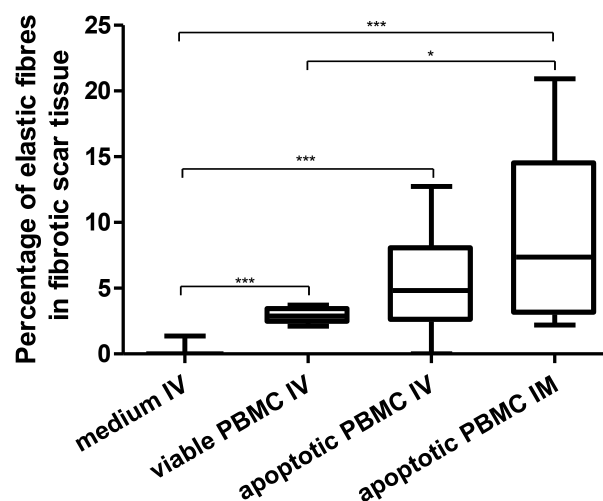


Figure 8: shows the accumulation of elastic fibres in the border zone between viable myocardium and scar tissue. Injection of apoptotic cells significantly increased elastin accumulation.

These higher amounts of elastic fibre accumulation stands in relation to higher levels of cells staining positively for Insulin-like growth factor I (IGF-I) and Fibroblast growth factor 2 (FGF-2) in treated animals compared to controls: rats injected with apoptotic

PBMC suspensions evidenced 36.0±3.3 cells staining positively for IGF-I per high power field (HPF) and 49.8±5.2 for FGF-2 per HPF in immunohistological analyses of cardiac specimens obtained 72 hours after induction of AMI. In comparison, only 7.0±1.6 IGF-I and 31.5±2.3 FGF-2 positive cells were detectable in control animals.

In order to further specify the association of elastin and collagen production within myocardial scar tissue and mechanisms induced by apoptotic cells, RT-PCR analyses were conducted. When exposing fibroblasts to cell culture supernatants obtained from apoptotic PBMC or apoptotic cells co-incubated with viable cells, elastin expression increased only slightly by 1.2 to 1.4 fold. The expression of collagen type III and IV increased moderately by 1.9 to 2.5 fold compared to controls (Fig. 3I). Supernatants derived from apoptotic cells also increased the expression of IL-8 (4-6.5 fold), MMP1 (18-31 fold), MMP3 (10-16 fold) and MMP9 (4-7 fold) in fibroblasts indicating a mechanistic cross-talk between apoptotic cells, their secretome and resident cells that could be accounted for alterations in the compositions of the extracellular matrix.

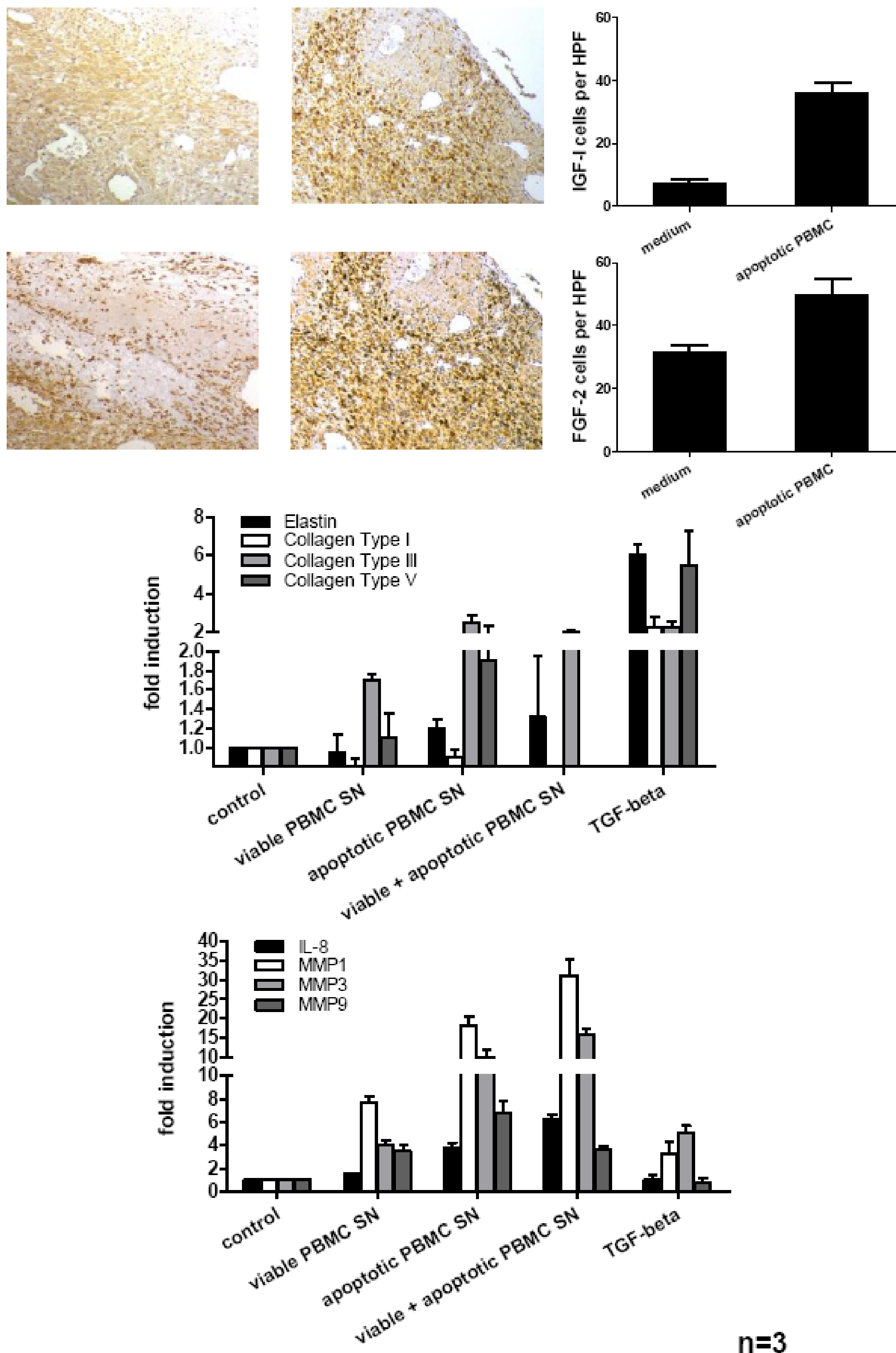


Figure 9 show immunohistological tissue sections stained for IGF-I and FGF-2 in controls and treated animals. Below data from RT-PCR analyses is shown. Human fibroblasts incubated with supernatants (SN) derived from apoptotic cells increased expression of elastin, collagen type III and IV, IL-8, MMP1, MMP3 and MMP9 transcripts.

Different secretion patterns of cytokines, chemokines and growth factors in apoptotic cells

Based on the finding in RT-PCR analysis that the expression of transcripts for IL-8 and MMPs was up-regulated in apoptotic PBMC, culture supernatants obtained from irradiated apoptotic and non-irradiated viable cells were analysed for 274 cytokines and growth factors by membrane arrays. Considerable differences were observed (amongst others) for IL-8, VEGF, MMP3, MMP9, IL-16, ENA-78 and MIP-1alpha (see table below). The results were analysed using Image J software. The secretion levels were expressed as relative to the respective positive control in column 1 and 2 (VIABLE SN, APO SN). The fold increase over supernatants obtained from non-irradiated cells was expressed in column 3 (APO SN divided by VIABLE SN).

Cytokines	VIABLE SN	APO SN	Fold increase	Cytokines	VIABLE SN	APO SN	Fold increase
Eotaxin-2	0,08	0,01	0,07	CTACK	0,02	0,04	1,79
IGF-I	0,07	0,06	0,90	ICAM-1	0,25	0,47	1,86
Leptin	0,06	0,06	1,02	I-TAC	not detect.	not detect.	not detect.
PDGF-BB	0,92	0,67	0,73	TECK	not detect.	not detect.	not detect.
Eotaxin-3	not detect.	not detect.	not detect.	Dtk	not detect.	not detect.	not detect.
IL-10	not detect.	not detect.	not detect.	ICAM-3	not detect.	not detect.	not detect.
LIGHT	not detect.	not detect.	not detect.	Lymphotactin	0,05	0,07	1,41
RANTES	0,97	1,74	1,80	TIMP-1	0,30	0,44	1,46
FGF-6	0,07	0,06	0,81	EGF-R	0,08	0,03	0,45
IL-13	not detect.	not detect.	not detect.	IGFBP-3	0,13	0,15	1,16
MCP-1	0,23	0,23	0,99	MIF	0,19	0,43	2,24
SCF	not detect.	not detect.	not detect.	TIMP-2	0,26	0,45	1,72
FGF-7	not detect.	0,06	APO SN only	ENA-78	0,21	0,56	2,66
IL-15	not detect.	not detect.	not detect.	IGFBP-6	0,14	0,35	2,43
MCP-2	0,15	0,07	0,48	MIP-1α	0,05	0,13	2,45
SDF-1	not detect.	not detect.	not detect.	Thrombopoietin	0,02	0,06	2,43
Fit-3 Ligand	not detect.	not detect.	not detect.	Fas/TNFRSF6	0,18	0,33	1,85
IL-16	0,15	0,75	4,99	IGF-I SR	0,03	0,05	1,89
MCP-3	0,07	0,09	1,19	MIP-1β	0,16	0,16	0,97
TARC	0,13	not detect.	VIABLE SN only	TRAIL R3	0,15	0,36	2,40
Angiogenin	0,67	0,92	1,37	Acrp30	1,22	2,19	1,80
Fractalkine	not detect.	not detect.	not detect.	FGF-4	0,21	0,34	1,61
IL-1α	0,09	0,02	0,27	IL-1 R4/ST2	0,05	0,04	0,87
MCP-4	not detect.	not detect.	not detect.	MIP-3β	not detect.	not detect.	not detect.
TGF-β1	0,11	0,05	0,48	TRAIL R4	not detect.	not detect.	not detect.
BDNF	0,42	0,44	1,05	AgRP	0,07	0,09	1,39

GCP-2	not detect.	not detect.	not detect.	FGF-9	0,18	0,22	1,24
IL-1β	0,17	0,06	0,36	IL-1 RI	0,06	0,19	2,99
M-CSF	0,11	0,11	1,00	MSP-α	0,27	0,45	1,67
TGF-β 3	not detect.	0,08	APO SN only	uPAR	0,27	0,41	1,48
BLC	not detect.	not detect.	not detect.	Angiopoietin-2	0,15	0,30	1,98
GDNF	not detect.	not detect.	not detect.	GCSF	not detect.	not detect.	not detect.
IL-1ra	0,27	0,43	1,62	IL-11	not detect.	not detect.	not detect.
MDC	0,16	0,29	1,83	NT-4	0,08	0,09	1,08
TNF-α	0,10	0,05	0,53	VEGF	0,08	0,25	3,34
BMP-4	0,16	0,20	1,28	Amphiregulin	0,10	0,13	1,40
GM-CSF	not detect.	not detect.	not detect.	GITR-Ligand	0,08	0,12	1,58
IL-2	not detect.	not detect.	not detect.	IL-12 p40	0,22	0,55	2,49
MIG	not detect.	not detect.	not detect.	Osteoprotegerin	0,06	0,10	1,82
TNF-β	0,14	0,13	0,92	VEGF-D	0,08	0,23	2,86
BMP-6	0,14	not detect.	VIABLE SN only	Axl	0,13	0,13	0,96
I-309	0,27	0,25	0,91	GITR	0,13	0,16	1,22
IL-3	0,24	0,24	0,99	IL-12 p70	0,08	0,11	1,42
MIP-1δ	0,15	0,11	0,71	Oncostatin M	0,11	0,19	1,78
CK β 8-1	0,15	0,16	1,09	bFGF	0,03	0,06	1,95
IFN-γ	not detect.	not detect.	not detect.	GRO	0,65	1,06	1,63
IL-4	not detect.	not detect.	not detect.	IL-17	not detect.	not detect.	not detect.
MIP-3α	0,25	0,16	0,64	PIGF	0,15	0,24	1,61
CNTF	not detect.	not detect.	not detect.	b-NGF	0,03	0,05	1,61
IGFBP-1	not detect.	not detect.	not detect.	GRO-α	0,25	0,33	1,36
IL-5	not detect.	not detect.	not detect.	IL-2 R alpha	0,09	0,22	2,54
NAP-2	0,48	0,77	1,61	sgp130	0,15	0,29	1,94
EGF	0,74	1,00	1,34	BTC	0,11	0,13	1,23
IGFBP-2	0,10	0,10	1,08	HCC-4	0,07	0,11	1,47
IL-6	0,34	0,05	0,15	IL-6 R	0,34	0,85	2,47
NT-3	0,16	0,18	1,13	sTNF RII	0,35	0,77	2,20
Eotaxin	not detect.	not detect.	not detect.	CCL-28	not detect.	not detect.	not detect.
IGFBP-4	0,10	0,20	2,05	HGF	0,18	0,16	0,89
IL-7	not detect.	not detect.	not detect.	IL-8	0,43	1,48	3,44
PARC	not detect.	not detect.	not detect.	sTNF-RI	0,12	0,12	0,99
Endoglin	not detect.	not detect.	not detect.	Furin	0,12	0,13	1,10
IL-21R	not detect.	not detect.	not detect.	LYVE-1	0,15	0,17	1,09
PDGF AA	0,34	0,40	1,18	Osteopontin	0,10	0,07	0,71
VE-Cadherin	not detect.	not detect.	not detect.	Trappin-2	0,05	0,09	1,87
ErbB3	0,08	0,07	0,92	Galectin-7	not detect.	not detect.	not detect.
IL-5 R alpha	0,04	0,07	1,74	Marapsin	0,07	0,06	0,84
PDGF-AB	0,18	0,20	1,06	PAI-I	0,19	0,30	1,60
VEGF R2	0,06	not detect.	VIABLE SN only	TREM-1	0,05	0,06	1,29
E-Selectin	0,09	0,12	1,25	GDF-15	0,13	0,13	1,03
IL-9	0,11	0,11	0,99	MICA	0,07	0,07	0,97
PDGF-R alpha	not detect.	not detect.	not detect.	Platelet Factor 4	0,11	0,14	1,23
VEGF R3	0,18	0,20	1,06	TSH	0,05	0,06	1,40

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Fas Ligand	0,13	0,07	0,57	Growth Hormon	0,05	0,04	0,78
IP-10	0,14	0,16	1,16	MICB	0,09	0,08	0,92
PDGF-R beta	not detect.	not detect.	not detect.	PSA-total	0,07	0,08	1,15
ICAM-2	0,28	0,46	1,66	TSLP	not detect.	not detect.	not detect.
LAP	0,24	0,33	1,38	Adiposin	0,29	0,43	1,49
PECAM-1	0,11	0,10	0,96	IL-10 R alpha	0,09	0,15	1,64
Activin A	not detect.	not detect.	not detect.	MMP-2	0,08	0,12	1,47
IGF-II	0,17	0,22	1,29	RAGE	0,05	0,10	2,07
Leptin R	0,05	0,07	1,37	VCAM-1	0,05	0,07	1,35
Prolactin	0,09	0,07	0,86	BCAM	0,09	0,12	1,39
ALCAM	0,17	0,22	1,30	IL-22	0,07	0,09	1,35
IL-1 R II	0,06	0,05	0,77	MMP-7	0,12	0,13	1,10
LIF	0,14	0,15	1,09	RANK	0,09	0,07	0,88
SCF R	0,27	0,28	1,04	VEGF-C	0,15	0,14	0,91
B7-1(CD80)	0,14	0,08	0,58	CD30	0,12	0,16	1,27
IL-10 R beta	not detect.	not detect.	not detect.	IL-28A	0,19	0,21	1,12
L-Selectin	0,21	0,23	1,07	MMP-8	0,19	0,30	1,54
SDF-1beta	not detect.	not detect.	not detect.	Resistin	0,08	0,08	0,97
BMP-5	0,24	0,17	0,73	XEDAR	0,07	0,10	1,39
IL-13 R alpha 2	0,17	0,25	1,46	CD40	0,06	0,05	0,89
M-CSF R	0,20	0,40	1,99	IL29	0,17	0,21	1,27
Siglec-5	0,63	0,87	1,37	MMP-10	0,05	0,08	1,51
BMP-7	not detect.	not detect.	not detect.	SAA	0,14	0,16	1,18
IL-18 BP alpha	0,14	0,09	0,65	Fcr RIIB/C	0,06	0,07	1,01
MMP-1	0,20	0,28	1,39	IL-31	0,05	0,08	1,64
TGF-alpha	0,12	0,10	0,85	NCAM-1	0,19	0,21	1,10
Cardiotrophin-1	not detect.	not detect.	not detect.	Siglec-9	0,06	0,05	0,84
IL-18 R beta	0,17	0,16	0,93	Ferritin	0,12	0,20	1,66
MMP-13	0,11	0,13	1,13	Insulin	0,10	0,07	0,65
TGF beta 2	0,19	0,21	1,13	Nidogen-1	0,26	0,30	1,16
CD14	0,36	0,57	1,55	TACE	0,06	0,10	1,58
MMP-3	0,08	0,20	2,58	FLRG	not detect.	not detect.	not detect.
MMP-9	0,10	0,20	1,93	Luteinizing Hormone	0,10	0,11	1,15
Tie-1	not detect.	not detect.	not detect.	NrCAM	0,08	0,11	1,30
CXCL- 16	0,31	0,45	1,48	TIM-1	0,04	0,04	0,85
IL-2 R beta	0,11	0,18	1,73	Follistatin	0,14	0,15	1,04
MPIF-1	not detect.	not detect.	not detect.	LIMPII	0,03	0,04	1,20
Tie-2	0,06	0,08	1,31	NRG1-beta 1	0,13	0,12	0,92
DR6 (TNFRSF21)	0,12	0,15	1,18	TRAIL R2	0,13	0,10	0,80
IL-2 R gamma	0,12	0,13	1,13	ACE-2	not detect.	not detect.	not detect.
NGF R	not detect.	not detect.	not detect.	Cathepsin S	0,08	0,12	1,52
TIMP-4	0,22	0,28	1,30	DKK-3	0,06	0,06	1,09
beta IG-H3	0,09	0,08	0,82	HVEM	0,10	0,16	1,58
Cripto-1	not detect.	not detect.	not detect.	Alpha-Fetoprotein	not detect.	not detect.	not detect.
ErbB2	not detect.	not detect.	not detect.	CCL14a	0,06	0,12	2,13

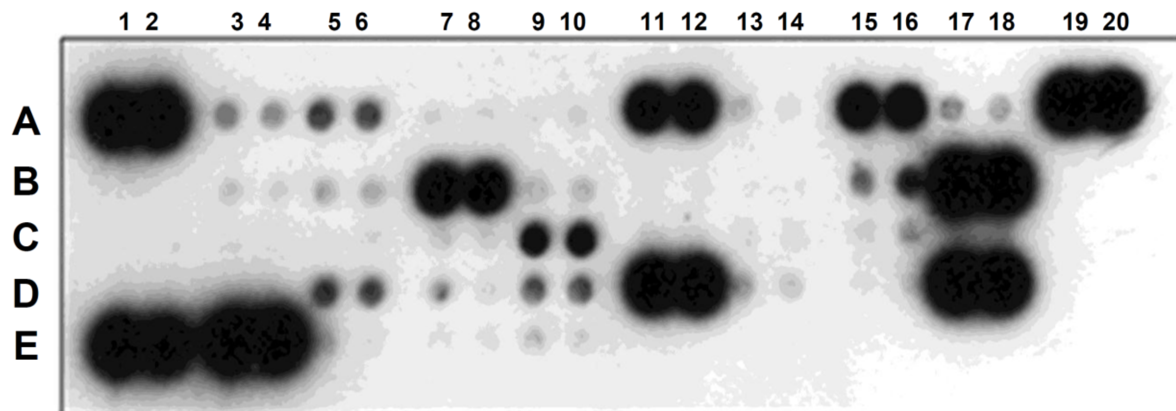
PSA-free	0,04	0,05	1,08	DKK-4	0,04	0,05	1,23
CA125	0,03	0,05	1,45	IL-13R1	0,10	0,12	1,17
CRP	0,09	0,13	1,44	Angiopoietin-1	0,12	0,20	1,63
Erythropoietin R	not detect.	not detect.	not detect.	CCL21	0,15	0,30	2,03
S-100b	not detect.	not detect.	not detect.	DPPIV	0,14	0,32	2,32
CA15-3	0,04	0,06	1,42	IL-17B	not detect.	not detect.	not detect.
DAN	0,04	0,04	1,07	Angiostatin	0,08	0,10	1,19
FSH	0,13	0,12	0,92	CD23	0,10	0,11	1,05
Shh N	0,07	0,09	1,25	E-Cadherin	not detect.	not detect.	not detect.
CA19-9	0,06	0,07	1,22	IL-17C	0,06	0,09	1,43
Decorin	0,14	0,19	1,37	ANGPTL4	not detect.	not detect.	not detect.
HB-EGF	0,07	0,07	0,90	CD40 Ligand	0,03	0,06	1,87
Thyroglobulin	not detect.	not detect.	not detect.	EDA-A2	not detect.	not detect.	not detect.
4-1BB	0,05	0,05	0,95	IL-17F	0,03	0,05	1,51
Carbonic Anhydrase IX	not detect.	not detect.	not detect.	Bate2 M	0,17	0,33	1,88
DKK-1	not detect.	0,04	APO SN only	CEA	0,15	0,16	1,02
hCGa, intact	not detect.	not detect.	not detect.	EG-VEGF	0,11	0,19	1,81
Ubiquitin+1	0,04	0,05	1,20	IL-17R	0,06	0,08	1,31
BCMA	not detect.	not detect.	not detect.	EpCAM	not detect.	not detect.	not detect.
CEACAM-1	0,08	0,05	0,55	Procalcitonin	not detect.	not detect.	not detect.

Table 1 shows a semi-quantitative analysis of soluble factors found in the supernatant of irradiated apoptotitc and non-apoptotic cells.

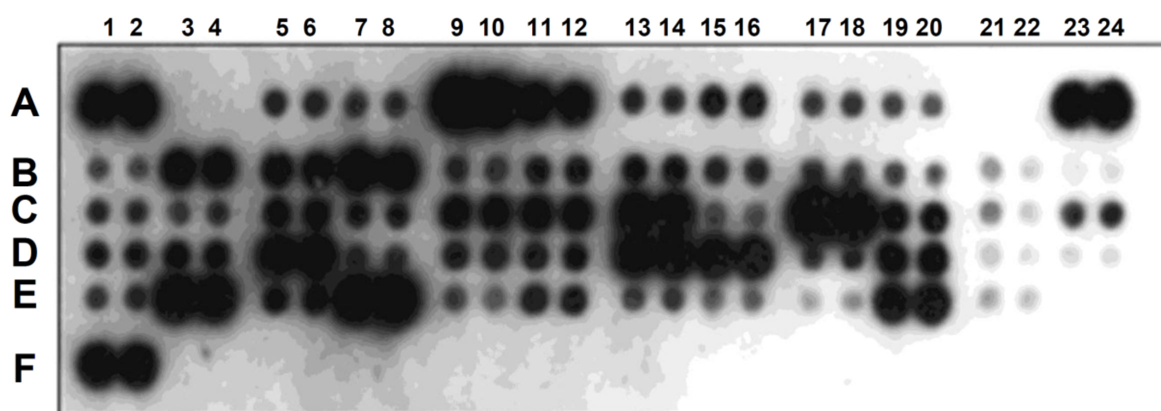
Results of experiments investigating the cardioprotective properties of supernatants obtained from apoptotic PBMC[39]

Analysis of soluble factors detectable in cell culture supernatants of irradiated human PBMC (termed APOSEC^{H})

In order to further determine the broad spectrum of soluble factors released by apoptotic cells we analysed the cell culture supernatant of irradiated cells by means of proteome membrane arrays and ELISA. As described previously, human PBMC were irradiated with 60 Gy and cells were incubated for 24 hours. After that incubation period, cell-free supernatants were harvested and analysed for cytokines, chemokines and growth factors that are associated with tissue repair mechanisms, angiogenesis, progenitor cell mobilization and homing to sites of injury.
To gain a better overview over the whole bandwidth of regenerative factors released by apoptotic cells, we screened the supernatants for cytokines and pro-angiogenic mediators using commercially available proteome membrane arrays.



A1,2: Positive control	B1,2: ---	C1,2: ---	D1,2: ---	E1,2: Positive control
A3,4: C5a	B3,4: IL-1a	C3,4: IL-10	D3,4: IL-32α	E3,4: RANTES
A5,6: CD40 ligand	B5,6: IL-1β	C5,6: IL-12 p70	D5,6: CXCL10	E5,6: CXCL12/SDF-1
A7,8: G-CSF	B7,8: IL-1ra	C7,8: IL-13	D7,8: CXCL11	E7,8: TNF-α
A9,10: GM-CSF	B9,10: IL-2	C9,10: IL-16	D9,10: MCP-1	E9,10: sTREM-1
A11,12: GRO-α	B11,12: IL-4	C11,12: IL-17	D11,12: MIF	E11,12: ---
A13,14: CCL1/I-309	B13,14: IL-5	C13,14: IL-17E	D13,14: MIP-1α	E13,14: ---
A15,16: sICAM-1	B15-16: IL-6	C15,16: IL-23	D15,16: MIP-1β	E15,16: ---
A17,18: IFN-γ	B17,18: IL-8	C17,18: IL-27	D17,18: PAI-1	E17,18: ---
A19-20: Positive control	B19,20: ---	C19,20: ---	D19-20: ---	E19,20: Negative control



A1,2: Positive control	B1,2: TF	C1,2: GDNF	D1,2: MIP-1a	E1,2: Serpin B5	F1,2: Positive control
A3,4: ---	B3,4: CXCL16	C3,4: GM-CSF	D3,4: MMP-8	E3,4: PAI-1	F3,4: ---
A5,6: Activin A	B5,6: CD26	C5,6: HB-EGF	D5,6: MMP-9	E5,6: Serpin F1	F5,6: ---
A7,8: ADAMTS-1	B7,8: EGF	C7,8: HGF	D7,8: NRG1-β1	E7,8: TIMP-1	F7,8: ---
A9,10: Angiogenin	B9,10: EG-VEGF	C9,10: IGFBP-1	D9,10: Pentraxin 3	E9,10: TIMP-4	F9,10: ---
A11,12: Angiopoietin-1	B11,12: CD105	C11,12: IGFBP-2	D11,12: PD-ECGF	E11,12: TSP-1	F11,12: ---
A13,14: Angiopoietin-2	B13,14: Endostatin	C13,14: IGFBP-3	D13,14: PDGF-AA	E13,14: TSP-2	F13,14: ---
A15,16: Plasminogen	B15,16: Endothelin-1	C15,16: IL-1β	D15,16: PDGF-AB/BB	E15,16: uPA	F15,16: ---
A17,18: Amphiregulin	B17,18: FGF acidic	C17,18: IL-8	D17,18: Persephin	E17,18: Vasohibin	F17,18: ---
A19,20: Artemin	B19-20: FGF basic	C19,20: TGF-β1	D19,20: CXCL4	E19,20: VEGF	F19,20: ---
A21,22: ---	B21,22: FGF-4	C21,22: Leptin	D21,22: PIGF	E21,22: VEGF-C	F21,22: ---
A23,24: Positive control	B23,24: FGF-7	C23,24: MCP-1	D23,24: Prolactin	E23,24: ---	F23,24: Negative control

Figure 10 shows a semi-quantitative evaluation of soluble factors that are detectable in the supernatant of irradiated apoptotic cells.

As shown in Table 2, human PBMC secreted high amounts of various paracrine mediators. Compared to non-irradiated controls, higher concentrations of IL-8, GRO-alpha, ENA-78, RANTES, sICAM-1, MIF, VEGF, IL-1ra and IL-16 were detected in a cell density dependent fashion. In comparison to the aforementioned factors, little if any secretion was detected for factors such as MCP-1, IL-10, IGF-1, HGF, FGF-2, TGF-β, SDF-1, G-CSF and GM-CSF (see Table 2).

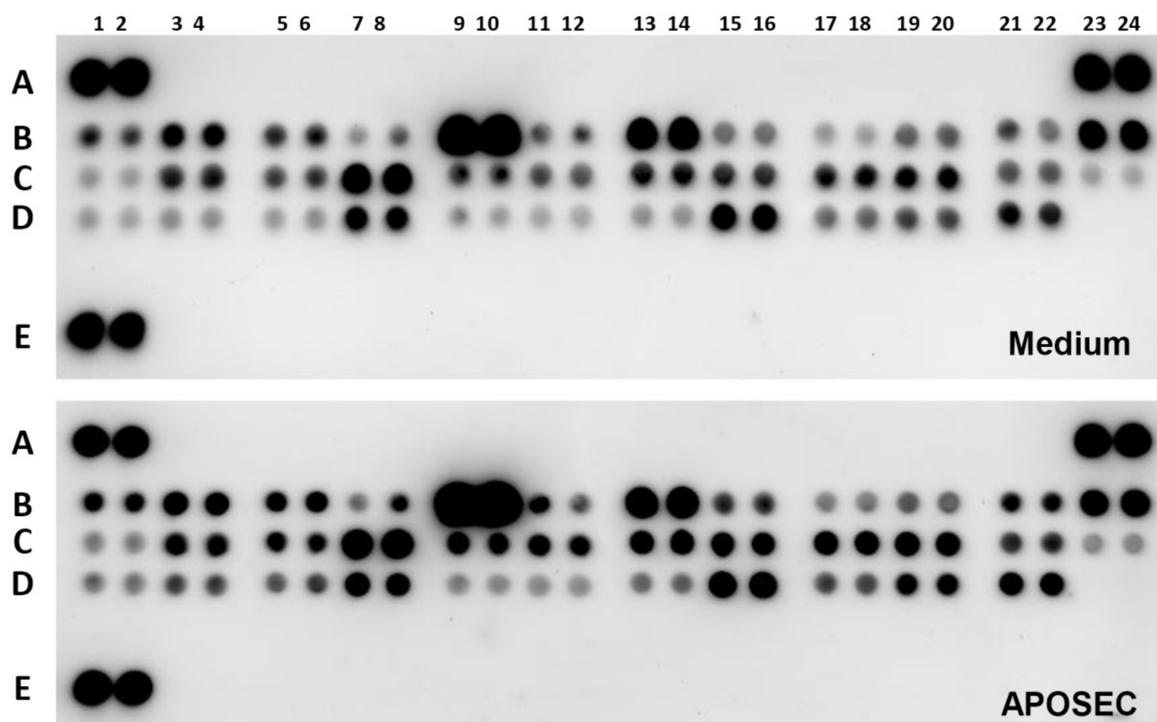
Analysis of soluble factors secreted by non-irradiated cells and irradiated apoptotic PBMC (APOSEC)

	viable PBMC			apoptotic PBMC			
soluble factors (ng/ml)	**$1*10^6$**	**$2.5*10^6$**	**$25*10^6$**	**$1*10^6$**	**$2.5*10^6$**	**$25*10^6$**	**sig.**
IL-8	1.74 ±0.40	1.93 ±0.09	10.49 ±3.53	1.22 ±0.29	2.30 ±0.13	18.01 ±2.87	*ns ns* ¥
GRO-alpha	0.17 ±0.09	0.36 ±0.09	2.06 ±1.58	0.07 ±0.02	0.48 ±0.09	3.95 ±0.93	*ns ns ns*
ENA-78	3.41 ±1.34	29.93 ±3.41	34.89 ±16.33	3.93 ±1.43	37.86 ±12.73	108.86 ±27.88	*ns ns* ¥
MCP-1	1.66 ±0.65	0.47 ±0.21	0.27 ±0.00	0.76 ±0.19	0.74 ±0.17	0.27 ±0.00	*ns ns ns*
RANTES	8.32 ±0.18	18.62 ±3.21	37.63 ±2.72	4.01 ±0.05	22.25 ±3.64	51.58 ±4.44	*ns ns ns*
HMGB1	0.63 ±0.39	3.44 ±2.11	33.57 ±6.45	2.74 ±0.27	6.46 ±1.12	20.51 ±3.62	† *ns ns*
MMP9	4.14 ±0.91	14.59 ±2.75	29.46 ±8.29	0.99 ±0.16	3.61 ±0.59	19.35 ±5.34	† ‡ *ns*
sICAM-1	0.14 ±0.04	1.43 ±0.25	7.43 ±0.85	0.42 ±0.25	2.09 ±0.42	9.40 ±1.29	*ns ns* ¥
$VEGF_{165}$	0.13 ±0.01	0.42 ±0.04	0.82 ±0.34	0.15 ±0.02	0.64 ±0.04	4.39 ±1.22	*ns ns* ¥
MIF	4.84 ±0.09	17.79 ±0.95	13.24 ±0.85	5.85 ±0.22	20.15 ±1.14	58.99 ±1.17	*ns ns* ¥
PAI-1	1.25 ±0.35	1.93 ±0.29	49.60 ±9.04	0.00 ±0.00	5.06 ±3.25	45.86 ±1.43	*ns ns ns*
IL-16	0.0 ±0.0	0.11 ±0.02	0.84 ±0.31	0.00 ±0.00	1.25 ±0.07	5.25 ±0.52	*ns* ‡ ¥
IL-1ra	0.35 ±0.09	0.52 ±0.17	2.16 ±0.96	0.13 ±0.04	0.41 ±0.17	6.43 ±1.33	*ns ns* ¥
IL-10	0.01 ±0.00	0.00 ±0.0	0.05 ±0.01	0.02 ±0.01	0.02 ±0.01	0.06 ±0.01	*ns ns ns*
IGF-I	0.00 ±0.00	0.01 ±0.0	0.03 ±0.02	0.00 ±0.00	0.01 ±0.01	0.03 ±0.03	*ns ns ns*
HGF	0.33 ±0.08	0.16 ±0.01	0.69 ±0.19	0.11 ±0.03	0.07 ±0.02	0.79 ±0.19	*ns ns ns*
FGF-2	0.56 ±0.02	0.53 ±0.00	0.59 ±0.01	0.48 ±0.01	0.53 ±0.02	0.55 ±0.02	*ns ns ns*
TGF-β	0.08 ±0.01	0.10 ±0.01	0.21 ±0.07	0.06 ±0.01	0.09 ±0.02	0.39 ±0.09	*ns ns ns*
SDF-1	0.17 ±0.0	0.19 ±0.0	0.22 ±0.03	0.16 ±0.01	0.15 ±0.07	0.12 ±0.04	*ns ns ns*
G-CSF	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	*ns ns ns*
GM-CSF	0.00 ±0.00	0.00 ±0.00	0.07 ±0.02	0.00 ±0.00	0.00 ±0.00	0.08 ±0.02	*ns ns ns*

Table 2: Analysis of soluble factors secreted by non-irradiated cells and irradiated apoptotic PBMC (APOSEC).
Cells were incubated in three different cell concentrations for 24 hours. Supernatants were analyzed for cytokines, chemokines and growth factors (n=5). † $p<0.05$ $1*10^6$ viable PBMC vs. $1*10^6$ apoptotic PBMC, ‡ $p<0.05$ $2.5*10^6$ viable PBMC vs. $2.5*10^6$ apoptotic PBMC, ¥ $p<0.05$ $25*10^6$ viable PBMC vs. $25*10^6$ apoptotic PBMC.

Up-regulation of anti-apoptotic and cytoprotective factors by supernatants of apoptotic cells

Based on the finding that irradiated apoptotic cells secreted a vast spectrum of cytokines and growth factors, we sought to investigate whether these mediators have a direct influence on cardiac myocytes. For these purpose, human cardiomyocytes were incubated together with $APOSEC^H$ in different concentrations. In order to gain an overview which anti-apoptotic or cytoprotective factors are involved in these mechanisms, we utilized a proteome membrane array (see Figure 11).



A1,2: pos con	B1,2: Bad	C1,2: TRAIL R17DR4	D1,2: PON2	E1,2: pos con
A3,4: ---	B3,4: Bax	C3,4: TRAIL R2/DR5	D3,4: P21/CIP1	E3,4: ---
A5,6: ---	B5,6: Bcl-2	C5,6: FADD	D5,6: P27/Kip1	E5,6: ---
A7,8: ---	B7,8: Bcl-x	C7,8: Fas/TNFRSF6	D7,8: Phospho-p53 (S15)	E7,8: ---
A9,10: ---	B9,10: Pro-Caspase-3	C9,10: HIF-1alpha	D9,10: Phospho-p53 (S46)	E9,10: ---
A11,12: ---	B11,12: Cleaved Caspase-3	C11,12: HO-1/HSP32	D11,12: Phospho-p53 (S392)	E11,12: ---
A13,14: ---	B13,14: Catalase	C13,14: HO-2	D13,14: Phospho-Rad17	E13,14: ---
A15,16: ---	B15-16: cIAP-1	C15,16: HSP27	D15,16: SMAC/Diablo	E15,16: ---
A17,18: ---	B17,18: c-IAP-2	C17,18: HSP60	D17,18: Survivin	E17,18: ---
A19,20: ---	B19,20: Claspin	C19,20: HSP70	D19-20: TNF RI/TNFRSF1A	E19,20: ---
A21,22: ---	B21,22: Clusterin	C21,22: HTRA2/Omi	D21,22: XIAP	E21,22: ---
A23-24: Pos con	B23,24: Cytochrome c	C23,24: Livin	D23,24: PBS	E23,24: ---

Figure 11 Membrane array analysis for apoptosis related proteins regulated by APOSEC^H in human primary cardiac myocytes. As shown, mainly Bcl-2 and Heat shock proteins were up-regulated after exposure to APOSEC^H.

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To verify the results obtained by proteome membrane array analysis, Western Blot assays were performed to evaluate up-regulation of the anti-apoptotic and cytoprotective factors Bcl-2 and BAG1[47, 48].

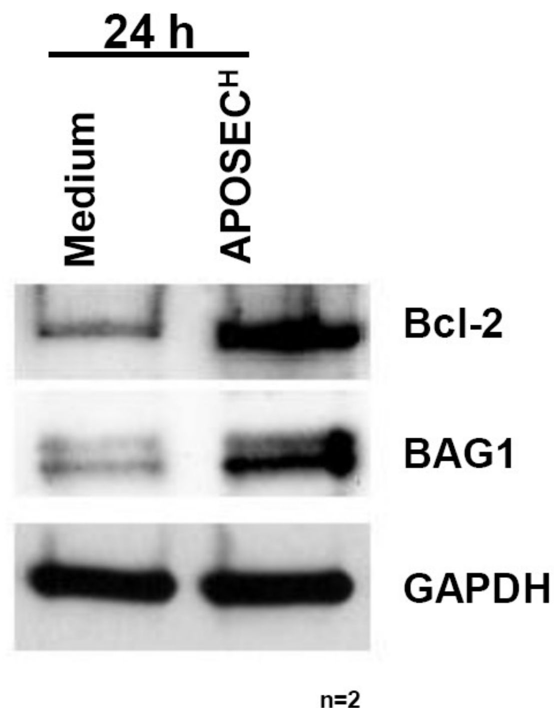


Figure 12 Expression of Bcl-2 and BAG1 in human cardiac myocytes after APOSEC treatment. 24 hours after start of co-incubation, expression of the anti-apoptotic proteins Bcl-2 and BAG1 was analysed by Western blotting. Proteins were normalized to the house-keeping gene GAPDH.

Furthermore, we investigated effects of APOSEC treatment on the triggering of signalling factors that were associated with cardioprotective mechanisms and cardiac (ischaemic) pre- and post-conditioning, e.g. AKT, Erk1/2, CREB and Heat shock protein 27 (Hsp27) [49-56].

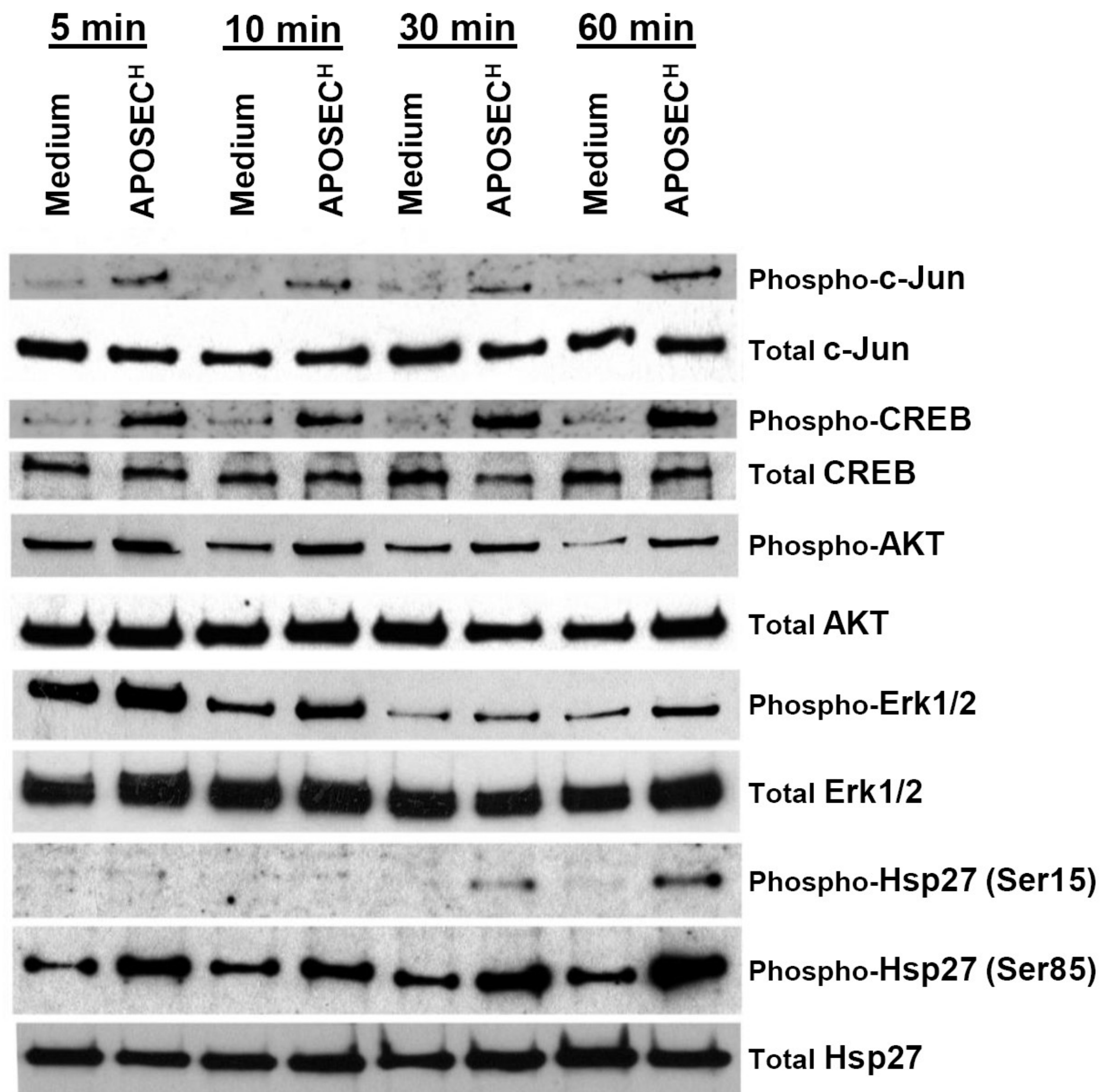


Figure 13 Human cardiac myocytes were treated with APOSEC^{H}, cell extracts were prepared after the indicated time intervals. Western Blot analysis shows increased phosphorylation of c-Jun, CREB, AKT, Erk1/2 and Hsp27.

In order to test a dose-dependent relationship of APOSEC treatment and the induction of cardioprotective signalling factors, further Western Blot assays were conducted.

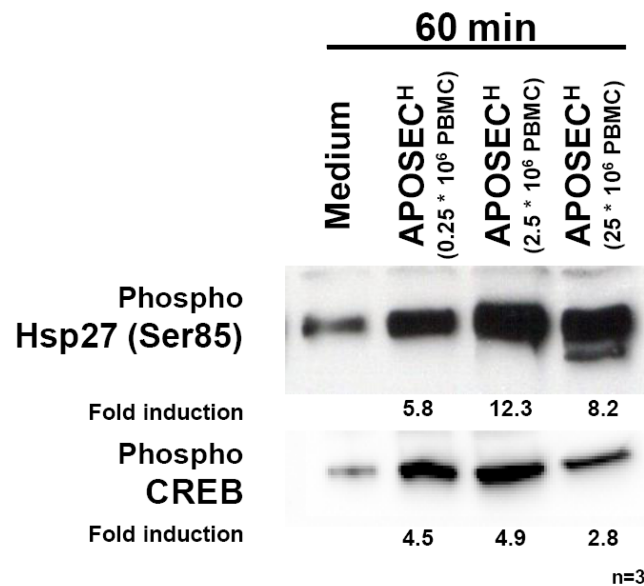


Figure 14 shows a dose-dependent increase in phosphorylation of Hsp27 and CREB. Highest levels were achieved when cardiac myocytes were incubated with APOSEC derived from $2.5 * 10^6$ apoptotic PBMC. Even APOSEC obtained from just $0.25 * 10^6$ apoptotic PBMC potently induced the phosphorylation of Hsp27 and CREB.

Moreover, we sought to determine whether APOSEC treatment confers direct cell protection to stressed cardiomyocytes. For this purpose, we utilized a cell starvation/ growth factor withdrawal assay of human cardiac myocytes. When APOSEC was added to these cultures in increasing concentrations, a dose-dependent effect was observed.

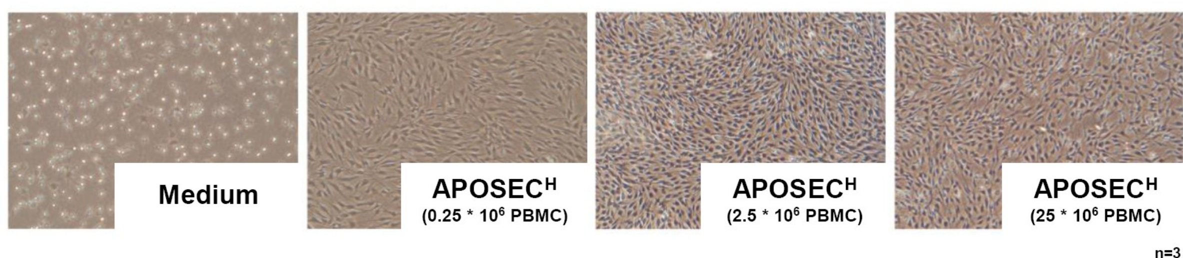


Figure 15 shows that growth factor withdrawal reduces cell viability of human cardiac myocytes. When APOSEC was added in increasing doses, cell viability was restored again.

APOSEC treatment in a rat model of AMI

Based on these *in vitro* findings that APOSEC up-regulates cytoprotective factors and increases survival of human cardiac myocytes, we sought to investigate intravenous APOSEC administration in a small animal model of acute myocardial infarction by ligation of the left anterior descending artery (LAD). The injection of fresh cell culture medium or supernatants obtained from non-irradiated viable cells served as controls in this experimental setting.
Three days after induction of AMI and injection of APOSEC^R, fresh medium or supernatants of non-irradiated cells, rat hearts were explanted, fixed in formalin solution and analysed microscopically by means of histology (H&E staining). Compared to the two control groups, hearts of APOSEC^R injected animals evidenced less myocardial damage and also less signs of cellular infiltration in the ischaemic areas of the myocardium.

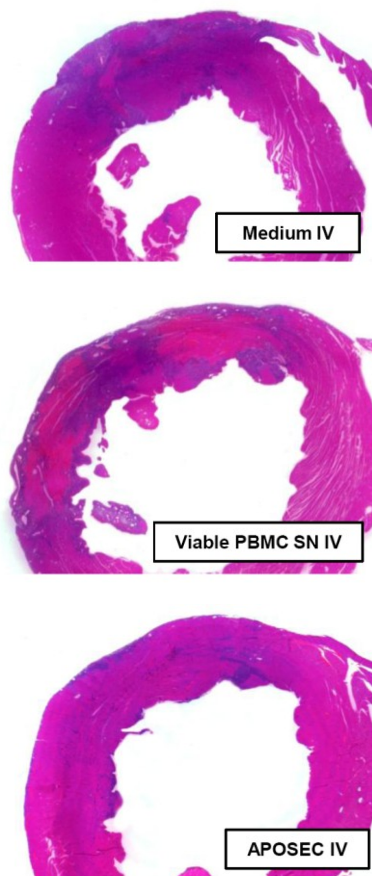


Figure 16 shows representative images of rat hearts three days after AMI. Hearts from APOSEC^R injected animals show less immune cell infiltrates and myocardial damage.

Image J software was used to quantify the extent of myocardial necrosis by means of planimetry. Animals that were injected with fresh cell culture medium showed a mean area of necrosis of 20.56% ±1.71 (SEM, expressed as % of the left ventricle). Rats with injection of supernatants derived from non-irradiated viable cells evidenced mean values of 21.08% ±2.76. This damage was reduced to 10.81% ±2.58 in animals that were treated with APOSEC derived from rat cells (p=0.017 and 0.03 vs. controls).

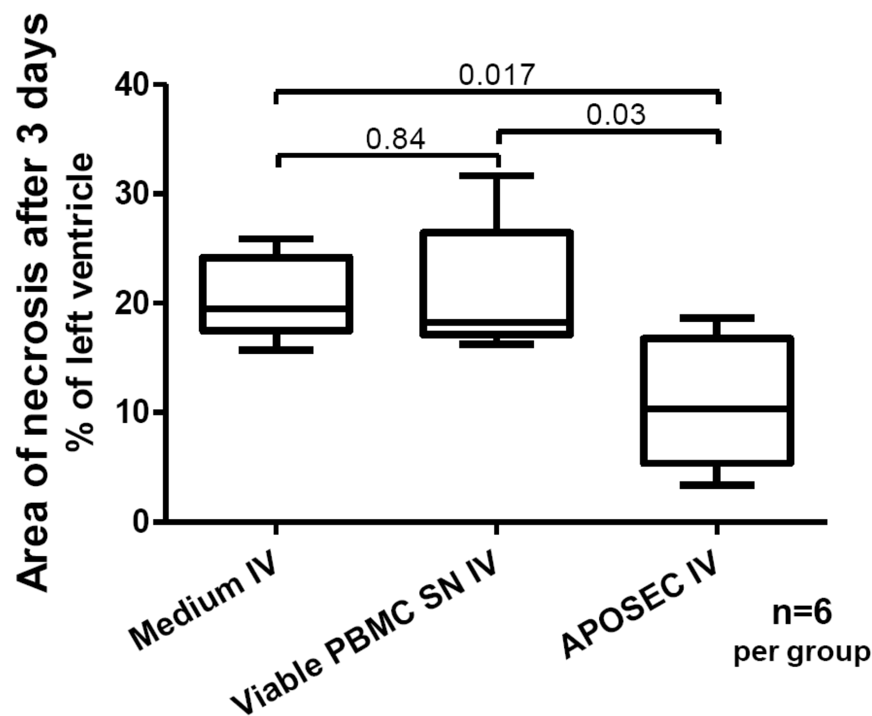


Figure 17 shows results obtained by planimetry three days after induction of AMI.

In accordance with our previous results (injection of cell suspensions of apoptotic PBMC after AMI) we found denser cellular infiltrates in the ischaemic myocardium of APOSEC treated animals compared to the two control groups.

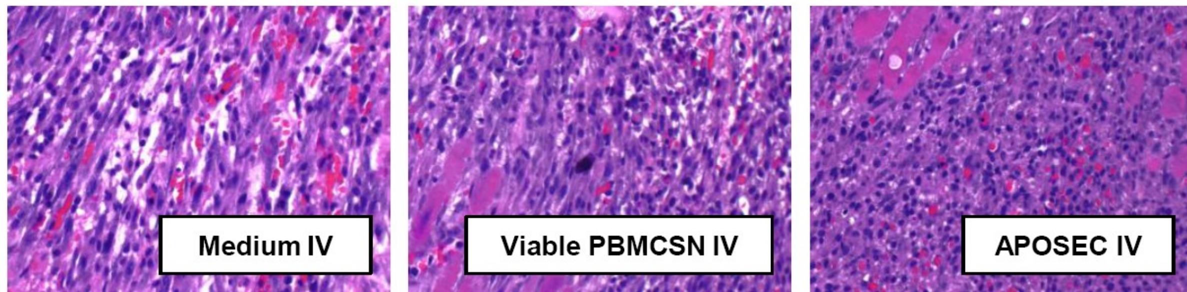


Figure 18 shows H&E-stained specimens of rat myocardium three days after induction of AMI. The cellular infiltrate in the ischaemic myocardium appears to be much more consolidated in APOSEC^{R} injected animals.

Immunohistology was utilized to further characterize the cellular infiltrate in the ischaemic myocardium. Myocardial specimens obtained three days after AMI were analysed for the markers CD68 and c-kit as cell populations bearing these epitopes were also enriched in rats that were injected with suspensions of apoptotic PBMC (see previous results).

In accordance, higher levels of infiltrating cells staining positive for CD68 were detected in specimens of rats that were injected with APOSEC^{R}. In total, 60.8 ±4.8 positive cells per high power field (HPF) were found in controls, 75.6 ±7.6 in rats injected with supernatants of non-irradiated cells and 114.2 ±11.6 in animals that were treated with APOSEC^{R}.

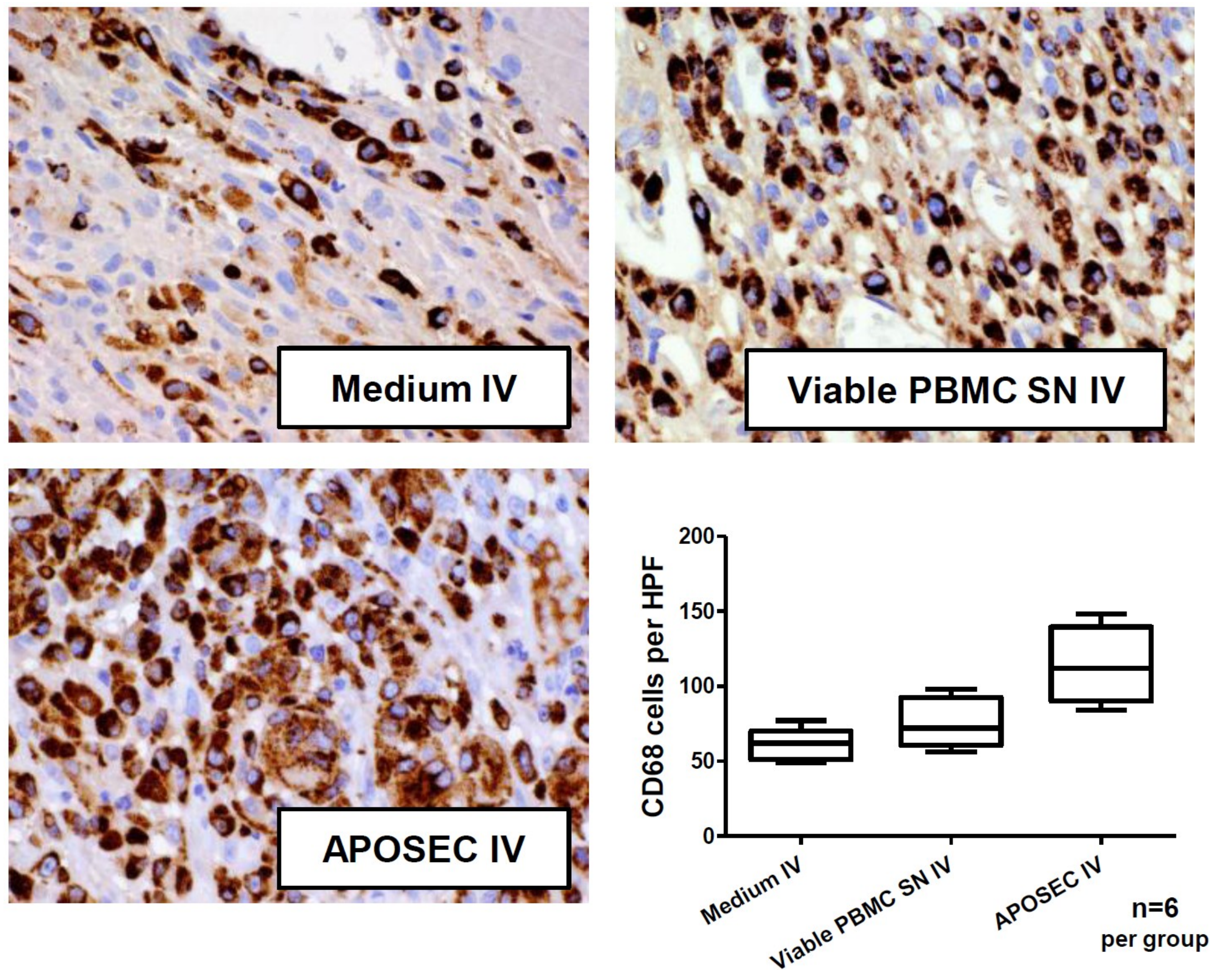


Figure 19 shows immunohistological images of specimens stained for the marker CD68 and a boxplot analysis of the obtained results.

Moreover, an immunohistological analysis was conducted for the marker c-kit. In our previous experiments, higher numbers of cells bearing that marker were found in the epicardial regions of animals that were injected with apoptotic cells. A similar result was found in rats that were treated with APOSEC.

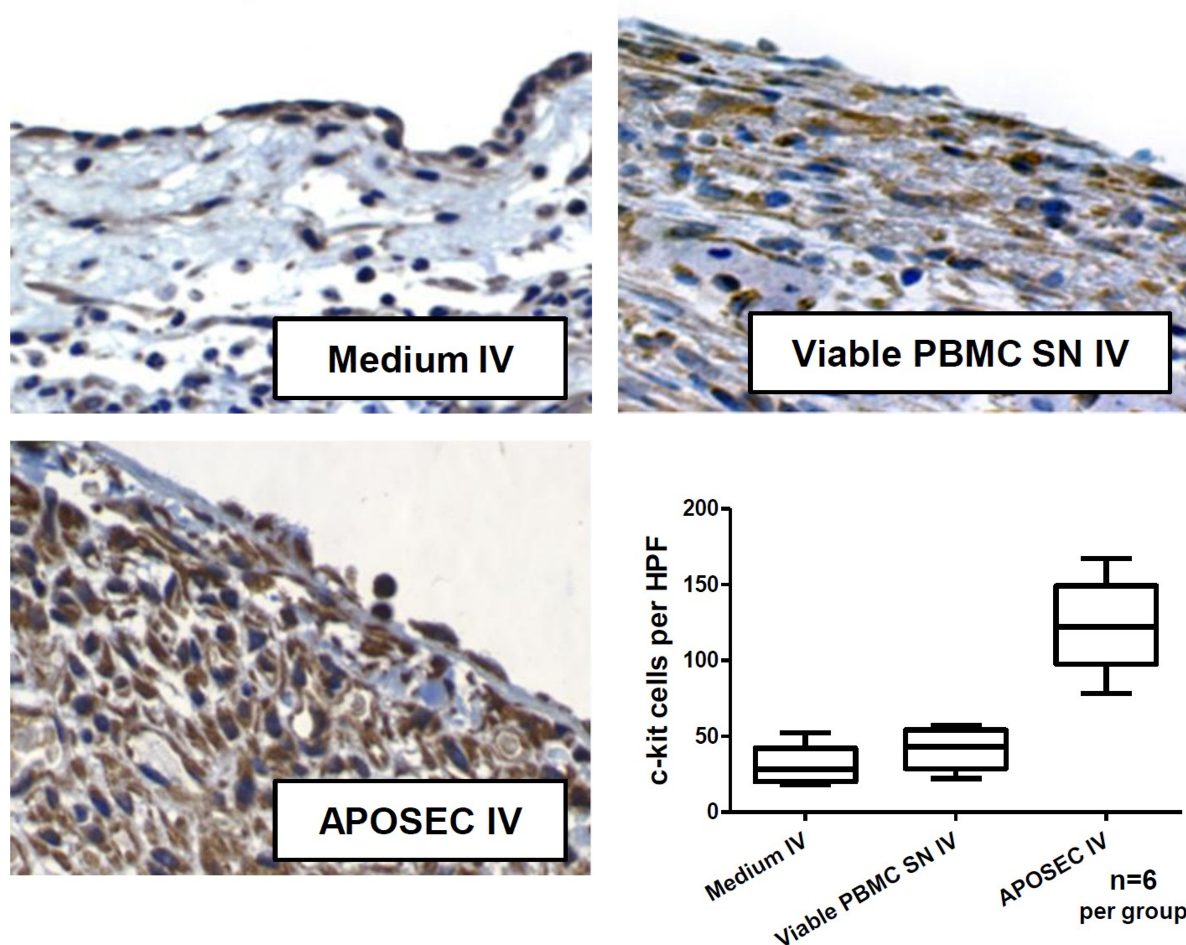
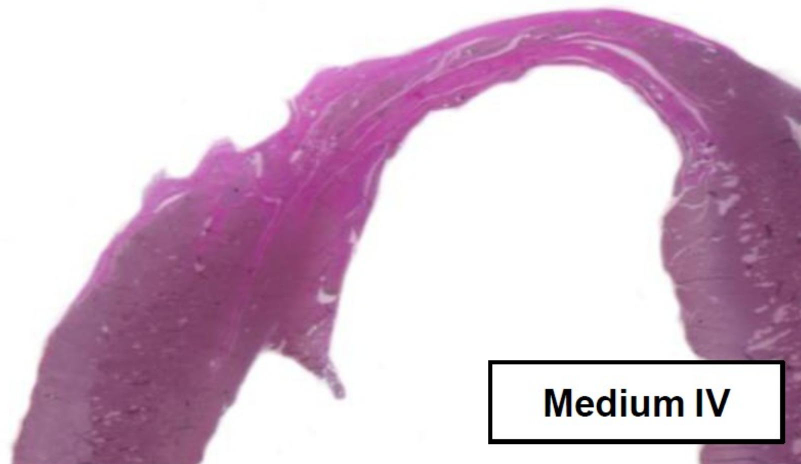
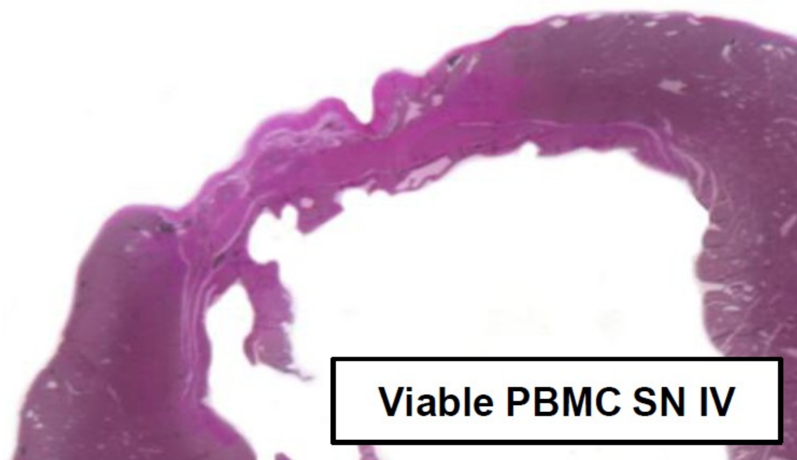


Figure 20 shows immunohistological staining for the marker c-kit and a boxplot analysis of the obtained data.

Only a few positive cells were found in myocardial specimens of control animals and rats injected with supernatants of non-irradiated cells with mean levels of 30.4 ±5.9 and 41.6 ±6.1 cells per HPF. In APOSEC^{R} injected rats, these levels increased to 123.0 ±14.3 c-kit positive cells in epicardial regions of the infarcted myocardium.
After homogenisation of cardiac specimens, the quantity of c-kit and CD68 positive cells was also determined by flow cytometry. Both cell populations were enriched in APOSEC^{R}-injected animals, as evidenced by a mean increase of 37% of CD68 cells and of 107% of c-kit positive cells compared to control animals

In order to investigate medium to long term results of APOSEC treatment in regards to ventricular remodelling, a second subgroup of animals was scarified six weeks after induction of AMI by LAD ligation.





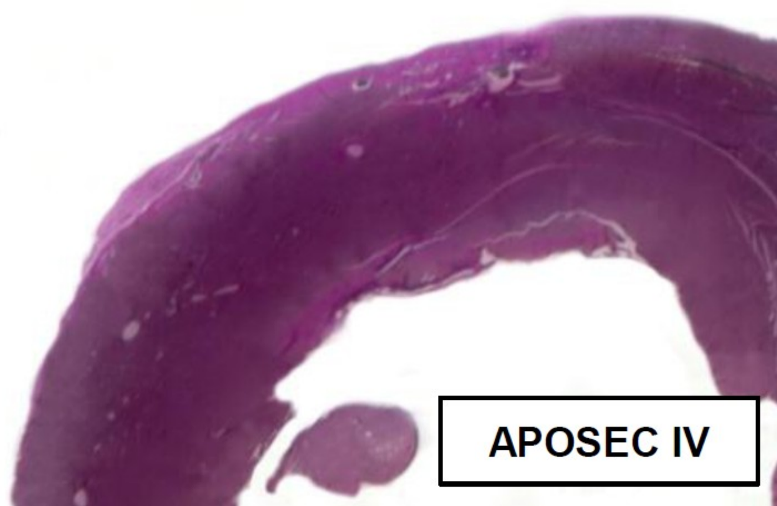


Figure 21 shows myocardial specimens obtained six weeks after induction of AMI. Hearts of APOSEC treated rats evidence less scar formation and less ventricular remodelling compared to controls.

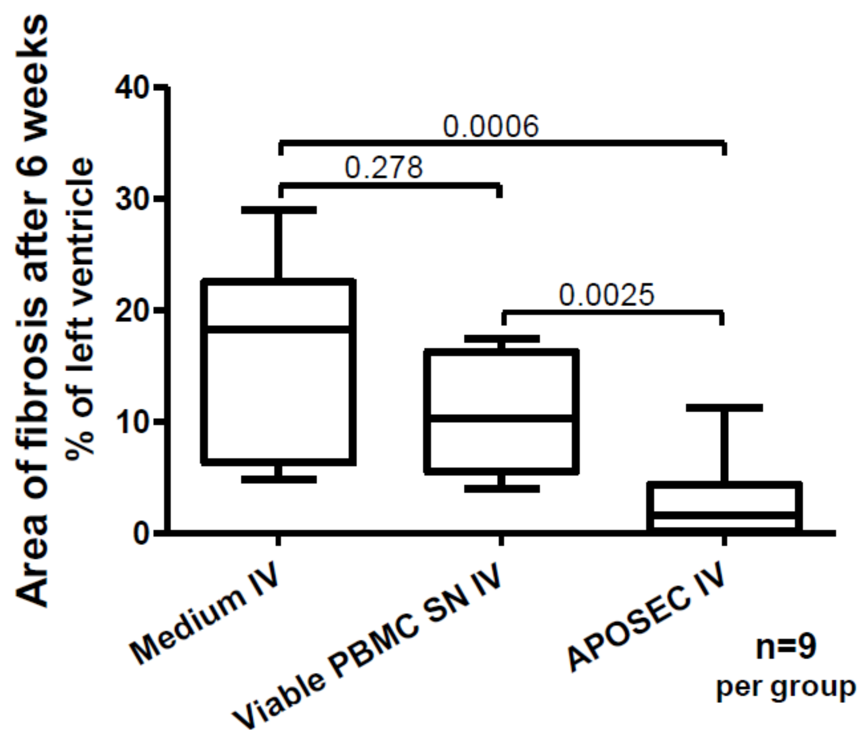


Figure 22 shows results obtained by planimetric analysis of rat heart specimens stained according to an Elastics van Gieson protocol. Results indicate a significant reduction of scar area compared to controls.

Assessment of cardiac function by means of echocardiography

In order to document functional changes of cardiac function after AMI induction, echocardiography was utilized and values of ejection fraction (EF), shortening fraction (SF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameters (LVESD) were recorded.

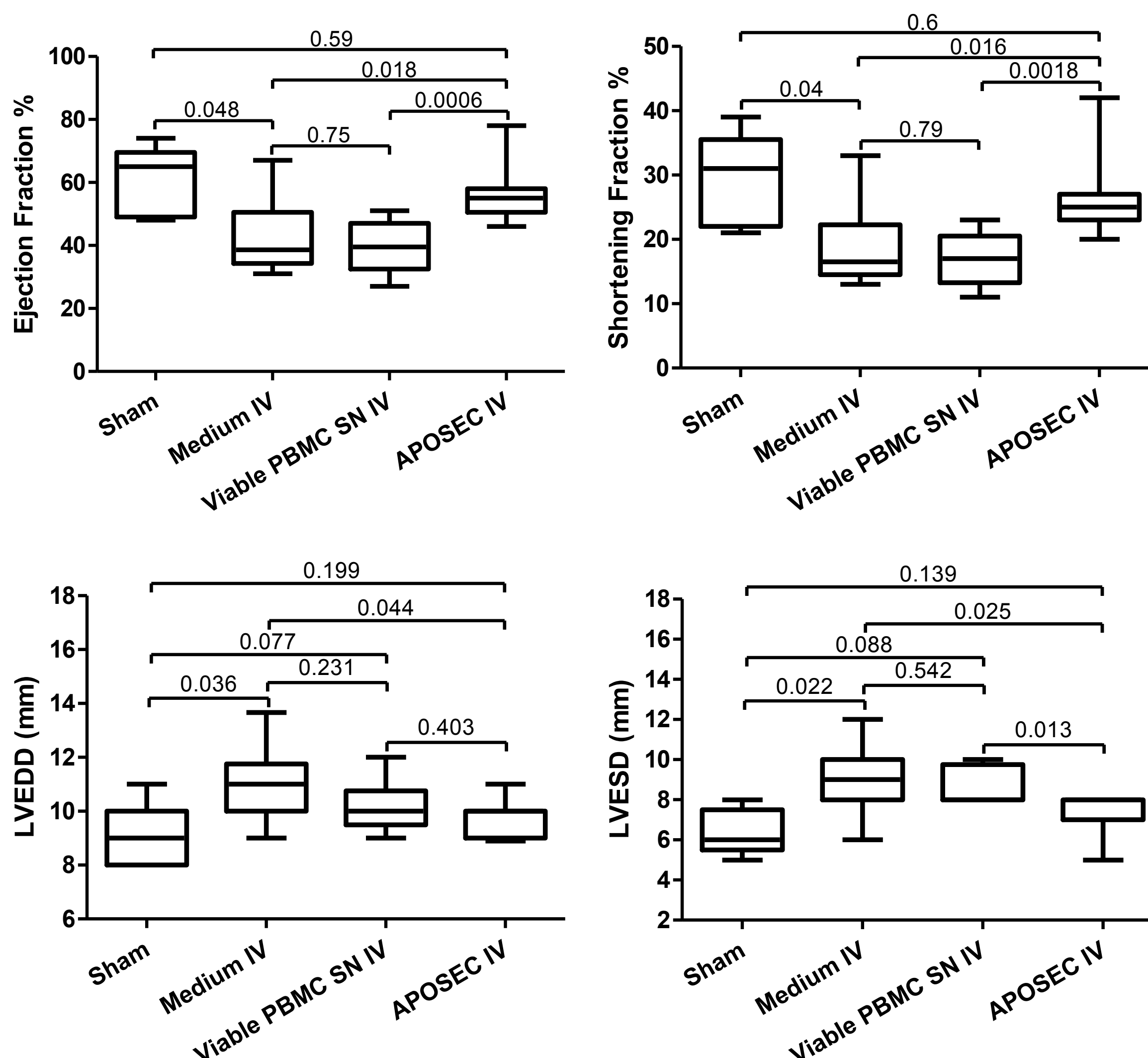


Figure 23 shows results obtained by echocardiography six weeks after LAD ligation and induction of AMI. Functional parameters of the heart (EF, SF, LVEDD, LVESD) were improved in APOSEC injected animals in comparison to medium or viable cell supernatant injected rats.

Six weeks after induction of AMI, the mean ejection fractions (EF), shortening fractions (SF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were determined to be 43.04%±4.17 (EF), 19.00%±2.29 (SF), 10.96mm±0.51 (LVEDD) and 9.00mm±0.63 (LVESD) in animals that were injected with culture medium alone and 39.38%±2.89 (EF), 16.88%±1.45 (SF), 10.17mm±0.33 (LVEDD) and 8.63mm±0.32 (LVESD) in rats which were injected with the supernatants of non-irradiated viable PBMC. Interestingly, APOSEC injected rats evidenced significantly improved functional parameters: 56.22%±3.05 (LVEF; p=0.018 vs. medium and p=0.0006 vs. viable cell supernatants),

26.33%±2.11 (SF; p=0.016 vs. medium and p=0.0018 vs. viable cell supernatants), 9.77mm±0.23 (LVEDD; p=0.044 vs. medium) and 7.33mm±0.33 (LVESD; p=0.025 vs. medium and p=0.013 vs. viable cell supernatants). Mean levels of cardiac function of healthy animals without induction of myocardial infarction (sham operation) were as follows: 60.40%±4.95 (LVEF), 29.20%±3.26 (SF), 9.00mm±0.55 (LVEDD) and 6.40mm±0.51 (LVESD).

Large animal AMI model

Based on these results obtained *in vitro* and in small animal experiments, we set up a large animal experiment of closed chest reperfused myocardial infarction using a porcine model investigating the therapeutic potential of APOSEC. For this purpose, $APOSEC^P$ was administered 40 minutes after onset of myocardial ischaemia in two different doses, in a low dose group with reconstituted cell culture supernatants obtained from $250*10^6$ apoptotic PBMC and in a high dose group with supernatants obtained from $1*10^9$ apoptotic PBMC.

For a short term analysis of the cardioprotective effects induced by $APOSEC^P$ treatment, a subgroup of animals was sacrificed 24 hours after reperfused myocardial infarction. The explanted hearts were evaluated using a staining protocol of tetrazolium cloride (TTC) and Evans blue solution. Necrotic myocardial tissue remained unstained.

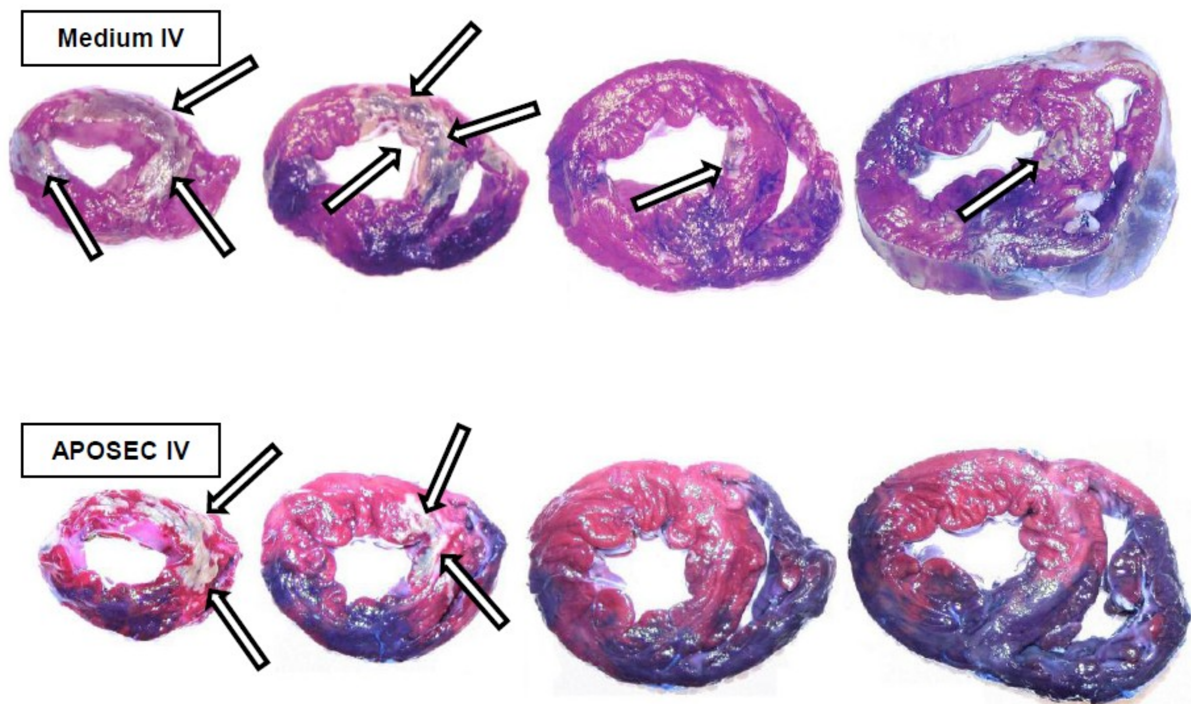


Figure 24 shows representative images of porcine hearts explanted 24 hours after reperfused myocardial infarction stained with tetrazolium chloride (TTC) and Evans blue solution. Arrows indicate necrotic tissue

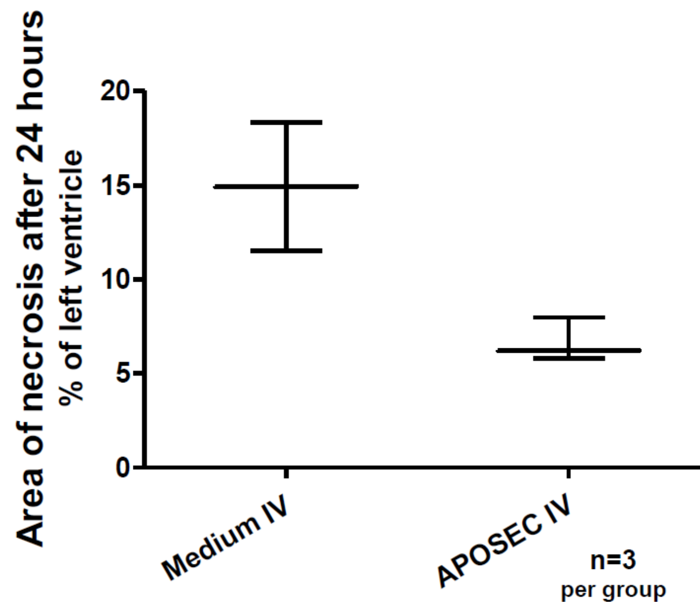


Figure 25 shows results obtained by a planimetric analysis of porcine hearts demonstrating a reduction of myocardial injury by APOSEC^{P} treatment after 24 hours.

In order to determine the reduction of myocardial injury by APOSEC treatment, an ELISA assay investigating Troponin I release over the first 24 hours after AMI was conducted.

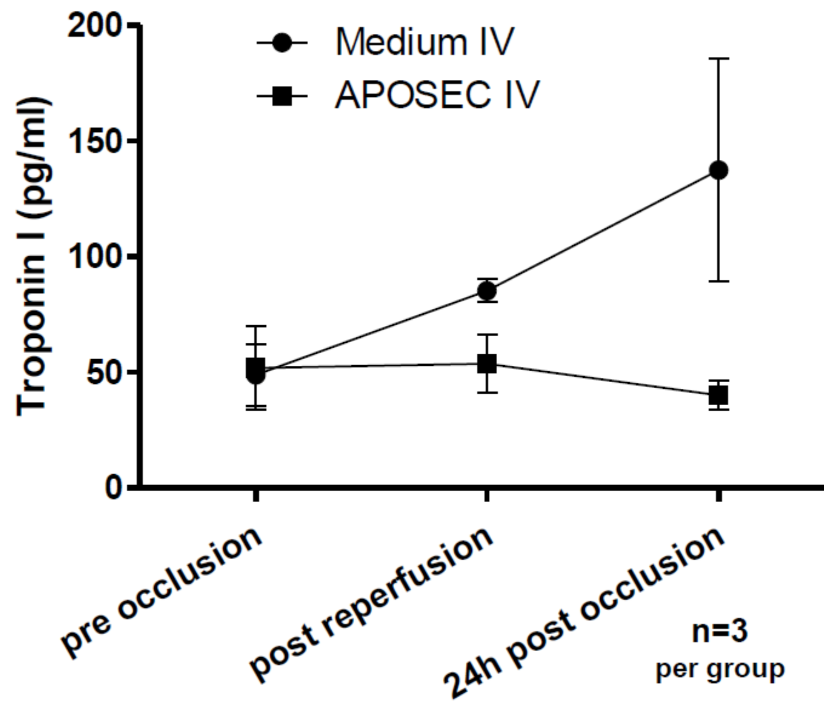
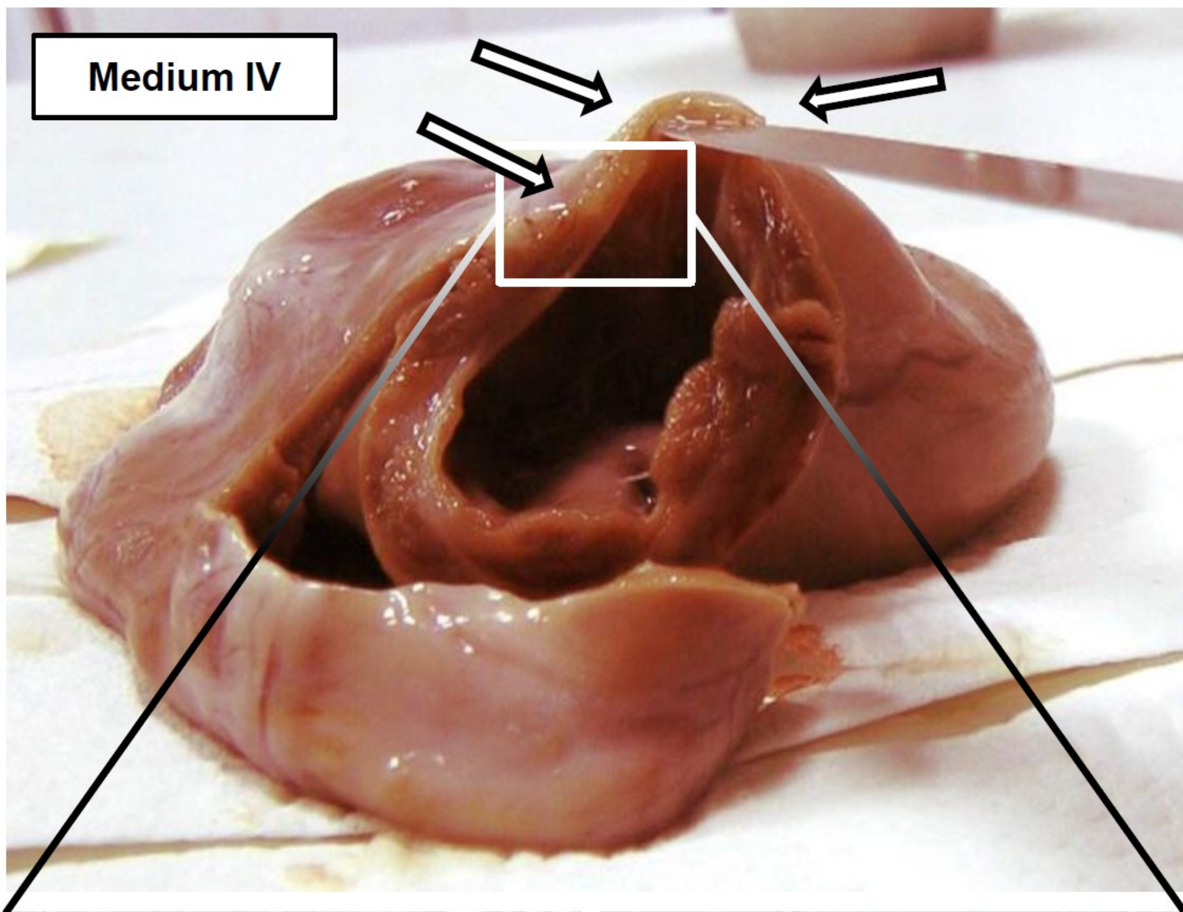
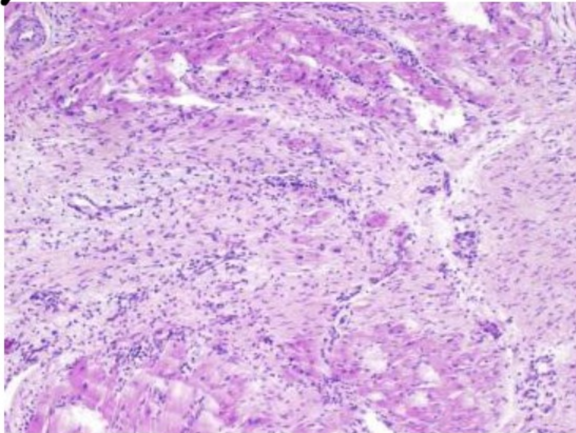
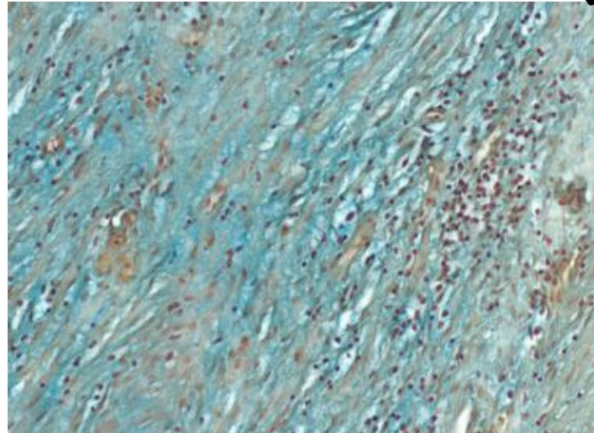


Figure 26 shows that plasma Troponin I levels of APOSEC[P] infused pigs remained at base line whereas an increase was detected in medium injected control animals.


Medium IV





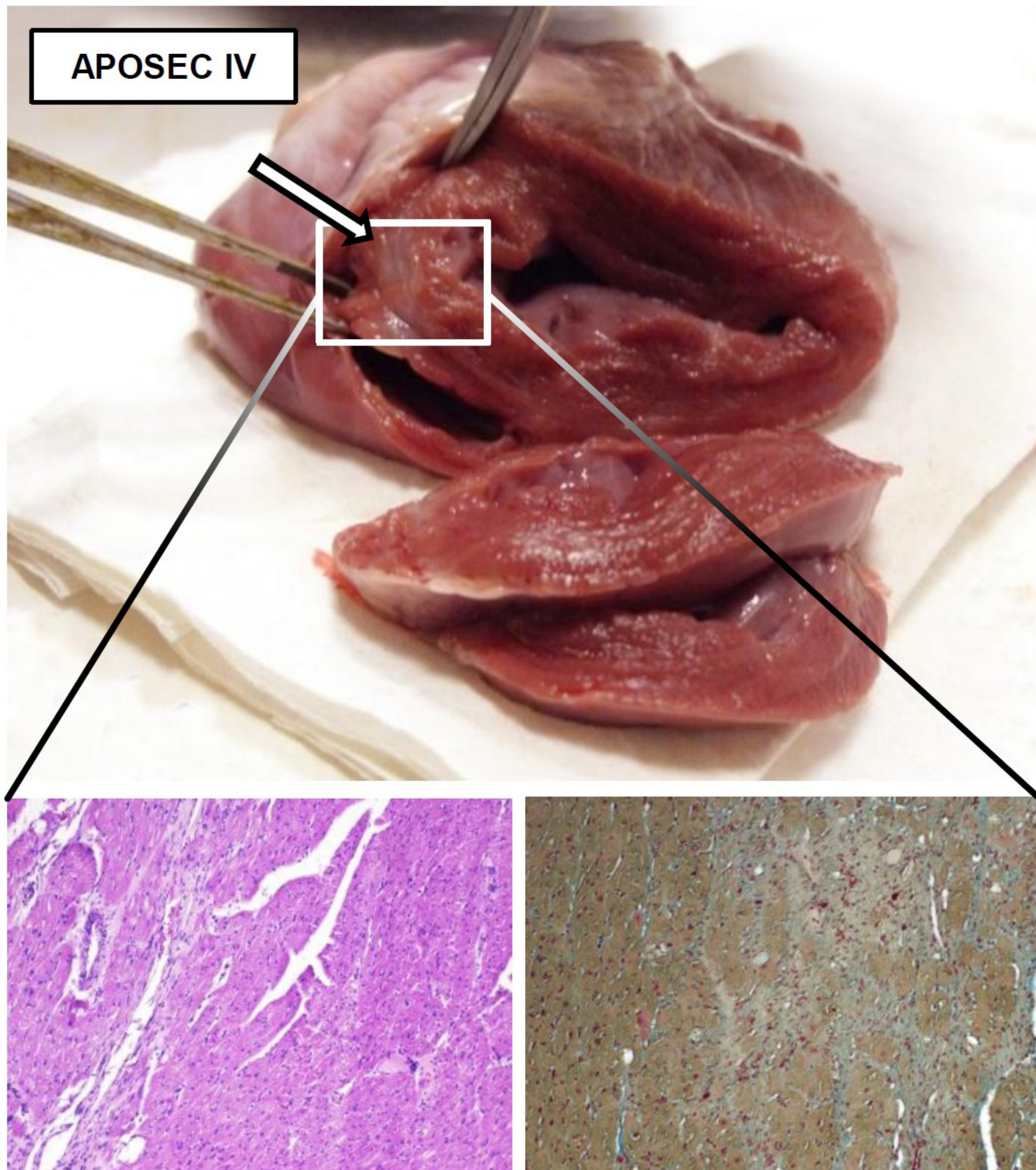


Figure 27 shows representative images of porcine hearts explanted 30 days after AMI. Hearts of APOSEC^{P}-injected pigs evidenced only very marginal scar tissue formation in the myocardium compared to control animals (Medium IV) where large infarcts were common. H&E-stained and Movat's pentachrome-stained specimens of the infarcted myocardium shown in the lower part of the figure indicate less signs of collagen deposition and more viable cardiomyocytes within the scar tissue of the left ventricle compared to control animals.

Cardiac MRI evaluation 3 and 30 days after AMI

	Parameters	Medium control (n=8)	$250 \cdot 10^6$ apoptotic PBMC (low dose APOSEC, n=7)	$1 \cdot 10^9$ apoptotic PBMC (high dose APOSEC, n=7)
after 3 days	weight (kg)	31.86 ±9.1	30.86 ±1.6 *ns*	33.33 ±1.3 *ns*
	age (days)	90 ±0	90 ±0 *ns*	90 ±0 *ns*
	LVEDV (ml)	67.59 ±2.7	64.19 ±5.4 *ns*	63.73 ±1.6 *ns*
	LVESV(ml)	38.42 ±2.5	35.96 ±3.0 *ns*	33.93 ±2.1 *ns*
	LVSV (ml)	29.17 ±1.3	28.23 ±3.2 *ns*	29.77 ±1.8 *ns*
	LVEF (%)	43.38 ±1.9	43.63 ±2.8 *ns*	46.65 ±2.9 *ns*
	HR/min.	111 ±6	109 ±5 *ns*	111 ±13 *ns*
	CO (l/min.)	3.24 ±0.1	3.03 ±0.3 *ns*	3.28 ±0.3 *ns*
	CI (l/min/m^2)	3.64 ±0.1	3.59 ±0.4 *ns*	3.82 ±0.4 *ns*
	Infarct %	18.17 ±1.7	14.01±1.9 ns	8.66 ±1.5 **
after 30 days	weight (kg)	39.43 ±0.5	37.00 ±1.9 *ns*	48.83 ±0.7 ***
	age (days)	120 ±0	120 ±0 *ns*	120 ±0 *ns*
	LVEDV (ml)	54.74 ±4.1	53.43 ±3.2 *ns*	65.99 ±3.5 *ns*
	LVESV(ml)	32.93 ±4.0	31.89 ±2.9 *ns*	28.71 ±3.5 *ns*
	LVSV (ml)	21.84 ±1.8	21.54 ±1.9 *ns*	37.29 ±1.7 ***
	LVEF (%)	40.54 ±3.6	40.64 ±3.2 *ns*	57.05 ±3.3 **
	HR/min.	114 ±7	108 ±7 *ns*	107 ±5 *ns*
	CO (l/min.)	2.44 ±0.1	2.28 ±0.1 *ns*	3.98 ±0.2 ***
	CI (l/min/m^2)	2.46 ±0.1	2.40 ±0.1 *ns*	3.51 ±0.2 ***
	Infarct %	12.60 ±1.3	11.50 ±1.5 ns	6.92 ±1.4 *

ns no significance versus control
* $p<0.05$ versus control
** $p<0.01$ versus control
*** $p<0.001$ versus control

Table 3 shows parameters obtained three and 30 days after reperfused myocardial infarction. Cardiac MRI analysis was conducted and parameters of cardiac function were obtained from pigs treated with low and high dose APOSEC and from control animals that were infused with reconstituted cell culture medium (left ventricular end-diastolic diameter, LVEDD; left ventricular end-systolic diameter, LVESD; left ventricular stroke volume, LVSV; left ventricular ejection fraction, LVEF; heart rate, HR; cardiac index, CI; cardiac output, CO).

BARI score evaluations

In order to determine that all animals evidenced a comparable perfusion of the myocardium prior to occlusion of the LAD and a comparable extent of myocardium at risk, a modified BARI score was calculated based on angiographic images of the LAD the left circumflex artery (LCX). No significant differences were detected between the three groups.

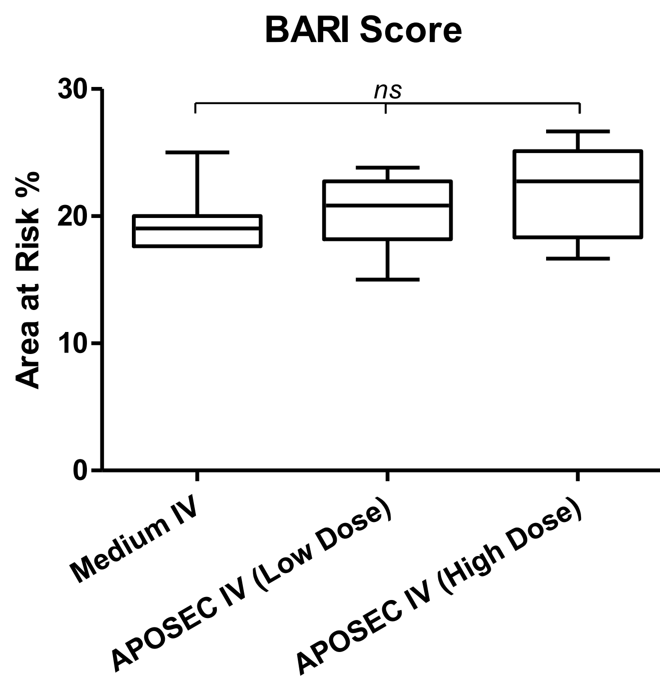


Figure 28 shows results obtained by BARI score calculation, no significant differences were observed between groups.

Discussion

Over the last decades, clinical research in cardiovascular medicine has focused on establishing early reperfusion after occlusion of a coronary artery and by doing so salvaging myocardium at risk for ischaemic cell death. Nevertheless, current treatment strategies do not address the key problem of myocardial ischaemia which is the loss of viable myocardium, composed of cardiomyocytes, vascular cells, and connective tissue cells. The consequence of this loss of vital contractile tissue is that patients continue to experience frequent hospitalisation after AMI and in many cases develop signs of heart failure.

Due to the detrimental processes associated with ischaemic tissue injury, great hopes were put in stem cell research and its clinical application for cardiovascular medicine. Although small animal experiments investigating the regenerative potential of stem cell therapy evidenced very convincing results, clinical trials showed only moderately beneficial effects results compared to control patients or these effects lasted only for a short period of time [11, 12].

Based on the hypothesis stated by Thum and Anker *et al.* [15] that apoptotic stem cells transplanted after AMI might modulate the inflammatory response and prevent ventricular remodelling, we sought to prove this in *in vitro* and *in vivo* experiments. Instead of bone marrow cells we used the more easily obtainable “cellular material” of PBMC based on two assumptions: first, the beneficial potential bone marrow cells, peripheral blood cells or their supernatants can be assumed to be of no major difference [31] and second, in order to promote the clinical application of cell therapy after AMI using easier obtainable cell types we have focused on PBMC instead of stem/progenitor cells. In our previous work we showed that apoptotic PBMC evidenced immunomodulatory effects *in vitro* and preserved cardiac function after AMI in a small animal model [22]. Based on these results we sought to further investigate how apoptotic cells prevent ventricular remodelling after AMI. Furthermore, we investigated additional ways of cell administration (i.e. intravenously and intramyocardially). Again, we found higher numbers of macrophages and cells expressing the markers c-kit and VEGF receptor 2 three days after AMI. This seems to be a pivotal point, as macrophages mediate the changeover from the initial phase

of AMI to tissue stabilization, indicating a faster healing process within the ischaemic myocardium. This phenomenon of cell accumulation was detectable even to a much greater extent when the irradiated apoptotic cells where injected directly into the ischaemic tissue by intramyocardial injection. Another major finding was the fact that the treatment with apoptotic PBMC suspensions also reduced the extent of infarct dimension in this experimental AMI model. This reduced loss of vital myocardial also correlated with improved functional parameters in echocardiography. Animals injected with irradiated apoptotic cell suspensions evidenced a significant improvement of all tested parameters of cardiac function (ejection fraction, shortening fraction, ventricular diameters and volumes).

When analysing the composition of the extracellular matrix of the cardiac tissue, it was also of great interest that the configuration of the fibrotic scar was evidently altered in animals injected with apoptotic cells compared to controls. In specimens stained according to the Elastica van Gieson protocol, a considerable accumulation of elastic fibres especially in the border zone between vital cardiomyocytes and the fibrotic scar was detected. This alteration in the ratio of elastic and collagenous fibres could be a major factor contributing to the improvement of cardiac function parameters in rats injected with apoptotic cells. This was even more pronounced when these cell suspensions of apoptotic PBMC were injected directly into the ischaemic myocardium.

Due to this interesting finding, we sought to further investigate which factor might be accountable for the increased expression of elastic fibres. In the cellular infiltrate in the ischaemic myocardium of animals injected with suspension of apoptotic PBMC, higher numbers of cells staining positively for Insulin-like growth factor I (IGF-I) and Fibroblast growth factor 2 (FGF-2) were found in an immunohistological analysis. It has been reported that these two growth factors contribute directly to the synthesis of elastic fibres within the extracellular matrix and also regulate cardiac repair mechanisms after AMI [57-61]. There are a number of possible mechanisms by which a more favourable ratio of elastic and collagenous fibres in the cardiac scar tissue could delay the onset of ventricular dysfunction and remodelling after AMI. A cardiac scar tissue showing more elastic properties and being more resistant could function as a kind of shock absorbing cushion that might reduce the tractile effects on the scar tissue during systole. The recoil of the elastic

fibres within the scar could provide a portion of passive energy that returns the scar size to pre-systolic dimensions. These mechanistic characteristic are important for preventing or reducing the risk for ventricular remodelling and by doing so might serve as an explanation for the preservation cardiac function in this experimental setting [62, 63].

Based on these convincing results we sought to further investigate the mechanisms of cardioprotective effects induced by apoptotic cells. When performing proteome membrane analysis in order to define the secretome of irradiated apoptotic PBMC compared to non-irradiated cells, an up-regulation of many pro-angiogenic cytokines, chemokines and growth factors was found. These findings also correlated with the results obtained in our previous study where we could show that irradiation and induction of apoptosis induced the expression of IL-8 and MMP9 transcripts in RT-PCR [22].

There has been a shift of opinion in the field of regenerative medicine since recent publications showed that soluble factors secreted by bone marrow cells during cell culture initiated proliferation and migration of coronary artery endothelial cells, endothelial tube formation and cell sprouting in a mouse aortic ring assay. Interestingly, supernatants obtained from peripheral blood cells showed no major differences in their potential to induce cell proliferation or to confer cytoprotective signalling [31]. In another publication it was shown that endothelial progenitor cell conditioned medium (termed EPC-CM) increased the formation of capillary outgrowth in a rat aortic ring model and enhanced the survival of serum-starved HUVEC [33]. The protective effects of EPC-CM were also demonstrated in an ischaemic hind limb model. The injection of EPC-CM increased the blood flow, muscle mitochondrial activity and also functional improvement in this experimental setting. More recently, the research group of Kalka showed that EPC-CM increased the resistance of HUVEC to oxidative stress by inducing anti-oxidative enzymes. Moreover, the resistance to stimuli inducing apoptosis *in vitro* was increased as well as cells showed an increased expression of the anti-apoptotic factor Bcl-2. Based on these results the authors concluded that EPC-CM contains cytoprotective proteins that confer anti-apoptotic and cytoprotective effects to stressed cells. Interestingly, the neutralization of factors found in the conditioned medium of EPC such as VEGF, IL-8

and MMP9 did not significantly reverse the cytoprotective effect of the EPC-CM. They argued that EPC secrete a broad bandwidth of factors which can cause many synergistic effects and may exert strong cytoprotective properties by increasing cellular anti-oxidant enzymes and increase the expression of pro-survival genes.

The mechanism described for cell culture supernatants obtained from bone marrow cells or (stressed) EPC shows many similarities to APOSEC. Here we could show that the conditioned culture medium obtained from irradiated apoptotic PBMC (APOSEC) induces cytoprotective mechanisms in cultured cardiomyocytes and protected them from starvation induced cell death. However, in contrary to the production of bone marrow cell derived supernatants, which requires a bone marrow biopsy, or the generation of EPC-CM, which requires rather elaborate laboratory techniques to expand these cell types, PBMC can be obtained quite simply from venous whole blood via venal puncture.

Due to the fact that the loss of vital myocardial tissue was significantly reduced three days after AMI in both animal models, we hypothesized that the main mechanism of action of APOSEC is in fact cytoprotection. This was substantiated by *in vitro* experiments, the incubation of human cardiac myocytes with APOSEC induced a rapid activation of several important survival factors described in the literature for cardioprotection and cardiac pre- and port-conditioning such as AKT, Erk1/2, p38 MAPK (all part of the ischaemia reperfusion injury salvage kinase pathway, RISK), c-JUN, cAMP-response element binding protein (CREB) and Hsp27 [56, 64, 65]. Moreover, Bcl-2 and BAG1, two major anti-apoptotic mediators were up-regulated in human cardiomyocytes exposed to APOSEC within 24 hours of cell culture.

Prompt restoration of coronary perfusion within the shortest time is currently considered to be the optimal standard of cardiac care for patients suffering from ST-elevation myocardial infarction (STEMI) according to the guidelines issued by the European Society of Cardiology and the American Heart Association (AHA) [66, 67]. In order to test the applicability of APOSEC infusions we implemented a closed chest large animal model of reperfused AMI which shows the most similarities to the clinical scenario AMI in human patients. In addition to this experimental simulation of standardized treatment of AMI in humans, APOSEC infusions were administered 40 minutes after the start of the LAD balloon occlusion. To further demonstrate a dose

dependent response, APOSEC was administered in two different concentrations, i.e. resuspended lyophilised supernatants obtained from $1{*}10^9$ and $250{*}10^6$ apoptotic PBMC. The time intervals of 40 minutes until intravenous administration of APOSEC and 90 minutes until reperfusion were selected to accord with the clinical scenario of earliest possible intravenous therapy with approximately 30 - 40 minutes delay from symptom onset to diagnosis of AMI and start of intravenous therapy and 90 minutes until primary coronary intervention (PCI) and reperfusion. Short (three days) and long term (30 days) MRI results evidenced that one single (high dose) infusion of APOSEC led to a significant improvement of cardiac function and to a significant reduction of infarct dimension. Administration of low dose APOSEC evidences only marginally improved functional values compared to controls indicating a dose-dependent effect of this treatment.

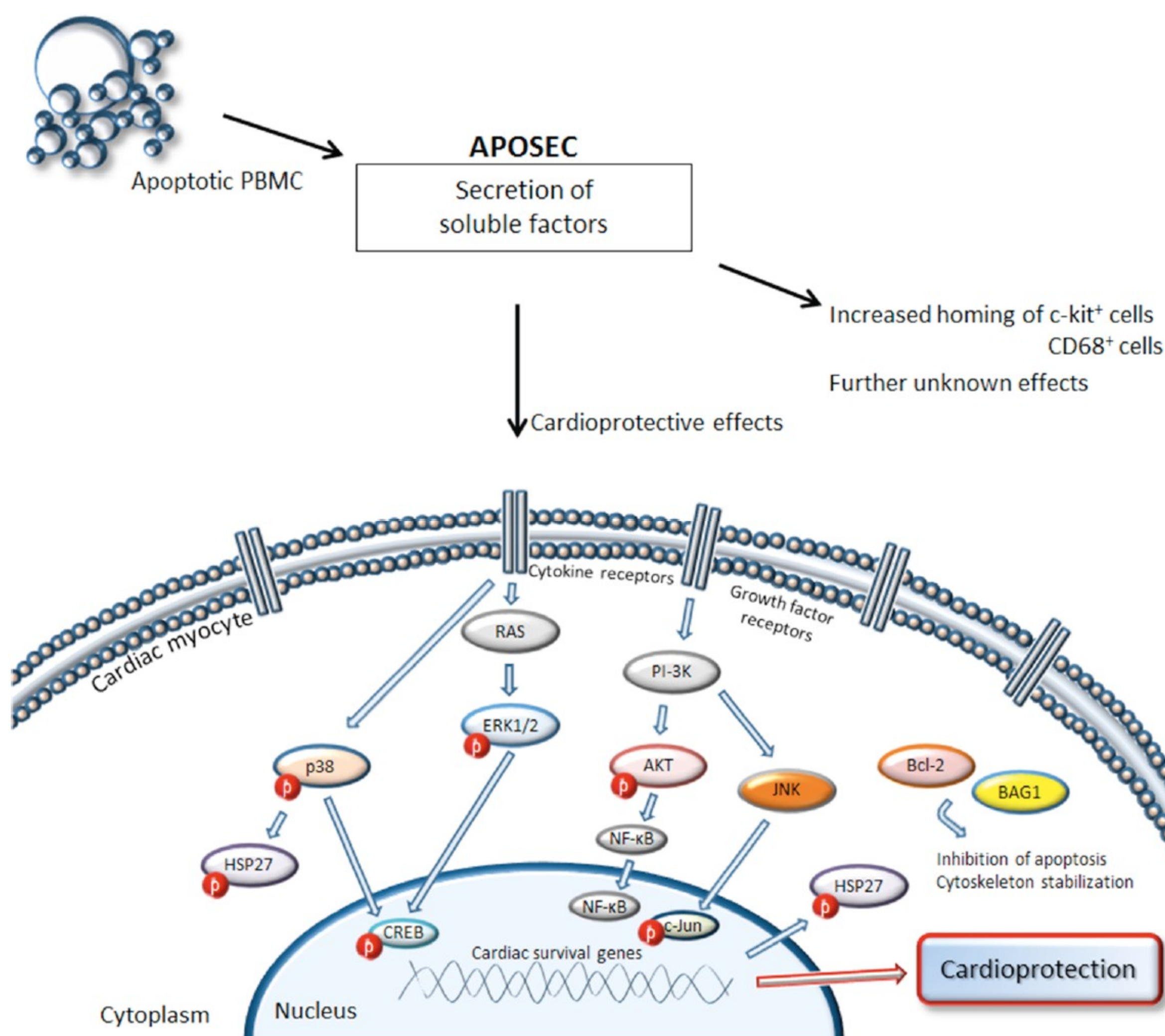


Figure 29 shows an illustration of the proposed mechanism of action that is induced by APOSEC treatment.

Conclusion

We demonstrated that cell culture supernatants of irradiated apoptotic PBMC can serve as an important therapeutic adjunct in the setting of AMI. Of major relevance is the fact that these supernatants containing soluble factor released by apoptotic cells can be lyophilised and stored as a powdery compound. The process of lyophilisation can increase the practicability of this therapeutic compound. In the clinical scenario of AMI, APOSEC preparations can be reconstituted in physiological saline solution and can be administered just like conventional intravenous infusion therapy. Comparable blood derived products such as intravenous immunoglobulin (IVIG) or coagulation factors have confirmed their clinical usefulness over the last decades [68-72]. Compared to these derivatives, APOSEC is a product made of soluble factors secreted by irradiated PBMC. This “biological” combines the following clinically favourable features: a) an easily obtainable cell material (PBMC) for production of APOSEC via venous blood withdrawal; b) low antigenicity due the cell-free nature of APOSEC and c) “off the shelf” use of APOSEC in the clinical setting of AMI requiring only intravenous administration in contrast to previous clinical trials investigating stem cells.

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MEDICAL UNIVERSITY OF VIENNA

Curriculum vitae

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PERSONAL BACKGROUND

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EDUCATION

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2007/02 – present	Research Fellow at the Department of Cardio-Thoracic Surgery, General Hospital Vienna, Medical University of Vienna, Austria
2004/10 – 2010/07	Medical Student at the Medical University of Vienna, Austria
2003/10 – 2004/09	Social Service at the Police Department of Vienna's 19th district
2003/06	Matura (High School Graduation) with Distinction

CLINICAL TRAINING

2010/06	Clinical Clerkship at the Department of Dermatology General Hospital Vienna
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2010/04	Clinical Clerkship at the Department of Ophthalmology, Krankenhaus Rudolfstifung Wien
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2009/12	Clinical Clerkship at the Department of Pediatrics, Landesklinikum Donauregion Tulln
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2009/10	Clinical Clerkship at the Department of Neurology, Landesklinikum Donauregion Tulln
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2006/08	Clinical Clerkship at the Department of Trauma Surgery, LKH Bad Ischl
2006/07	Clinical Clerkship at the Department of Surgery, Evangelisches Krankenhaus, Vienna

CONGRESSES AND MEETINGS

08/2011	European Society of Cardiology Congress 2011, Paris, France
06/2011	52. Österreichischer Chirurgenkongress, Wien
05/2011	Österreichischer Kardiologenkongress, Salzburg
04/2011	Annual Meeting of the International Society for Heart and Lung Transplantation (ISHLT), San Diego, USA
03/2011	Kardiologie 2011, Innsbruck
12/2010	2nd EACTS Meeting on Cardiac and Pulmonary Regeneration, Wien
10/2010	Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik, Austrotransplant, Villach
09/2010	41. Jahrestagung der Österreichischen Gesellschaft für Innere Medizin, Salzburg
08/2010	European Society of Cardiology Congress 2010, Stockholm, Schweden

06/2010	Österreichischer Kardiologenkongress, Salzburg
10/2009	Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik, Austrotransplant, Seefeld in Tirol
09/2009	40. Jahrestagung der Österreichischen Gesellschaft für Innere Medizin, Wien
09/2009	Hanseatische Klinikkonferenz Pneumologie, Hamburg, Deutschland
09/2009	Kardiovaskuläre Medizin, Hamburg, Deutschland
06/2009	50. Österreichischer Chirurgenkongress, Wien
06/2009	Österreichischer Kardiologenkongress, Salzburg
04/2009	Frühjahrstagung der Österreichischen Gesellschaft für Hämatologie und Onkologie, Salzburg
03/2009	Kardiologie 2009, Innsbruck
02/2009	53rd Annual Meeting of the Society of Thrombosis and Haemostasis Research, Wien
11/2008	1st EACTS Meeting on Cardiac and Pulmonary Regeneration, Bern, Schweiz
09/2008	39. Jahrestagung der Österreichischen Gesellschaft für Innere Medizin, Graz
05/2008	Österreichischer Kardiologenkongress, Salzburg
05/2008	49. Österreichischer Chirurgenkongress, Innsbruck
02/2008	EAACI-Ga^{2}len Davos Meeting, Pichl/Schladming
10/2007	Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik, Austrotransplant, St. Wolfgang

PUBLICATIONS

17 Peer reviewed papers (6 first authorships, 11 co-authorships, Impact Factor 41,099)
1 Case report
2 Research update letters
47 Abstracts
12 Oral presentations
11 Poster presentations
1 Book

Peer reviewed Papers, Case Reports, Letters

Lichtenauer M, Mildner M, Hoetzenecker K, Zimmerman M, Podesser BK Sipos W, Berényi E, Dworschak M, Tschachler E, Gyöngyösi M, Ankersmit HJ.

Secretome of apoptotic peripheral blood cells (APOSEC) confers cytoprotection to cardiomyocytes and inhibits tissue remodelling after acute myocardial infarction: a preclinical study.

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Lichtenauer M, Mildner M, Baumgartner A, Hasun M, Werba G, Beer L, Altmann A, Roth G, Gyöngyösi M, Podesser BK Ankersmit HJ.

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Administration of anti-thymocyte globulin (ATG) preserves cardiac function after experimental myocardial infarction

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Secretome of Apoptotic Peripheral Blood Cells (APOSEC) Confers Cytoprotection to Cardiomyocytes and Inhibits Tissue Remodeling after Acute Myocardial Infarction

05/2011 — Jahrestagung der österreichischen Gesellschaft für Kardiologie (ÖKG), Salzburg

Injection of Apoptotic Peripheral Blood Mononuclear Blood Cells (PBMC) Increases Elastin Expression in Cardiac Scar Tissue after Myocardial Infarction

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Intravenous and Intramyocardial Injection of Irradiated Apoptotic Peripheral Blood Mononuclear Cells (PBMC) Preserves Ventricular Function after Myocardial Infarction

05/2011 — Jahrestagung der österreichischen Gesellschaft für Kardiologie (ÖKG), Salzburg

Anti-Thymocyte Globulin (ATG) Reduces Damage Caused by Ischaemia and Preserves Cardiac Functionafter Experimental Myocardial Infarction

04/2011 — Annual Meeting of the International Society for Heart and Lung Transplantation (ISHLT), San Diego, USA

Secretome of apoptotic peripheral blood cells (APOSEC) confers cytoprotection to cardiomyocytes and inhibits tissue remodelling after acute myocardial infarction

08/2010 — European Society of Cardiology Congress 2010, Stockholm
Basic Science Reception of the ESC Council on Basic Cardiovascular Science

Irradiated cultured apoptotic peripheral blood mononuclear cells preserve ventricular function after myocardial infarction by inducing immunesuppressive and pro-angiogenic mechanisms

08/2010 — European Society of Cardiology Congress 2010, Stockholm

Irradiated cultured apoptotic peripheral blood mononuclear cells preserve ventricular function after myocardial infarction by inducing immunesuppressive and pro-angiogenic mechanisms

06/2010	Jahrestagung der Österreichischen Gesellschaft für Kardiologie 2010 *Irradiated Apoptotic Peripheral Blood Mononuclear Cells Preserve Ventricular Function After Myocardial Infarction: Implication of the Way of Cell Administration*
09/2009	40. Jahrestagung der Österreichischen Gesellschaft für Innere Medizin *Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium*
02/2008	EAACI-Ga^{2}len Davos Meeting 2008 *Alpha-Gal on Bioprostheses in Cardiac Surgery*

Book:

Apoptotic Cells and Myocardial Infarction: Irradiated apoptotic peripheral blood mononuclear cells preserve ventricular function after myocardial infarction (ISBN 3639194586)

MEMBERSHIPS

2011/04	International Society for Heart and Lung Transplantation (ISHLT)
2011/01	European Society of Cardiology – Working Group Cell Biology (ESC)
2010/11	Austrian Society of Cardiology (ÖKG)
2008/10	Austrian Society of Internal Medicine (ÖGIM)
2008/02	EAACI - European Academy of Allergology and Clinical Immunology
2007/08	Austrian Society of Transplantation, Transfusion and Genetics

AWARDS AND GRANTS

2011/05	Österreichischer Kardiologenpreis (1.Platz Basic Science) - €2500
2011/01	Leistungsstipendium Medizinische Universität Wien - €1500
2010/08	Traval Award of the ESC Council on Basic Cardiovascular Science - €500
2008/12	Research Scholarship – Medical University Vienna - €1500
2003/06	Matura (High School Graduation) with Distinction

TEACHING ACTIVITY

since 2007/02 Teaching Assistant at the Department of Thoracic Surgery, General Hospital Vienna, Medical University Vienna, Univ. Doz. Dr. Hendrik Jan Ankersmit

METHODS

Cultivation of human and animal cell lines
ELISA
Histology and Immunohistology
Flow Cytometry
Preparation of ethic committee proposals

REVIEWING ACTIVITY

Scandinavian Journal of Clinical Investigation

Xenotransplantation

COMPUTER SKILLS

MS Word
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SPSS
GraphPad Prism
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Image J
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ORIGINAL CONTRIBUTION

Intravenous and intramyocardial injection of apoptotic white blood cell suspensions prevents ventricular remodelling by increasing elastin expression in cardiac scar tissue after myocardial infarction

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Abstract Congestive heart failure developing after acute myocardial infarction (AMI) is a major cause of morbidity and mortality. Clinical trials of cell-based therapy after AMI evidenced only a moderate benefit. We could show previously that suspensions of apoptotic peripheral blood mononuclear cells (PBMC) are able to reduce myocardial damage in a rat model of AMI. Here we experimentally examined the biochemical mechanisms involved in preventing ventricular remodelling and preserving cardiac function after AMI. Cell suspensions of apoptotic cells were injected intravenously or intramyocardially after experimental AMI induced by coronary artery ligation in rats. Administration of cell culture medium or viable PBMC served as controls. Immunohistological analysis was performed to analyse the cellular infiltrate in the ischaemic myocardium. Cardiac function was quantified by echocardiography. Planimetry of the infarcted hearts showed a significant reduction of infarction size and an improvement of post AMI remodelling in rats treated with suspensions of apoptotic PBMC (injected either intravenously or intramyocardially). Moreover, these hearts evidenced enhanced homing of macrophages and cells staining positive for c-kit, FLK-1, IGF-I and FGF-2 as compared to controls. A major finding in this study further was that the ratio of elastic and collagenous fibres within the scar tissue was altered in a favourable fashion in rats injected with apoptotic cells. Intravenous or intramyocardial injection of apoptotic cell suspensions results in attenuation of myocardial remodelling after experimental AMI, preserves left ventricular function, increases homing of regenerative cells and alters the composition of cardiac scar tissue. The higher expression of elastic fibres provides passive energy to the cardiac scar tissue and results in prevention of ventricular remodelling.

Keywords Myocardial infarction · Apoptosis · Cytokines · Cell therapy · Elastin · Collagen

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Introduction

Within the last decades early reperfusion therapy significantly reduced mortality following acute myocardial infarction (AMI) and also improved survival and prognosis of patients. However, the development of chronic

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ischaemic heart disease and congestive heart failure represents one of the most frequent causes of hospitalisation in developed countries, particularly in patients with a large myocardial infarction [7, 41]. New therapeutic concepts were developed to reduce the risk of heart failure after an ischaemic event, ranging from the use of steroids in the 70s up to cell therapeutic strategies which were assessed in clinical trials over the last few years [6, 23, 34, 44]. More recently, new concepts were developed in cardiovascular research as how to modulate the paracrine and cellular response after myocardial ischaemia and by doing so reducing the loss of vital myocardium [15, 35]. It was shown that tumour necrosis factor alpha (TNF-alpha) acts as a major player in the post AMI paracrine microenvironment in a bidirectional way by not only causing transient contractile dysfunction but also by inducing cardioprotective effects. Concomitantly, regulating factors for TNF are up-regulated in stressed cardiac myocytes [8]. Moreover, TNF-alpha signalling also interacts with scar tissue formation after AMI by targeting fibroblasts, angiogenesis and also the system of matrixmetalloproteinases (MMP) and their inhibitors [2, 19, 36, 40]. More recently, studies further elucidated how paracrine factors mediate not only the tight regulation of post AMI inflammation but also cardioprotection after ischaemia which subsequently influences the formation of scar tissue and ventricular remodelling after AMI [5, 25].

These observations indicate that a rapid transition to the reparative phase after AMI mediated by cytokines, macrophages and myofibroblasts is a prerequisite for the preservation of cardiac function after ischaemia. Nevertheless, the fibrotic scar represents a thin, weakened area that is unable to withstand the pressure and volume load on the heart in the same manner as healthy myocardium would. The intraventricular pressure stresses the infarcted area with each contraction of the heart, the scar expands and the left ventricular chamber dilates over time [33]. Recent research has suggested that cardiac function may be improved after AMI by modifying the composition of myocardial scar tissue. One way of doing so could be to alter the ratio of elastic and collagenous fibres within the scar tissue in a positive way which would support the contractility of the damaged heart. Elastin is one of the major insoluble extracellular matrix components and consists of a core of tropoelastin surrounded by fibrillin and microfibrils. The elastin fibre network provides (scar) tissue with a critical property of elasticity and resilient recoil and maintains the architecture against repeated expansion. The elasticity of the myocardium highly depends on the ratio of muscle fibres to fibrotic tissue composed of cross-linked collagen [22]. A higher ratio of elastin expression in the myocardial scar may be able to alter the composition of the ventricular scar tissue in a way to preserve the elasticity of the infarcted heart. Based on these aspects, Mizuno et al. [27, 28] have shown that endothelial cells transfected with a plasmid vector containing the elastin gene increased elastic fibre expression within the scar significantly and preserved ventricular function in a rat model of AMI.

Another approach to prevent ventricular remodelling was investigated in an abundance of (pre-)clinical trials of stem cell therapy after AMI. Recently, this concept came under critical investigation since most clinical trials showed only inconsiderable beneficial results compared to control patients or these effects lasted only for a short period of time [3, 26]. Based on these results we sought to investigate an alternative approach namely whether injection of apoptotic cell suspensions shortly after AMI can prevent ventricular remodelling by combining immunomodulatory and pro-angiogenic effects. In 2005, this therapeutic principle was first described in a hypothesis by Thum et al. [38]. Several reports have substantiated this perception by showing the protective potential of stressed or apoptotic cell suspensions in various ischaemic disease entities [1, 11]. Moreover, experimental pre-treatment of spontaneously hypertensive rats with apoptotic cells reduced severe renal ischaemia reperfusion injury [39]. In a model of acute inflammatory lung injury, the administration of apoptotic cells enhanced the resolution of inflammation via increased TGF-beta secretion [17]. Of mechanistic relevance are also reports that demonstrate that infusion of apoptotic cells lead to allogeneic hematopoietic cell engraftment in a transplantation model and to a delay of lethal acute graft-versus-host disease (GVHD) [4, 31]. Moreover, infusion of donor apoptotic cells increased heart graft survival in a solid organ transplantation model [37].

However, the exact mechanism by which apoptotic cells can alter immune reactions after AMI and interfere with post-AMI tissue remodelling still remains to be elucidated. In our previous research, we have provided evidence that irradiation and induction of apoptosis in human peripheral blood mononuclear cells (PBMC) increased mRNA transcripts of Interleukin-8 and MMP9, two important factors for progenitor cell liberation from the bone marrow and their homing to sites of ischaemia [1]. Therefore, we sought to further elucidate the pleiotropic effects which apoptotic cells can induce when injected as a suspension in an experimental model of AMI. Here we show that suspensions of irradiated apoptotic PBMC injected either intravenously or intramyocardially cause progenitor cell homing to sites of myocardial ischaemia, prevent ventricular remodelling and alter the composition of cardiac scar tissue after AMI. This new concept evidences many convenient characteristics which are particularly of relevance compared to the therapeutic strategies mentioned earlier.

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Methods

Acquisition of syngeneic rat PBMC suspensions for in vivo experiments

Animal experiments were approved by the committee for animal research, Medical University of Vienna (vote: BMBWK-66.009/0278-BrGT/2005). All experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Syngeneic rat PBMC for in vivo experiments were separated by density gradient centrifugation from whole blood obtained from prior heparinized rats by direct punctuation of the heart. Cells were separated by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. Apoptosis of PBMC was induced by Caesium-137 irradiation (Department of Transfusion Medicine, General Hospital Vienna) with 45 Gray (Gy). Cells were resuspended in serum-free UltraCulture Medium (UltraCulture, Lonza, Switzerland) and cultured in a humidified atmosphere for 18 h. Induction of apoptosis was measured by Annexin-V/propidium iodine (FITC/PI) co-staining (Becton–Dickinson, Franklin Lakes, NJ, USA) on a flow cytometer. Annexin-positivity of PBMC was determined as >80% and these cells were consequently termed apoptotic PBMC (irradiated apoptotic PBMC, IA-PBMC).

Experimental rat model of acute myocardial infarction

Acute myocardial infarction was induced in adult male Sprague–Dawley rats (weight 300–350 g) by ligating the left anterior descending artery (LAD) as described previously [1]. In short, animals were anaesthetized intraperitoneally with a mixture of xylazine (1 mg/100 g bodyweight) and ketamine (10 mg/100 g bodyweight) and ventilated mechanically. A left lateral thoracotomy was performed and a ligature using 6-0 prolene was placed around the LAD beneath the left atrium. Immediately after the onset of ischaemia, cell suspensions of 8.5×10^6 cells viable or apoptotic PBMC suspended in 0.3 mL UltraCulture Medium were injected in the femoral vein. Furthermore, apoptotic cells (8.5×10^6 cells) were also injected directly into the myocardium at five different sites of the peri-infarct zone. Injection of cell culture medium alone and sham operation served as controls in this experimental setting.

Histology and immunohistochemistry

Animals were killed either 72 h or 6 weeks after experimental infarction. Hearts were explanted and then sliced at three layers at the level of the largest extension of infarcted area ($n = 6$ for 72 h analyses, $n = 10$–12 for 6 weeks analyses). Slices were fixed in 10% neutral buffered formalin and embedded in paraffin. The tissue samples were stained with hematoxylin–eosin (H&E) and Elastica van Gieson (EVG). Immunohistochemical evaluation 72 h after AMI was performed using the following antibodies directed to CD68 (MCA 341R, AbD Serotec, Kidlington, UK), c-kit (sc-168, Santa Cruz Biotechnology, CA, USA), FLK1 (sc-6251, Santa Cruz Biotechnology, CA, USA), IGF-I (sc-9013, Santa Cruz Biotechnology, CA, USA) and FGF-2 (sc-79, Santa Cruz Biotechnology, CA, USA). Tissue samples were evaluated on an Olympus AX70 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) at 200× magnification and captured digitally using Meta Morph v4.5 Software (Molecular Devices, Sunnyvale, USA). Image J planimetry software (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, USA) was utilised to determine the area of necrosis after 3 days and the size of myocardial infarct after 6 weeks. The extent of infarcted myocardial tissue (% of left ventricle) was calculated by dividing the area of the circumference of the infarcted area by the total endocardial and epicardial circumferenced areas of the left ventricle. Planimetric evaluation after 6 weeks was carried out on tissue samples stained with EVG for better comparison of vital myocardium and fibrotic areas. Infarction size was expressed as a percentage of the total left ventricular area. EVG stained tissue specimens were analysed microscopically for the ratio of elastic and collagen fibres. Using Image J Planimetry software, the ratio was calculated by dividing the area occupied by elastic fibres by the total area of scar tissue.

Assessment of cardiac function by echocardiography

Six weeks after induction of myocardial infarction, rats were anaesthetized with 100 mg/kg ketamine and 20 mg/kg xylazine. The sonographic examination was conducted on a Vivid 7 system (General Electric Medical Systems, Waukesha, USA). Analyses were performed by an experienced observer blinded to the treatment groups to which the animals were allocated. M-mode tracings were recorded from a parasternal short-axis view and functional systolic and diastolic parameters were obtained (shortening fraction, SF; left ventricular end-diastolic diameter, LVEDD; left ventricular end-systolic diameter, LVESD). Shortening fractional was calculated as follows: $SF(\%) = ((LVEDD - LVESD)/LVEDD) \times 100$.

Cell culture of human PBMC

Human peripheral blood mononuclear cells (PBMC) were obtained from young healthy volunteers after informed

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consent. All experimental procedures were approved by the Regional Committee for Medical Research Ethics (ethics committee vote: EK-Nr 2010/034) and were conducted in compliance with the Declaration of Helsinki Principles. Cells were separated by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. Apoptosis of PBMC was induced by Caesium-137 irradiation with 60 Gray (Gy) for in vitro experiments. Cells were resuspended in serum-free UltraCulture Medium and cultured in a humidified atmosphere for 24 h at a density of 2.5×10^6 cells/mL, $n = 5$. Induction of apoptosis was measured by Annexin-V/propidium iodine (FITC/PI) co-staining (Becton–Dickinson, Franklin Lakes, NJ, USA) on a flow cytometer. Annexin-positivity of PBMC was determined to be >80% in order to characterise apoptotic cells.

Fibroblast cell culture, RNA isolation and cDNA preparation

Cell Culture supernatants were obtained from viable PBMC, IA-PBMC and mixed co-cultures of viable cells and IA-PBMC after 24 h (cell density 2.5×10^6 suspended in UltraCulture Medium). 1×10^5 human primary fibroblasts (Cascade Inc., Portland, USA) were exposed to supernatants obtained from viable PBMC, apoptotic PBMC and mixed cultures of viable and apoptotic cells for 24 h. Fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Gaithersburg, USA) supplemented with 10% foetal bovine serum (FBS, PAA, Linz, Austria), 25 mM L-glutamine (Gibco BRL, Gaithersburg, USA) and 1% penicillin/streptomycin (Gibco) and seeded in 12 well plates. After RNA extraction of fibroblasts (using RNeasy, QiAGEN, Vienna, Austria) following the manufacturer's instruction, cDNAs were transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, USA) as indicated in the instruction manual.

Quantitative real time polymerase chain reaction (RT-PCR)

mRNA expression was quantified by real time PCR with LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. The primers for Elastin (forward: 5′-CCTACTTACGGGGTTGG-3′, reverse: 5′-GCCGAGCAGACAAGAA-3′), Collagen Type I (forward: 5′-GTGCTAAAGGTGCCAATGGT-3′, reverse: 5′-CTCCTCGCTTTCCTTCCTCT-3′), Collagen Type III (forward: 5′-GTCCATGGATGGTGGTTTTC-3′, reverse: 5′-CACCTTCATTTGACCCCATC-3′), Collagen Type V (forward: 5′-GTCCATACCCGCTGGAAA-3′, reverse: 5′-TCCATCAGGCAAGTTGTGAA-3′), IL-8 (forward: 5′-CTCTTGGCAGCCTTCCTGATT-3′, reverse: 5′-TATGCACTGACATCTAAGTTCTTTAGCA-3′), MMP1 (forward: 5′-GGTCTCTGAGGGTCAAGCAG-3′, reverse: 5′-CCGCAACACGATGTAAGTTG-3′), MMP3 (forward: 5′-TGCTTTGTCCTTTGATGCTG-3′, reverse: 5′-GGCCCAGAATTGATTTCCTT-3′), MMP9 (forward: 5′-GGGAAGATGCTGGTGTTCA-3′, reverse: 5′-CCTGGCAGAAATAGGCTTC-3′) and β-2-microglobulin (β2M, forward: 5′-GATGAGTATGCCTGCCGTGTG-3′, reverse: 5′-CAATCCAAATGCGGCATCT-3′) were designed as described previously [18]. The relative expression of the target genes was calculated by comparison with the house keeping gene β2M using a formula described by Pfaffl et al. [32]. The efficiencies of the primer pairs were determined as described [18].

Evaluation of cytokines and growth factors secreted by apoptotic PBMC

Membrane arrays for the detection of cytokines and growth factors in pooled cell culture supernatants derived from non-irradiated and IA-PBMC (cultured at a density of 2.5×10^6/ml, cells obtained from 4 healthy volunteers) were performed to scan the protein content (AAH-CYT-G4000, RayBiotech, Norcross, USA). Experiments were performed according to the manufacturer's protocol.

Statistical methods

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, USA). All data are given as mean ± standard error of the mean (SEM). The Wilcoxon-Mann–Whitney-Test was utilised to calculate significances between the groups. p values <0.05 were considered statistically significant (p values were expressed as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Histological and immunohistological analysis of explanted hearts 3 days after AMI

Since the inflammatory response after acute ischaemia determines the road map to ventricular dilation, we performed histological analysis after 72 h after AMI in order to study short term effects of intravenous (IV) or intramyocardial (IM) injection of apoptotic cell suspensions. H&E stained specimens obtained from control AMI animals evidenced a mixed cellular infiltrate in the wound areas in accordance with granulation tissue with abundance of neutrophils, macrophages/monocytes, lymphomononuclear cells admixed to dystrophic cardiomyocytes within

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the first 72 h after AMI (Fig. 1a,b). In contrast, rats treated with apoptotic PBMC suspensions evidenced a dense monomorphic infiltrate in wound areas (Fig. 1c,d). Furthermore, the total area of necrosis was significantly smaller in rats injected with apoptotic cells in comparison to controls (Fig. 1e). Immunohistological analysis revealed that the cellular infiltrate in both treatment groups (intravenous and intramyocardial injection) was composed of abundant CD68+ monocytes/macrophages that was much weaker in the control group (Fig. 1f–j). Cell counts quantified per high power-field (HPF) were 28.6 ± 2.4 (±SEM) in control, 36.0 ± 3.5 (±SEM) in animals injected with non-irradiated viable cells compared to 55.3 ± 3.4 and 76.5 ± 5.9 (± SEM) in rats treated with apoptotic cells (IV or IM, respectively). In addition, most of the medium-sized monocytoid cells were identified to be highly positive for c-kit (CD117) and vascular endothelial growth factor receptor 2 (FLK-1). High power-field (HPF) cell counts for c-kit were 68.0 ± 3.1(±SEM) in controls, 77.0 ± 4.6 (±SEM) in the group injected with viable cell versus 121.2 ± 9.4 (±SEM) in IV and 168.6 ± 12.4 (±SEM) in IM injected rats (Fig. 1k–o). For FLK-1, values were 58.3 ± 5.6 (±SEM) in controls, 86.0 ± 7.0 (±SEM) for viable cell injected rats, 170.3 ± 7.1 (±SEM) for IV and 202.0 ± 9.4 (±SEM) for IM injected rats (Fig. 1p–t). Expression of these three cellular markers was more accentuated in rats injected with apoptotic cell suspensions compared to the control AMI group. (see representative images Fig. 1, $n = 5$–6 per group).

Histological analysis 6 weeks after induction of AMI

Cardiac tissue specimens obtained 6 weeks after ligation of the LAD and stained according to the Elastica van Gieson protocol showed that hearts from rats injected with apoptotic PBMC suspensions showed a highly significant reduction of infarction area. Figure 3a shows representative images which evidence that rats treated with apoptotic cells demonstrate a significantly greater extent of viable myocardium within the anterior free wall of the left ventricle. In contrast, collagen deposition and scar formation extended through almost the entire left ventricular wall thickness in rats receiving control medium. Compared to controls and viable injected animals the left ventricular geometry was almost completely preserved in rats treated with irradiated apoptotic cell suspensions. The area of fibrosis as calculated by planimetry was 25% (percent of the left ventricle) in medium injected controls, 14% in viable cell injected animals compared to 6% (IV) and 8% (IM) in rats treated with apoptotic PBMC suspensions ($p < 0.01$ vs. controls, $p < 0.05$ vs. viable cell injected animals, $n = 10$–12 per group) (Fig. 2a,b).

Evaluation of functional parameters by echocardiography

Cardiac function was evaluated by means of echocardiography. Six weeks after ligation of the LAD the mean left ventricular shortening fractions (SF), left ventricular end-diastolic diameters (LVEDD), left ventricular end-systolic diameters (LVESD) were determined to be 19% ± 1 (SF), 10.4 mm ± 0.2 (LVEDD), 8.5 mm ± 0.2 (LVESD) in animals that received culture medium alone (±SEM). In animals injected with viable cells similar values were obtained: 18% ± 2 (SF), 11.0 mm ± 0.4 (LVEDD), 9.0 mm ± 0.5 (LVESD),

Noteworthy, rats with intravenous injection of apoptotic cell suspensions evidenced significantly improved functional parameters: 25% ± 3 (SF), 8.9 mm ± 0.3 (LVEDD), 6.8 mm ± 0.4 (LVESD). In animals with direct intramyocardial injection of apoptotic cells these parameters were improved as well: 26% ± 2 (SF), 9.8 mm ± 0.4 (LVEDD), 7.4 mm ± 0.5 (LVESD). Mean levels of cardiac function of healthy rats without myocardial infarction (sham operated) were as follows: 29% ± 2 (SF), 9.2 mm ± 0.4 (LVEDD), 6.5 mm ± 0.3 (LVESD), ($p <$ 0.05 vs. viable cell injected animals and medium injected controls, $n = 10$–12 per group) (Fig. 2c–e).

These findings indicate a significant improvement or preservation of cardiac function in both treatment groups that were injected with apoptotic cell suspensions. Rats that were injected with viable non-irradiated PBMC or fresh culture medium showed signs of dilation which were accompanied by a considerable loss of ventricular function.

Alterations in the composition of the fibrotic scar tissue

Six weeks after AMI the composition of the fibrotic scar tissue was analysed microscopically (Elastica van Gieson staining). Especially within the border zone between viable myocardium and scar tissue a highly remarkably accumulation of elastic fibres was detected in animals injected with suspensions of apoptotic cells compared to controls (Fig. 3a–d). A planimetric analysis revealed that the fibrotic scar in apoptotic cell (IV and IM) injected rats was composed by 5.5% ± 1.1 and 8.9% ± 2.2 of elastic fibres compared to 0.2% ± 0.1 in controls and 2.9% ± 0.2 in viable injected animals (Fig. 3e), ($p < 0.001$ vs. control, $n = 10$–12 per group). This finding stands in relation to higher levels of cells stained positive for insulin-like growth factor I (IGF-I) and fibroblast growth factor 2 (FGF-2) in treated animals (Fig. 3i–k) compared to controls (Fig. 3f–h). In animals that were injected with apoptotic cell suspensions 36.0 ± 3.3 cells staining positive for IGF-I and 49.8 ± 5.2 for FGF-2 were found in immunohistological analyses of myocardial specimens 72 h after

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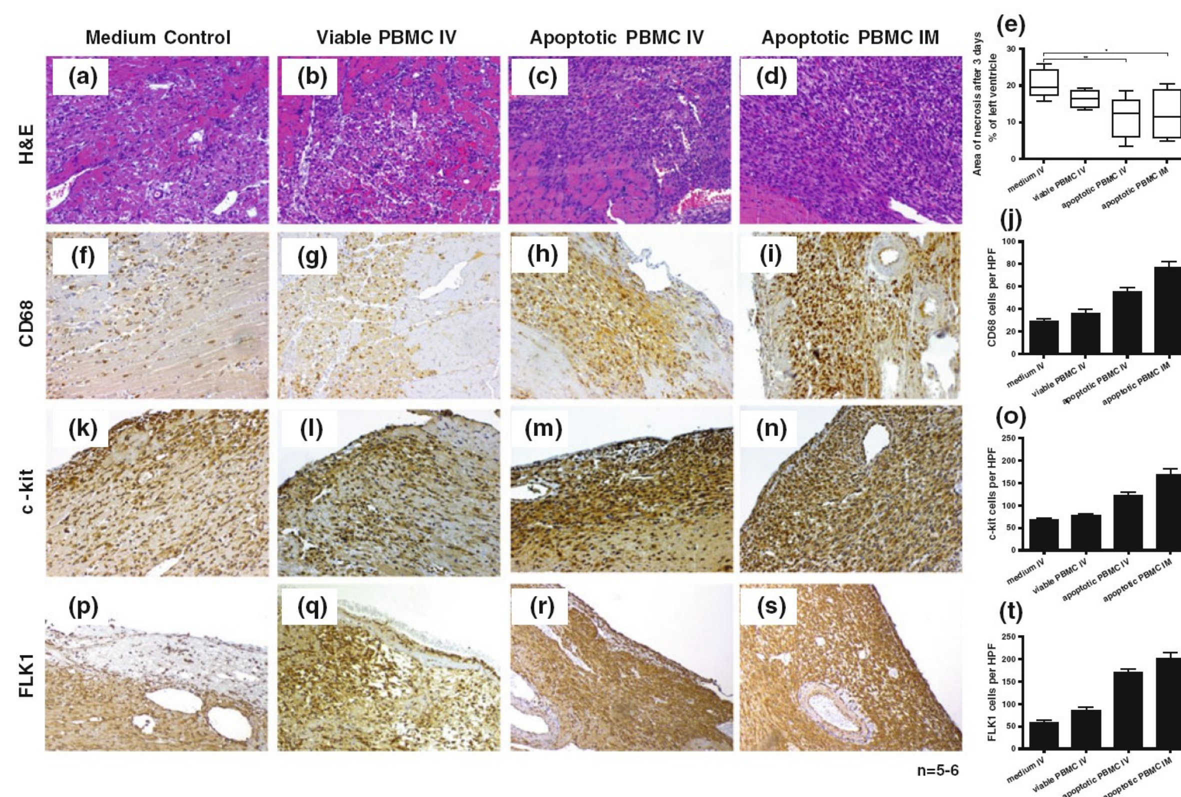

Fig. 1 Results 3 days after AMI induction: H&E stained rat myocardium 3 days after LAD ligation. The cellular infiltrate appears to be more consolidated in apoptotic PBMC treated animals (**a–d**). The total area of necrosis was significantly reduced in both treatment groups compared to controls (**e**). Below, myocardial tissue stained for CD68 is shown (**f–i**). Quantification of positively stained cells per high power-field (HPF) indicated higher numbers of macrophages in both treatment groups (**h–j**). When tissue specimens were stained for c-kit (**k–n**), more of these cell populations could be detected in the epicardial zone in rats injected with apoptotic cells (**m–o**). Concomitantly, more cells staining positive for FLK-1/VEGF receptor 2 were found in the same areas (**p–t**)

LAD ligation. In controls only 7.0 ± 1.6 IGF-I and 31.5 ± 2.3 FGF-2 positive cells were detectable.

In order to specify the association of elastin and collagen production within myocardial scar tissue and mechanisms induced by apoptotic cells RT-PCR analyses were conducted. When exposing fibroblasts to cell culture supernatants obtained from apoptotic PBMC or apoptotic cells co-incubated with viable cells elastin expression increased only slightly by 1.2- to 1.4-fold. The expression of collagen type III and IV increased moderately by 1.9- to 2.5-fold compared to controls (Fig. 3l). Supernatants derived from apoptotic cells also increased the expression of IL-8 (4- to 6.5-fold), MMP1 (18- to 31-fold), MMP3 (10- to 16-fold) and MMP9 (4- to 7-fold) in fibroblasts indicating a mechanistic cross-talk between apoptotic cells, their secretome and resident cells that could be accounted for alterations of the compositions of the extracellular matrix (Fig. 3m).

Analysis of paracrine factors secreted by non-irradiated and irradiated apoptotic cells

Based on the finding that the expression of transcripts for IL-8 and MMPs were up-regulated in apoptotic cells we screened the supernatant obtained from irradiated and non-irradiated cell for 274 cytokines and growth factors. Considerable differences were observed (amongst others) for IL-8, VEGF, MMP3, MMP9, IL-16, ENA-78 and MIP-1alpha (see supplementary table).

Discussion

Over the last decade, cardiovascular research has focused on determining clinical protocols for stem cell therapy in order to salvage damaged myocardium after AMI. Here we present evidence that suspensions of apoptotic cells can

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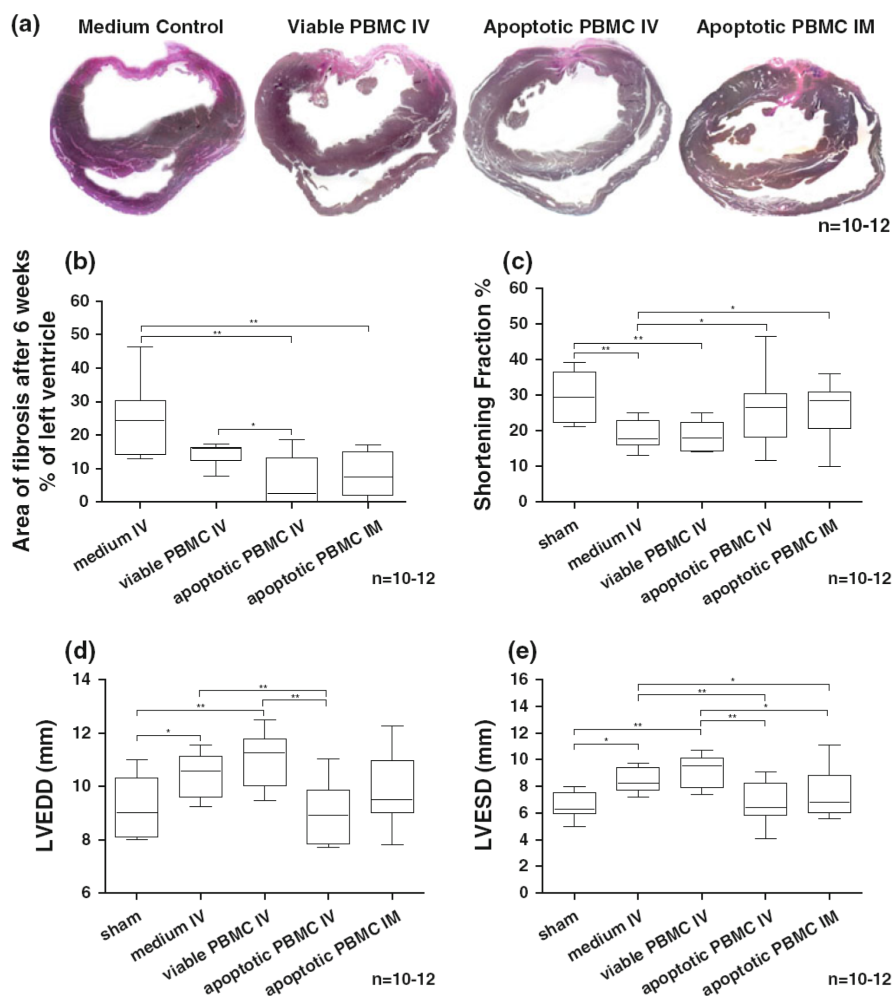

Fig. 2 Results of in vivo rat experiments after 6 weeks. **a** Hearts of apoptotic cell injected animals explanted 6 weeks after LAD ligation evidence less myocardial damage compared to controls. Hearts from medium or viable cell injected animals appear more dilated and show a greater extension of fibrotic tissue. **b** Statistical analysis of data obtained from planimetric evaluation of specimens collected 6 weeks after LAD ligation shows a mean scar extension of 25% in medium injected controls, 14% in viable cell injected animals compared to 6% (IV) and 8% (IM) in rats treated with apoptotic PBMC. **c–e** Results obtained by echocardiography 6 weeks after AMI (shortening fraction; left ventricular enddiastolic diameter, LVEDD; left ventricular endsystolic diameter, LVESD). Functional parameters were improved in comparison to medium or viable cell injected animals

serve as a potent therapeutic entity for preserving ventricular function after ischaemia when being injected shortly after occlusion of a coronary artery. We could show that apoptotic PBMC act in a cardioprotective manner by pleiotropic effects, first of all, the expression of the pro-angiogenic chemokine IL-8 and various matrixmetalloproteinases is induced in cells when being exposed to the conditioned medium of apoptotic PBMC. IL-8 and MMP9 are factors which are of great importance for reparative processes in the ischaemic heart by enabling progenitor cell populations of the bone marrow to enter the circulation and home to sites of myocardial damage [14, 20]. Consequently, more cells staining positive for c-kit and FLK-1/VEGF receptor 2 were detected in the myocardium of rats injected with apoptotic cells. This phenomenon was detectable even to a much greater extent when irradiated cells where injected directly into the ischaemic tissue. Furthermore, also higher numbers of macrophages where found in the ischaemic myocardium 3 days after induction of AMI, suggesting an accelerated repair phase in both treatment groups. When interpreting our data obtained by immunohistology correctly, we believe that injection of apoptotic cell suspensions shortly after AMI results in quicker changeover from an inflammatory phase to tissue infiltration of c-kit+/FLK-1 cells into the ischaemic myocardium causing increased angiogenesis in the early phase after AMI [30]. Previous work has confirmed that bone marrow progenitor cells improve cardiac function after AMI, regardless of whether transdifferentiation of the cells to cardiomyocytes occurs or not [29]. Fazel et al. defined the significant role of bone marrow derived c-kit+ cells as an essential population for cardiac repair. In a knock-out model of c-kit it has been shown that the triggering of this receptor is a necessary prerequisite for bone marrow progenitor cell mobilization after ischaemia since c-kit dysfunction led to a reduced myofibroblast repair response and

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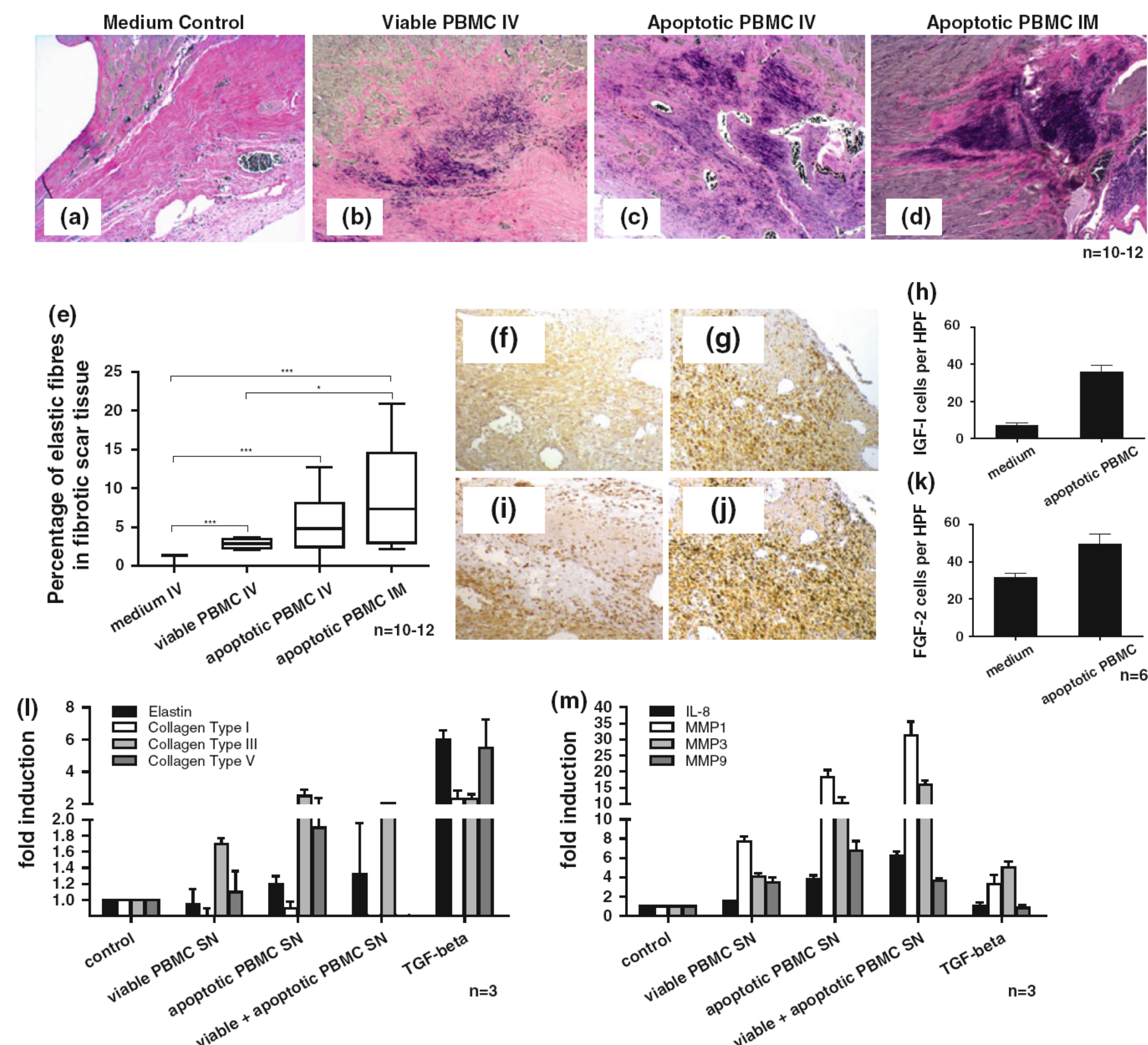

Fig. 3 Composition of fibrotic scar tissue. **a–d** Representative images of tissue specimens obtained 6 weeks after LAD ligation and stained according to the Elastica van Gieson protocol. Significantly more elastic fibres were found within the scar tissue of AMI rats injected with apoptotic PBMC. **f**, **g** Specimens stained for IGF-I in controls and treated animals. **i**, **j** Immunohistological staining for FGF-2. More cells staining positive for IGF-I and FGF-2 were found in hearts from rats injected with apoptotic cells. **l**, **m** Data from RT-PCR analyses. When fibroblasts were exposed to cell culture supernatants obtained from apoptotic PBMC the expression of elastin, collagen type III and IV, IL-8, MMP1, MMP3 and MMP9 was up-regulated

subsequently to cardiac failure after experimental AMI. It was also determined that c-kit activation requires the activity of MMP9 within the bone marrow compartment [9, 12, 13]. Regarding our own data this correlates with an up-regulation of MMP transcripts in fibroblasts when exposed to conditioned medium obtained from cell cultures of apoptotic cells.

Six weeks after ligation of the LAD, a further important finding was that the treatment with apoptotic cells also attenuated the extent of infarction in this experimental AMI model. This also became apparent in echocardiography. Animals injected with irradiated apoptotic cell suspensions evidenced a significant improvement of all tested functional parameters.

Also of great interest was the finding that the composition of the extracellular matrix within the scar tissue was evidently altered compared to the controls. In Elastica van Gieson stained specimens, a considerable accumulation of elastic fibres, especially, in the border zone between vital cardiomyocytes and the fibrotic scar was detected. This

alteration in the elastin/collagen ratio could be a major factor contributing to the improvement of cardiac function parameters in rats injected with apoptotic cells. This was even more pronounced when these cell suspensions were injected directly into the ischaemic myocardium. Together with the increased accumulation of elastic fibres we also found higher numbers of cells staining positive for insulin-like growth factor I (IGF-I) and fibroblast growth factor 2 (FGF-2). It has been reported that these two growth factors contribute directly to the synthesis of elastic fibres within the extracellular matrix and regulate cardiac repair mechanisms after AMI [10, 21, 24, 42, 43]. There are a number of possible mechanisms by which a favourable ratio of elastic and collagenous fibres in the myocardial scar tissue could delay the onset of ventricular dysfunction and remodelling. A cardiac scar tissue showing more elastic properties and being more resistant could function as a shock absorbing cushion thus reducing the tractive effects of the acute increase in intraventricular pressure on the scar during systole. The recoil of the elastic fragments in the scar could provide passive energy to return the scar size and ventricular chamber volume to precontractile dimensions. These mechanistic characteristics are important for preventing or reducing the risk for ventricular remodelling.

Conclusion

In summary, we believe that PBMC undergoing apoptotic cell death feature many pleiotropic effects which evidence multiple beneficial characteristics in the setting of (experimental) AMI. Based on these data we conclude that apoptotic cells induce the expression of pro-angiogenic factors necessary for attraction of regenerative cells to sites of ischaemia. An additional mechanistic concept has already been discussed in the scientific literature and states that apoptotic cells interact with surrounding cells causing immunomodulation [16]. A modulatory effect on invading inflammatory cells into the ischaemic myocardium could also prove to be beneficial by reducing the secondary damage caused by neutrophils in the early stages after AMI. The combination of these mechanisms leads to a quicker resolution of the inflammatory phase after AMI, induces the homing of CD68+, c-kit+ and FLK-1+ cells, accelerates reparative processes, alters the elastin/collagen ratio within the scar tissue in a beneficial way and in the end improves ventricular function.

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ORIGINAL CONTRIBUTION

Secretome of apoptotic peripheral blood cells (APOSEC) confers cytoprotection to cardiomyocytes and inhibits tissue remodelling after acute myocardial infarction: a preclinical study

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Abstract Heart failure following acute myocardial infarction (AMI) is a major cause of morbidity and mortality. Our previous observation that injection of apoptotic peripheral blood mononuclear cell (PBMC) suspensions was able to restore long-term cardiac function in a rat AMI model prompted us to study the effect of soluble factors derived from apoptotic PBMC on ventricular remodelling after AMI. Cell culture supernatants derived from irradiated apoptotic peripheral blood mononuclear cells (APOSEC) were collected and injected as a single dose intravenously after myocardial infarction in an experimental AMI rat model and in a porcine closed chest reperfused AMI model. Magnetic resonance imaging (MRI) and echocardiography were used to quantitate cardiac function. Analysis of soluble factors present in APOSEC was performed by enzyme-linked immunosorbent assay (ELISA) and activation of signalling cascades in human cardiomyocytes by APOSEC in vitro was studied by immunoblot analysis. Intravenous administration of a single dose of APOSEC resulted in a reduction of scar tissue formation in both AMI models. In the porcine reperfused AMI model, APOSEC led to higher values of ejection fraction (57.0 vs. 40.5%, $p < 0.01$), a better cardiac output (4.0 vs. 2.4 l/min, $p < 0.001$) and a reduced extent of infarction size (12.6 vs. 6.9%, $p < 0.02$) as determined by MRI. Exposure of primary human cardiac myocytes with APOSEC in vitro triggered the activation of pro-survival signalling-cascades (AKT, Erk1/2, CREB, c-Jun), increased anti-apoptotic gene products (Bcl-2, BAG1) and protected them from starvation-induced cell death. Intravenous infusion of culture supernatant of apoptotic PBMC

M. Lichtenauer and M. Mildner contributed equally to this work.

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attenuates myocardial remodelling in experimental AMI models. This effect is probably due to the activation of pro-survival signalling cascades in the affected cardiomyocytes.

Keywords Apoptosis · Myocardial infarction · Cardioprotection · Ischaemia/reperfusion

Introduction

Although prompt reperfusion within a narrow time window has significantly reduced early mortality following acute myocardial infarction (AMI), ischaemic heart failure remains widely prevalent and represents an increasing economic burden [42]. A new field in regenerative cardiovascular medicine emerged when investigators observed that distant stem cells were able to sense sites of damage and promote structural and functional repair after experimental myocardial infarction [6, 9, 10, 17, 29, 31, 38, 44]. This experimental insight into regeneration following AMI triggered the start of randomized, controlled clinical trials demonstrating that cell therapy could improve cardiac function in patients after AMI [14, 27, 36, 43]. In 2005, Thum et al. established "The Dying Stem Cell Hypothesis", namely, that therapeutic stem cells are already undergoing apoptosis while being infused into the infarcted area, thereby attenuating infarction-induced immunoactivation and remodelling via the induction of immunomodulatory mechanisms [33, 34, 39].

We have previously shown that infusion of cultured irradiated apoptotic peripheral blood mononuclear cell (PBMC) suspensions in a rat acute AMI model caused homing of regenerative FLK+/c-kit+ cells in the early phase of experimental AMI and restored long-term cardiac function [2, 26]. In contrast, infusion of cultured viable PBMC in the same setting had only marginal efficacy in preservation of cardiac function. Moreover, we found that induction of apoptosis in PBMC led to the massive secretion of Interleukin-8 (IL-8) and Matrixmetalloproteinase 9 (MMP9) proteins known to be responsible for neo-angiogenesis and recruitment of pro-angiogenic cells from the bone marrow (BM) to the infarcted myocardium [20, 24, 28].

Our data suggest two possible causes for this in vivo regenerative effect. Either infusion of apoptotic PBMC reduces the immune response after AMI by defined mechanisms [4, 34] or soluble factors secreted by apoptotic PBMC cause induction of neo-angiogenesis and cytoprotection in the acute phase of myocardial infarction. This latter speculation is supported by the recent publications providing evidence that bone marrow cells or endothelial progenitor cells secrete soluble proteins which induce regenerative mechanisms in a paracrine manner [7, 13, 25].

Having shown that infusion of apoptotic PBMC suspensions in an acute rat AMI model prevented ventricular remodelling, we investigated whether simply administering soluble factors derived from irradiated PBMC (*APOSEC*; this acronym stands for **apo**ptotic **sec**retome) showed similar properties and caused cardioprotection in the same acute rat AMI model. To further strengthen the clinical relevance of APOSEC, we tested this "biological" in cell cultures of cardiac myocytes in vitro and in a porcine closed chest reperfused AMI model in vivo. Here we provide evidence that APOSEC confers cytoprotection directly to cardiac myocytes, and that intravenous application of APOSEC is effective in preventing myocardial damage and tissue remodelling in a dose-dependent manner in the porcine reperfused AMI model.

Methods

Generation of cell culture medium derived from irradiated human apoptotic PBMC (APOSEC^{H}) for in vitro assays

Human PBMC were obtained from young healthy volunteers after informed consent (ethics committee vote: EK-Nr 2010/034). Cells were separated by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation as described previously [2]. Apoptosis of PBMC was induced by Caesium-137 irradiation (Department of Transfusion Medicine, General Hospital Vienna) with 60 Gray (Gy) for in vitro experiments. Induction of apoptosis was measured by Annexin-V/propidium iodine (FITC/PI) co-staining (Becton–Dickinson, Franklin Lakes, NJ, USA) on a flow cytometer. Irradiated and non-irradiated cells were resuspended in serum-free UltraCulture Medium (Lonza, Switzerland) and cultured for 24 h in various cell densities (1×10^6, 2.5×10^6 and 25×10^6 cells/ml, $n = 5$). After 24 h supernatants were collected and served as experimental entities for ELISA content analysis or were lyophilized as follows: supernatants were dialyzed against ammonium acetate (at a concentration of 50 mM) for 24 h at 4°C. The obtained liquid was sterile filtered (Whatman Filter 0.2 µm FP30/o,2 Ca–S, Dassel, Germany), frozen and lyophilized overnight (Lyophilizator Christ alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) (Fig. 1).

To avoid possible cross-species detrimental immune reactions, we opted to utilize APOSEC preparations solely in a syngeneic fashion.

For in vivo rat experiments, syngeneic rat PBMC were separated by density gradient centrifugation from whole blood obtained from heparinized rats. PBMC were irradiated by Caesium-137 (45 Gy) and cultured for 24 h at a

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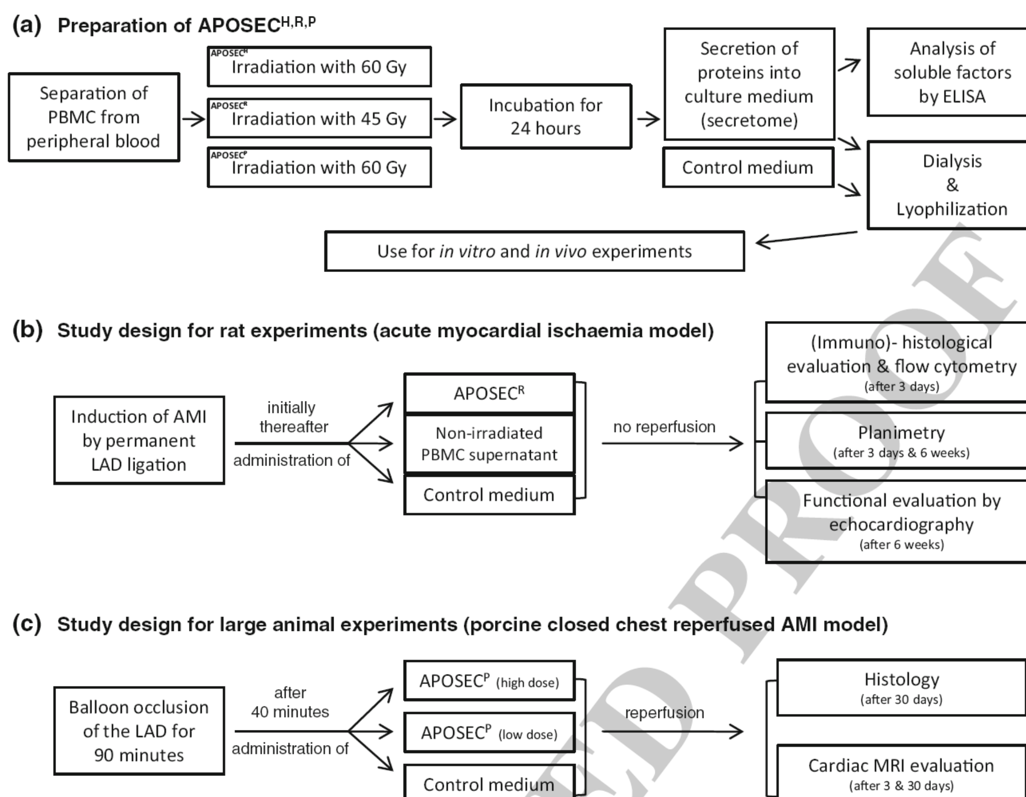

Fig. 1 Study design: **a** The preparation process of APOSEC, starting with cell separation, induction of apoptosis, cell culture, dialysis, lyophilization and its use in in vitro and in vivo experiments is shown. **b** Study design for in vivo rat experiments. A permanent ligation of the LAD was conducted in male Sprague–Dawley rats, initially thereafter APOSEC^R, supernatant derived from non-irradiated cells or control medium was injected intravenously into the femoral vein. Evaluations by immunohistology and flow cytometry were performed 3 days after LAD ligation. Planimetric analysis and echocardiography were conducted 6 weeks after MCI. **c** Experimental setting of closed chest reperfused AMI in a porcine model. Ischaemia was induced by balloon occlusion of the LAD for 90 min. 40 min after balloon inflation, APOSEC^P was administered intravenously. Cardiac MRI evaluations were performed 3 and 30 days after AMI

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cell density of 25×10^6 cells/ml (in UltraCulture Medium, Lonza, Switzerland). APOSEC for rat experiments (APOSEC^R) was further processed as described for APOSEC^H. Supernatants of non-irradiated rat PBMC served as controls.

For large animal experiments, blood was obtained from anaesthetized pigs by direct heart puncture. Three pigs were anaesthetized with an IV bolus of 10 mg/kg ketamine and 1.3 mg/kg azaperone and a left thoracic dermal incision was conducted. Direct heart puncture was then performed under sterile conditions using a hollow needle and blood was drawn with 50-mL syringes. Blood obtained during this procedure was transferred into heparinized plastic bags for blood products. PBMC were then obtained according to the protocol described above. CellGro serum-free medium (Cell Genix, Freiburg, Germany), a “Good Manufacturing Practice” certified culture medium, was utilized for porcine PBMC-derived APOSEC production (APOSEC^P). APOSEC for porcine experiments (APOSEC^P) was processed as described for APOSEC^H.

APOSEC^H content evaluation by membrane arrays and ELISA analysis

APOSEC^H was screened for cytokines and angiogenic factors using two commercially available array systems (Proteome Profiler Arrays, R&D Systems, Minneapolis, USA). Supernatant levels of cytokines secreted by irradiated and non-irradiated PBMC in various concentrations were measured by utilizing commercially available enzyme-linked immunosorbent assay (ELISA, Duoset, R&D Systems, Minneapolis, USA) kits for the quantification of IL-8, GRO-α, ENA-78, VEGF, IL-16, IL-10, TGF-β, sICAM-1, RANTES, IL-1ra, MIF, PAI-1, IGF-I, HGF, FGF-2, MCP-1, MMP9, SDF-1, G-CSF, GM-CSF and HMGB1 (IBL International GmbH, Hamburg, Germany).

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Acute rat ischaemic model and APOSEC^R treatment

Animal experiments were approved by the committee for animal research, Medical University of Vienna (vote: 66.009/0168-II/10b/2008). Acute myocardial infarction was induced in adult male Sprague–Dawley rats (weight 275–300 g) by ligating the LAD. A left lateral thoracotomy was performed and a ligature using 6-0 prolene was placed around the LAD beneath the left atrium. Immediately after the onset of ischaemia, lyophilized supernatants obtained from 8.5×10^6 either irradiated apoptotic PBMC or non-irradiated viable cells resuspended in 0.3 ml fresh Ultra-Culture Medium (Lonza, Basel, Switzerland) were injected in the femoral vein. Injection of cell culture medium alone and sham operation served as controls.

Rat immunohistochemistry and determination of myocardial infarction size by planimetry

All animals were sacrificed either 72 h or 6 weeks after experimental infarction. Hearts were explanted and then sliced into three layers at the level of the largest extension of infarcted area ($n = 6$ for 72 h analyses, $n = 9$ for 6 weeks analyses). The tissue samples were stained with hematoxylin–eosin (H&E) and Elastica van Gieson (EVG). Short-term immunohistological evaluation (72 h) was performed using antibodies directed to CD68 (MCA341R, AbD Serotec, Kidlington, UK) and c-kit (sc-168, Santa Cruz Biotechnology, CA, USA). Image J planimetry software (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, USA) was utilized to determine the size of myocardial infarct after 6 weeks.

Flow cytometry analysis, homing of CD68+ and c-kit+ cells

Three days after myocardial infarction rats treated with either APOSEC^R, viable cell derived supernatant or control medium were sacrificed. Infarcted areas of explanted hearts were cut into small cubes (1 mm) and incubated with collagenase (2.4 U/ml, Sigma, St Louis, Mo) for 12 h at 4°C. After digestion and washing, the cells were incubated with primary antibodies directed to CD68 (MCA341R, AbD Serotec, Kidlington, UK) and c-kit (sc-168, Santa Cruz Biotechnology, CA, USA). After an incubation period with a secondary antibody, cell suspensions were analysed for total CD68+ and c-kit+ cell numbers by flow cytometry (FACS Calibur, Becton–Dickinson, Franklin Lakes, USA).

Rat cardiac function assessment by echocardiography

Six weeks after induction of myocardial infarction, rats were anaesthetized with 100 mg/kg ketamine and 20 mg/kg xylazine. The sonographic examination was conducted on a Vivid 7 system (General Electric Medical Systems, Waukesha, USA). Analyses were performed by an experienced observer blinded to the treatment groups to which the animals were allocated. M-mode tracings were recorded from a parasternal short-axis view and functional systolic and diastolic parameters were obtained. Fractional shortening was calculated as follows: FS (%) = ((LVEDD − LVESD)/LVEDD) × 100%, where LVEDD and LVESD mean left ventricular end-diastolic and end-systolic diameter, respectively.

Porcine closed chest reperfused infarction model and APOSEC^P

A closed chest reperfused AMI infarction model was applied in a large animal setting [15, 16]. The experiments in the porcine infarction model were carried out at the Institute of Diagnostics and Oncoradiology, University of Kaposvar, Hungary. Animal experiments were approved by the University of Kaposvar (vote: 246/002/SOM2006, MAB-28-2005). After overnight fasting, the pigs (female Large Whites weighing approximately 30 kg) were sedated with 12 mg/kg ketamine hydrochloride, 1.0 mg/kg xylazine and 0.04 mg/kg atropine. After the administration of 200 IU/kg of heparin, a 6F guiding catheter (Medtronic Inc., Minneapolis, USA) was introduced into the left coronary ostium, and selective angiography of the left coronary arteries was performed using Ultravist contrast medium (Bayer Healthcare, Berlin, Germany). A Maverick balloon catheter (diameter: 3.0 mm, length: 15 mm; Boston Scientific, Natick, USA) was inserted into the left anterior descending artery (LAD) after the origin of the second major diagonal branch. The LAD was then occluded by inflating the balloon slowly at 4–6 atm ($n = 8$ in the control group, $n = 7$ in the treatment high dose and $n = 7$ in the treatment low-dose group), controlling the occlusion with angiography. Forty minutes after the start of the LAD occlusion, the lyophilized supernatant obtained from 250×10^6 (low-dose group), 1×10^9 (high-dose group) irradiated apoptotic porcine PBMC or lyophilized serum-free cell culture medium (CellGro Medium, Cell Genix, Freiburg, Germany) was resuspended in 250 ml of 0.9% physiologic sodium chloride solution and administered intravenously over the next 25 min. After 90 min of occlusion, the balloon was deflated and reperfusion was established. Control coronary angiography was performed to prove the patency of the infarct-related artery and to exclude arterial injury. Furthermore, all animals received 75 mg clopidogrel and 100 mg acetylsalicylic acid. After 24 h or after 30 days, euthanasia was performed by the administration of saturated potassium chloride. For the analysis conducted after 24 h, in situ double-staining with

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1% Evans blue dye and a 4% solution of 2,3,5-triphenyltetrazolium chloride (TTC) was performed to delineate areas at risk for ischaemia and infarcted (necrotic) areas, respectively. In short, after explantation the LAD was occluded again at same position where the balloon was situated before and both coronary arteries were perfused with Evans blue solution to delineate the area at risk and non-risk region. The hearts were cut into 7 mm thick slices starting from the apex towards the level of the occlusion (6–7 layers per heart). The slices were incubated in 500 ml of TTC solution at 37°C in a shaking water bath for 20 min. Subsequently, all slices underwent an overnight bleach cycle at room temperature using 4.5% formaldehyde. All slices were photographed using a digital camera (Panasonic HDC-HS700, Osaka, Japan) mounted on a fixed stand. Planimetry was performed using Image J software (Rasband, W.S., Image J, US National Institutes of Health, Bethesda, USA). Serum levels of Troponin I were determined by ELISA (Uscn Life Science Inc., Wuhan, China). After 30 days, specimens of infarcted myocardium were obtained after euthanasia, fixed in formaldehyde and embedded in paraffin for histological staining (H&E, Movat's pentachrome).

Bari scores for all animals were calculated based on LAD and LCX pre-occlusion angiograms according to the method previously described [1, 32].

Quantification of cardiac parameters after reperfused AMI in pigs by magnetic resonance imaging (MRI)

Three and thirty days after the reperfused AMI procedure, cardiac MRI was performed using a 1.5-T clinical scanner (Avanto, Siemens, Erlangen, Germany) together with a phased array coil and a vector ECG system. Cine MR images were acquired using a retrospectively ECG-gated, steady-state free precession cine MRI technique in short-axis and long-axis views of the heart using 1.2 ms echo time (TE), 40 ms repetition time (TR), 50° flip angle, 300 mm field-of-view, 8 mm slice thickness, and 256×256 image matrix. Sixteen short-axis images were acquired using ECG-gated, saturation recovery true fast imaging with steady-state precession (FISP) sequences. Delayed enhancement images were obtained after injection of 0.05 mmol/kg of contrast medium using an inversion recovery prepared, gradient-echo sequence. Short-axis and long-axis images were obtained 10–15 min after gadolinium injection. The images were analyzed using Mass 6.1.6 software (Medis, Leiden, The Netherlands). After segmentation of the left ventricular (LV) endocardial and epicardial borders, end-diastolic and end-systolic volumes and global LV ejection fraction were automatically calculated. The left ventricular and infarcted myocardial mass was determined from the cine and delayed enhancement MR images, respectively. The infarct size was determined relative to LV mass. Data analyses and interpretations were performed by an experienced observer blinded to all study results.

Human cardiomyocyte culture and immunoblot analysis

Primary human ventricular cardiac myocytes were obtained from CellSystems (CellSystems Biotechnologie, St. Katharinen, Germany) and cultured in cardiac myocyte medium (CellSystems) at 37°C. To investigate the cytoprotective activity of APOSEC, 3×10^5 human cardiac myocytes were seeded in 6-well plates and cultivated in either basal medium without serum and growth factors or in basal medium supplemented with APOSEC (PBMC cell density, 0.25×10^6, 2.5×10^6 and 25×10^6) for 24 h. For Western Blot analysis, 3×10^5 human cardiac myocytes were incubated with APOSEC (PBMC cell density for APOSEC production, 2.5×10^6) or with lyophilized UltraCulture Medium for 5, 10, 30 and 60 min and for 24 h. Immunodetection was performed with anti-phospho-c-Jun (1 µg/ml, New England Biolabs, Beverly, MA, USA), anti-phospho-CREB (1 µg/ml, New England Biolabs, Beverly, MA, USA), anti-phospho-AKT (1 µg/ml, New England Biolabs, Beverly, MA, USA), anti-phospho-Erk1/2 (1 µg/ml, New England Biolabs, Beverly, MA, USA), anti-phospho-Hsp27 (Ser15) (1 µg/ml, New England Biolabs, Beverly, MA, USA), anti-phospho-Hsp27 (Ser85), anti-BAG1 (C-16) (1 µg/ml, Santa Cruz Biotechnology, Heidelberg, Germany), anti-Bcl-2 (2 µg/ml, Acris, Herford, Germany), followed by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antisera (both 1:10,000; Amersham BioSciences). In parallel, identical blots were performed with the equivalent non-phosporylated factors as controls. In addition, an apoptosis membrane-array (R&D Systems) was performed with lysates from cardiac myocytes treated with either medium or APOSEC (PBMC cell density 2.5×10^6) for 24 h according to the manufacturer's instructions.

To identify some of the mayor soluble factors that are responsible for exerting the cytoprotective effect of APOSEC or a subset thereof, we sought to inhibit the activity of selected prominent cytokines by adding neutralizing antibodies. VEGF, IL-8, ENA-78 and MMP9 were selected based on the higher expression levels over controls and their known cytoprotective properties. The activity of these selected factors was blocked using the recommended concentration of the neutralizing antibodies (2 µg/ml anti-VEGF: AF-293-NA, 2 µg/ml anti-IL-8: MAB208, R&D Systems; 2 µg/ml anti-ENA-78: AB-254-PB and 2 µg/ml anti-MMP9: MAB911; all antibodies obtained from R&D Systems, USA). Blocking capacity of neutralizing

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antibodies was verified (see Supplementary Fig. 5). Mouse and goat isotype antibodies were used as controls (Mouse IgG (Clone 11711), MAB002, Goat IgG, AB-108-C, R&D Systems). For these neutralization assays, 3×10^5 human cardiac myocytes were incubated with APOSEC and neutralizing antibodies, a combination thereof or with lyophilized medium for 60 min (for phospho-CREB and phospho-Hsp-27) or for 24 h (for Bcl-2 and BAG1). Western blots were performed as described above.

Statistical methods

Statistical analysis was performed using Graph Pad Prism software (La Jolla, USA). All data are given as mean ± standard error of the mean (SEM). The Wilcoxon–Mann–Whitney test or Student's *t* test were utilized to calculate significances between the groups. The Bonferroni–Holm correction was used to adjust significance levels for ELISA results. In boxplot figures, whiskers indicate minimums and maximums, the upper edge of the box indicates the 75th percentile and the lower one indicates the 25th percentile. p values < 0.05 were considered statistically significant.

Results

An overview of the study design is shown in Fig. 1 and in a supplementary video file.

Analysis of soluble factors produced by irradiated human PBMC (APOSEC^H)

To induce apoptosis, PBMC (purity > 98%) were γ-irradiated (60 Gray) and cultured for 24 h. Supernatants of irradiated and non-irradiated cells were collected and secreted proteins associated with tissue repair and neoangiogenesis were determined by membrane arrays and ELISA. As shown in Table 1, after irradiation of PBMC, higher amounts of IL-8, GRO-alpha, ENA-78, RANTES, sICAM-1, MIF, VEGF, IL-1ra and IL-16 were detected in a cell density-dependent manner as compared to the

Table 1 Analysis of soluble factors secreted by non-irradiated cells and irradiated apoptotic PBMC (APOSEC)

Soluble factors (ng/ml)	Viable PBMC			Apoptotic PBMC			Sig.
	1×10^6	2.5×10^6	25×10^6	1×10^6	2.5×10^6	25×10^6	
IL-8	1.74 ± 0.40	1.93 ± 0.09	10.49 ± 3.53	1.22 ± 0.29	2.30 ± 0.13	18.01 ± 2.87	ns ns$^{¥}$
GRO-alpha	0.17 ± 0.09	0.36 ± 0.09	2.06 ± 1.58	0.07 ± 0.02	0.48 ± 0.09	3.95 ± 0.93	ns ns ns
ENA-78	3.41 ± 1.34	29.93 ± 3.41	34.89 ± 16.33	3.93 ± 1.43	37.86 ± 12.73	108.86 ± 27.88	ns ns$^{¥}$
MCP-1	1.66 ± 0.65	0.47 ± 0.21	0.27 ± 0.00	0.76 ± 0.19	0.74 ± 0.17	0.27 ± 0.00	ns ns ns
RANTES	8.32 ± 0.18	18.62 ± 3.21	37.63 ± 2.72	4.01 ± 0.05	22.25 ± 3.64	51.58 ± 4.44	ns ns ns
HMGB1	0.63 ± 0.39	3.44 ± 2.11	33.57 ± 6.45	2.74 ± 0.27	6.46 ± 1.12	20.51 ± 3.62	ns ns†
MMP9	4.14 ± 0.91	14.59 ± 2.75	29.46 ± 8.29	0.99 ± 0.16	3.61 ± 0.59	19.35 ± 5.34	ns†,‡
sICAM-1	0.14 ± 0.04	1.43 ± 0.25	7.43 ± 0.85	0.42 ± 0.25	2.09 ± 0.42	9.40 ± 1.29	ns ns$^{¥}$
$VEGF_{165}$	0.13 ± 0.01	0.42 ± 0.04	0.82 ± 0.34	0.15 ± 0.02	0.64 ± 0.04	4.39 ± 1.22	ns ns$^{¥}$
MIF	4.84 ± 0.09	17.79 ± 0.95	13.24 ± 0.85	5.85 ± 0.22	20.15 ± 1.14	58.99 ± 1.17	ns ns$^{¥}$
PAI-1	1.25 ± 0.35	1.93 ± 0.29	49.60 ± 9.04	0.00 ± 0.00	5.06 ± 3.25	45.86 ± 1.43	ns ns ns
IL-16	0.0 ± 0.0	0.11 ± 0.02	0.84 ± 0.31	0.00 ± 0.00	1.25 ± 0.07	5.25 ± 0.52	ns$^{‡, ¥}$
IL-1ra	0.35 ± 0.09	0.52 ± 0.17	2.16 ± 0.96	0.13 ± 0.04	0.41 ± 0.17	6.43 ± 1.33	ns ns$^{¥}$
IL-10	0.01 ± 0.00	0.00 ± 0.0	0.05 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.06 ± 0.01	ns ns ns
IGF-I	0.00 ± 0.00	0.01 ± 0.0	0.03 ± 0.02	0.00 ± 0.00	0.01 ± 0.01	0.03 ± 0.03	ns ns ns
HGF	0.33 ± 0.08	0.16 ± 0.01	0.69 ± 0.19	0.11 ± 0.03	0.07 ± 0.02	0.79 ± 0.19	ns ns ns
FGF-2	0.56 ± 0.02	0.53 ± 0.00	0.59 ± 0.01	0.48 ± 0.01	0.53 ± 0.02	0.55 ± 0.02	ns ns ns
TGF-β	0.08 ± 0.01	0.10 ± 0.01	0.21 ± 0.07	0.06 ± 0.01	0.09 ± 0.02	0.39 ± 0.09	ns ns ns
SDF-1	0.17 ± 0.0	0.19 ± 0.0	0.22 ± 0.03	0.16 ± 0.01	0.15 ± 0.07	0.12 ± 0.04	ns ns ns
G-CSF	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	ns ns ns
GM-CSF	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.02	ns ns ns

Cells were incubated in three different cell concentrations for 24 h. Supernatants were analyzed for cytokines, chemokines and growth factors ($n = 5$)

† $p < 0.05$ 1×10^6 viable PBMC versus 1×10^6 apoptotic PBMC

‡ $p < 0.05$ 2.5×10^6 viable PBMC versus 2.5×10^6 apoptotic PBMC

¥ $p < 0.05$ 25×10^6 viable PBMC versus 25×10^6 apoptotic PBMC

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supernatant of non-irradiated cells. In contrast, little if any secretion was detected for MCP-1, IL-10, IGF-1, HGF, FGF-2, TGF-β, SDF-1, G-CSF and GM-CSF (Table 1), indicating that some of the factors previously associated with cardioprotection might not play a relevant role in this experimental setting [35]. An overview of secreted factors is shown in Supplementary Fig. 1.

Diverted early inflammatory immune response and long-term preservation of ventricular function in AMI rats treated with APOSEC^{R}

Since the degree of the inflammatory response after AMI is an important factor which correlates to infarct size and outcome, we investigated whether APOSEC^{R} IV (derived from rat cells) modifies the extent of necrosis and the quality of infiltrating cells in the ischaemic myocardium. H&E-staining revealed that rat hearts treated with APOSEC^{R} showed a significant reduction of infarction area within 72 h after ligation of the LAD as compared to animals receiving medium or the supernatant from viable cells (Fig. 2a–d).

Compared to APOSEC-treated rats (Fig. 2g), control AMI animals evidenced a more mixed cellular infiltrate in the wound areas similar to granulation tissue within 72 h after AMI (Fig. 2e, f). Immunohistochemical analysis revealed that the cellular infiltrate in the APOSEC^{R} AMI rats was composed of abundant CD68+ monocytes/macrophages (Fig. 2j), significantly more than in the control groups (Fig. 2h, i).

In addition, most of these medium-sized monocytoid cells in the APOSEC^{R} cellular infiltrate were highly positive for c-kit (CD117) and vascular endothelial growth factor receptor 2 (FLK-1) (data not shown) (Fig. 2n). Expression of both markers was more accentuated in APOSEC^{R}-injected rats as compared to control groups (see representative images, Fig. 2l–o, $n = 6$ per group). To further strengthen these histological data, we obtained hearts 72 h after treatment with either APOSEC^{R} or control medium. After homogenisation of myocardium, infiltrating cells were separated and the quantity of c-kit+ and CD68+ cells was determined by flow cytometry analysis. Both the cell populations were enriched in APOSEC^{R}-injected animals, as evidenced by a mean increase of 37% of CD68+ cells and 107% of c-kit+ cells compared to control animals injected with medium ($n = 4$ per group). Figure 2p–r shows that APOSEC^{R}-injected rats demonstrate a significantly greater extent of viable myocardium within the anterior free wall of the left ventricle. In contrast, collagen deposition and scar formation extended through almost the entire left ventricular wall in rats receiving control medium.

Six weeks after LAD ligation, the mean left ventricular ejection fractions (LVEF), shortening fractions (SF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were determined to be 43.04% ± 4.17 (LVEF), 19.00% ± 2.29 (SF), 10.96 mm ± 0.51 (LVEDD) and 9.00 mm ± 0.63 (LVESD) in animals having received culture medium alone and 39.38% ± 2.89 (LVEF), 16.88% ± 1.45 (SF), 10.17 mm ± 0.33 (LVEDD) and 8.63 mm ± 0.32 (LVESD) in rats injected with the supernatant of non-irradiated cells (p values between 0.23 and 0.79). Remarkably, APOSEC IV-injected AMI rats evidenced significantly improved functional parameters: 56.22% ± 3.05 (LVEF; $p = 0.018$ vs. medium and $p = 0.0006$ vs. viable cell supernatant), 26.33% ± 2.11 (SF; $p = 0.016$ vs. medium and $p = 0.0018$ vs. viable cell supernatant), 9.77 mm ± 0.23 (LVEDD; $p = 0.044$ vs. medium) and 7.33 mm ± 0.33 (LVESD; $p = 0.025$ vs. medium and $p = 0.013$ vs. viable cell supernatant). Mean levels of cardiac function of healthy rats without myocardial infarction were as follows: 60.40% ± 4.95 (LVEF), 29.20% ± 3.26 (SF), 9.00 mm ± 0.55 (LVEDD) and 6.40 mm ± 0.51 (LVESD). Figure 2t, u shows echocardiographic data for each group.

APOSEC^{P} IV protects the myocardium from ischaemic injury in a pig model of closed chest reperfused AMI

BARI scores did not differ significantly between the groups (see Supplementary Fig. 4), indicating a comparable distribution of the area a risk after balloon occlusion.

Hearts explanted from animals infused with APOSEC^{P} evidenced less myocardial necrosis as shown by tetrazolium chloride staining after 24 h compared to controls (Fig. 3a–c). Additionally, troponin I release was less than in animals treated with resuspended lyophilized medium as controls (Fig. 3d).

Intravenous high-dose APOSEC^{P} caused a significant short and long-term reduction of myocardial damage as determined by cardiac MRI (Table 2). These results were corroborated by a significant hemodynamic improvement as compared to controls. In the high-dose group, APOSEC infusion reduced the extent of myocardial infarction by approximately 50%, from 18.17 to 8.66% after 3 days ($p = 0.0019$) and from 12.60 to 6.92% after 30 days ($p = 0.015$), respectively. Conversely, LVEF, cardiac output and cardiac index were improved significantly compared to controls or low-dose treatment. Figure 3 (see also Supplementary Fig. 2 and supplementary video file) illustrates representative transverse sections of a high-dose APOSEC^{P}-injected animal (Fig. 3h) and a control (Fig. 3e). H&E-stained (Fig. 3f, i) and Movat's pentachrome-stained (Fig. 3g, j) myocardial specimens obtained

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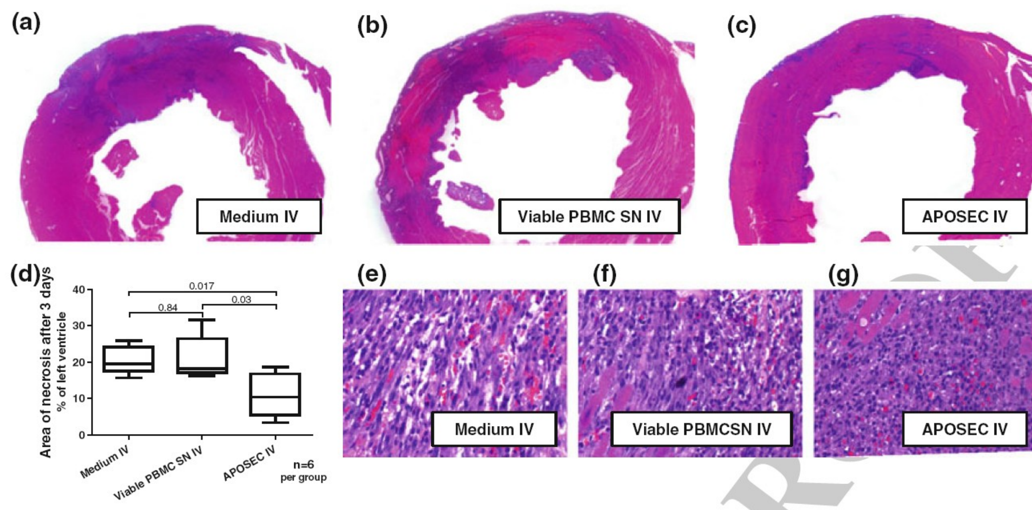

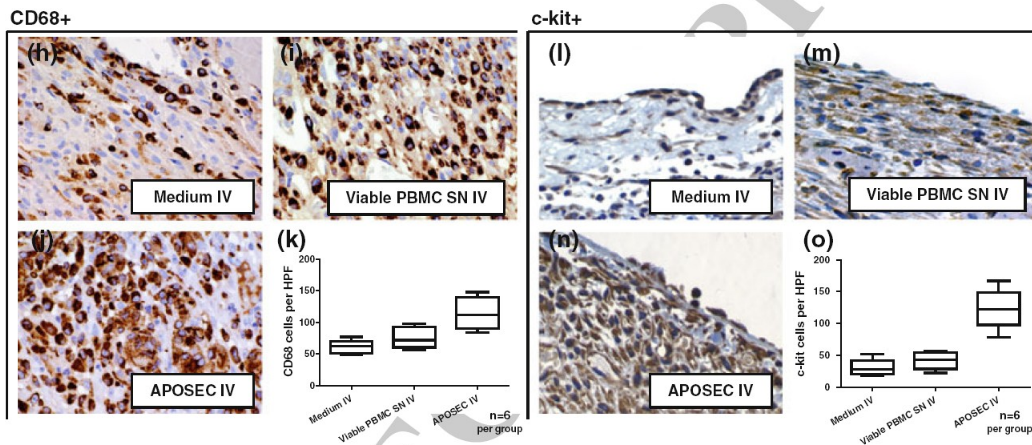

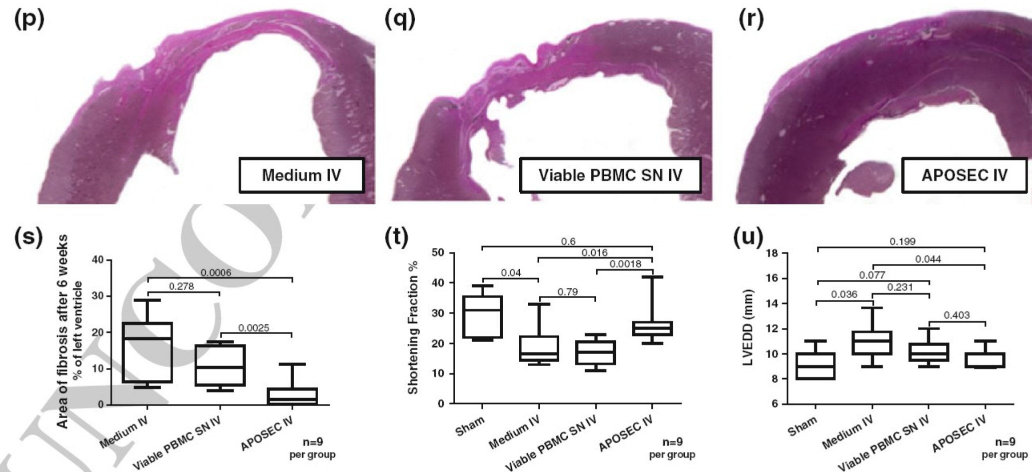

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◀ **Fig. 2** Results of in vivo rat experiments. **a–d** Hearts of APOSEC^R-injected animals 3 days after LAD ligation evidenced less myocardial necrosis compared to controls. **e–g** H&E-stained rat myocardium 3 days after LAD ligation. The cellular infiltrate appears to be more consolidated in APOSEC^R-injected animals. **h–j** Myocardial tissue stained for CD68. **k** Quantification of positively stained cells per high power field (HPF). **l–n** Specimen obtained 3 days after AMI stained for c-kit, **o** shows results of cell quantification per HPF. **p–r** shows size of myocardial infarction 6 weeks after LAD ligation. **s** Planimetric analyses indicate a significant reduction of scar area compared to controls. **t, u** show results obtained by echocardiography 6 weeks after AMI. Functional parameters (SF, LVEDD) were improved in comparison to medium or viable cell supernatant injected animals

30 days after AMI showed less collagen deposition and more viable myocardium in treated pigs as compared to controls.

APOSEC^H induces cytoprotective and anti-apoptotic mechanisms in cardiac myocytes in vitro

Strategies to abrogate the death cascade at the level of intracellular signalling kinases and inhibitors of apoptosis were previously defined in the ischaemia–reperfusion (I/R) and ischaemic postconditioning literature [18, 19, 37, 40], suggesting that targeting anti-apoptotic mechanisms of cellular protection at the time of ischaemia may offer a potential approach for reducing reperfusion-induced myocyte cell death. To investigate whether APOSEC^H is able to activate pro-survival pathways in cultured cardiac myocytes, we performed Western blot analysis. APOSEC^H induced phosphorylation of AKT, p42/p44 extra-cellular signal-regulated kinases (Erk1/2), p38 MAPK, Hsp27, c-Jun and CREB in human primary cultured cardiomyocytes within 60 min (Fig. 4a) and in dose-dependent fashion (Fig. 4b). Moreover, the expression of several anti-apoptotic proteins such as Bcl-2 and BAG1 was up-regulated in APOSEC^H-treated cardiac myocytes after 24 h (Fig. 4c and Supplementary Fig. 3).

Furthermore, APOSEC^H circumvented cardiomyocyte cell death in a starvation assay even at low doses such as APOSEC obtained from 0.25×10^6 irradiated apoptotic PBMC (Fig. 4d). Neutralization of selected factors, e.g. VEGF, IL-8, ENA-78, MMP9 alone or in combination did not result in an obvious decrease with regard to CREB and Hsp27 phosphorylation nor Bcl-2 or BAG1 up-regulation (Fig. 4e).

Discussion

Current medical and invasive therapies after AMI do not address the central problem associated with the massive loss of cardiomyocytes, vascular cells, and interstitial cells, and in consequence these patients continue to experience frequent hospitalizations and premature death. Additionally, although reperfusion after coronary occlusion is undoubtedly prerequisite for tissue salvage, additional myocardial injury can occur. The sudden reinitiation of blood flow leads to an inflammatory response, which results in further endothelial and myocardial injury. No drug has successfully passed clinical trials for cardiac injury despite the 30 years that have passed since the phenomenon of "myocardial injury through reflow" was first acknowledged [11]. This myocardial reperfusion injury is triggered by several distinct soluble and/or cellular components of the inflammatory network [12, 23]. In our previous work we abrogated myocardial remodelling by infusing cultured irradiated apoptotic PBMC [2, 26] and we could demonstrate that an alternative approach, namely, intravenous infusion of soluble factors derived from irradiated PBMC (APOSEC) reduced progressive collagen deposition and scar formation and improved ventricular function in a rodent model of acute myocardial ischaemia and in a large animal closed chest reperfused infarction model.

Recently, there has been a change of thinking in the field of regenerative medicine since publications showed that soluble factors from bone marrow cells, termed BMC-SN, initiated proliferation and migration of coronary artery endothelial cells, endothelial tube formation and cell sprouting in a mouse aortic ring assay [25]. Other investigators demonstrated that endothelial progenitor cell-conditioned medium (EPC-CM) enhanced the formation of capillary outgrowth in a rat aortic ring model and enhanced survival of serum-starved human umbilical cord endothelial cells (HUVEC) [7]. In vivo relevance was demonstrated in that EPC-CM caused increased blood flow, muscle mitochondrial activity and functional improvement in an ischaemic hindlimb model. More recently Kalka et al. have shown that EPC-CM causes resistance of HUVEC to oxidative stress by inducing anti-oxidative enzymes, and resistance to apoptosis induction in vitro by increasing the Bcl-2/BAX ratio. From these results, the group deduced that EPC-CM contains massive amounts of cytoprotective proteins causing anti-apoptotic and growth factor-mediated effects. Most importantly and similar to our results, neutralization of selective or combined cytokines such as VEGF, HGF, IL-8 and MMP9 did not significantly reverse the cytoprotective effect of EPC-CM. The group concluded that EPC secrete factors which cause broad synergistic effects and exert strong cytoprotective properties on differentiated endothelium through modulation of intracellular antioxidant and defensive mechanisms and pro-survival genes [45].

The mechanism described in the above paragraph for BMC-SN and EPC-CM is similar to that which we describe in this paper concerning secreted factors from PBMC,

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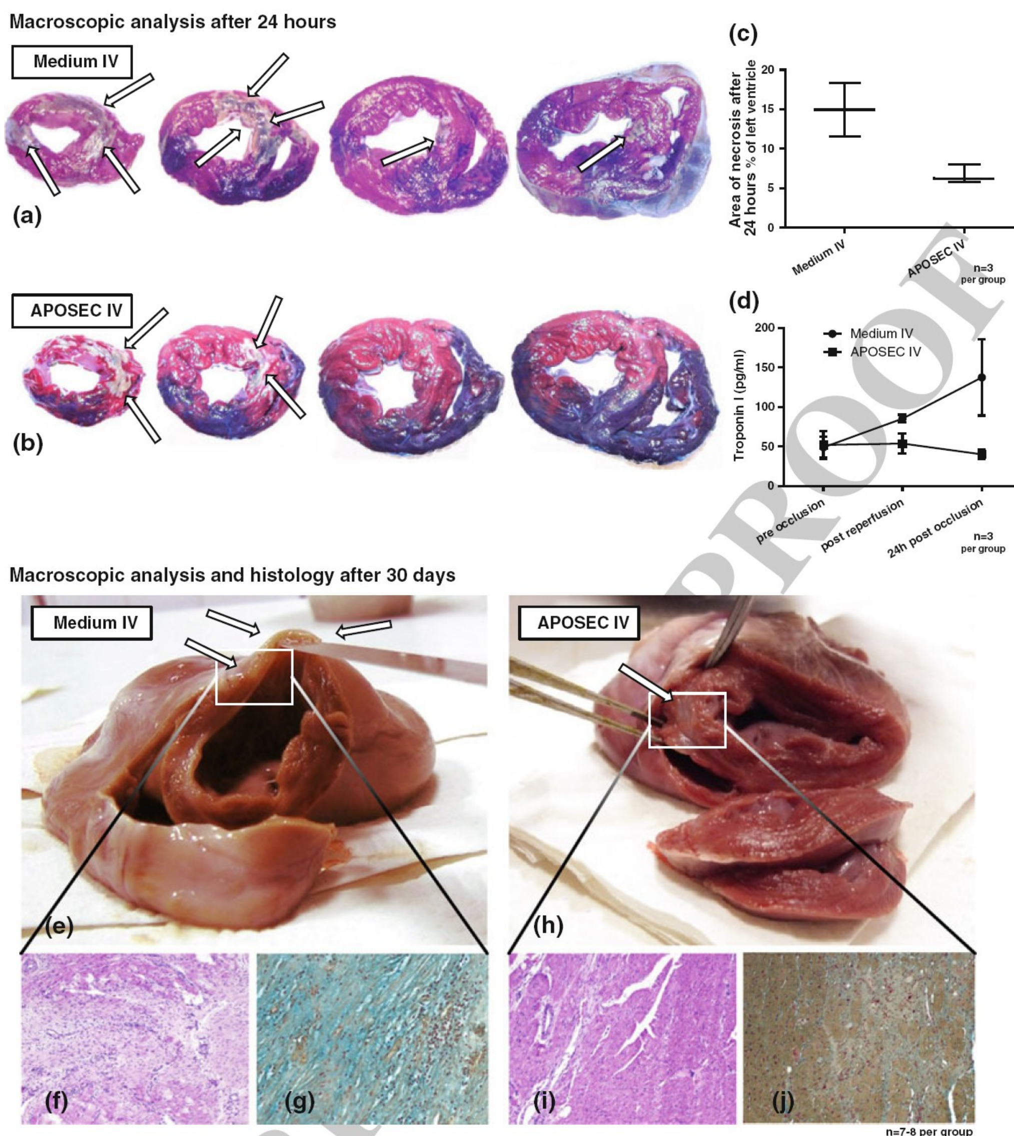

Fig. 3 Results of the porcine closed chest reperfused AMI model. **a, b** Show representative images of porcine hearts explanted 24 h after myocardial infarction stained with tetrazolium chloride and Evans blue solution. The area at risk is stained *red* and necrotic areas remained unstained (*white/grey*, *arrows*). **c** shows the extent of necrosis for controls and APOSEC^{P}-treated animals. **d** shows ELISA data indicating less troponin I release in treated animals. **e, h** Show representative images of hearts explanted 30 days after AMI. Hearts of APOSEC^{P}-injected pigs evidenced only marginal formation of scar tissue in the myocardium compared to control animals where large infarcts were common. H&E-stained **(f, i)** and Movat's pentachrome-stained **(g, j)** specimens of the infarcted myocardium shown in the lower part of Fig. 3 indicate fewer signs of collagen deposition and more viable cardiomyocytes within the scar tissue **(i, j)** compared to control animals **(f, g)**

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which documents the first evidence that culture medium from apoptotic PBMC (APOSEC) induces cytoprotective mechanisms in cultured cardiomyocytes in vitro. However, unlike the BMC-SN process, which requires bone marrow stem cell acquisition and preparation, PBMC can be obtained from venous whole blood, which can be much more easily obtained via simple venal puncture.

Based on the fact that infarct dimensions were reduced compared to controls in both in vivo models even within the first 3 days after AMI, we believe that the main mechanism of action induced by APOSEC treatment is conferring cardioprotective effects to cardiomyocytes at risk. In support of this hypothesis, we tested whether APOSEC could induce the activation of intracellular

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Table 2 Cardiac MRI evaluation 3 and 30 days after AMI

	Parameters	Medium control ($n = 8$)	250×10^6 apoptotic PBMC (low-dose APOSEC, $n = 7$)	1×10^9 apoptotic PBMC (high-dose APOSEC, $n = 7$)
After 3 days	Weight (kg)	31.86 ± 9.1	30.86 ± 1.6 ns	33.33 ± 1.3 ns
	Age (days)	90 ± 0	90 ± 0 ns	90 ± 0 ns
	LVEDV (ml)	67.59 ± 2.7	64.19 ± 5.4 ns	63.73 ± 1.6 ns
	LVESV(ml)	38.42 ± 2.5	35.96 ± 3.0 ns	33.93 ± 2.1 ns
	LVSV (ml)	29.17 ± 1.3	28.23 ± 3.2 ns	29.77 ± 1.8 ns
	LVEF (%)	43.38 ± 1.9	43.63 ± 2.8 ns	46.65 ± 2.9 ns
	HR/min	111 ± 6	109 ± 5 ns	111 ± 13 ns
	CO (l/min)	3.24 ± 0.1	3.03 ± 0.3 ns	3.28 ± 0.3 ns
	CI (l/min/m^2)	3.64 ± 0.1	3.59 ± 0.4 ns	3.82 ± 0.4 ns
	Infarct %	18.17 ± 1.7	14.01 ± 1.9 ns	8.66 ± 1.5**
After 30 days	Weight (kg)	39.43 ± 0.5	37.00 ± 1.9 ns	48.83 ± 0.7***
	Age (days)	120 ± 0	120 ± 0 ns	120 ± 0 ns
	LVEDV (ml)	54.74 ± 4.1	53.43 ± 3.2 ns	65.99 ± 3.5 ns
	LVESV(ml)	32.93 ± 4.0	31.89 ± 2.9 ns	28.71 ± 3.5 ns
	LVSV (ml)	21.84 ± 1.8	21.54 ± 1.9 ns	37.29 ± 1.7***
	LVEF (%)	40.54 ± 3.6	40.64 ± 3.2 ns	57.05 ± 3.3**
	HR/min	114 ± 7	108 ± 7 ns	107 ± 5 ns
	CO (l/min)	2.44 ± 0.1	2.28 ± 0.1 ns	3.98 ± 0.2***
	CI (l/min/m^2)	2.46 ± 0.1	2.40 ± 0.1 ns	3.51 ± 0.2***
	Infarct %	12.60 ± 1.3	11.50 ± 1.5 ns	6.92 ± 1.4*

Three and 30 days after ischaemia/reperfusion injury, MRI was conducted and parameters of cardiac function were obtained from pigs treated with low and high-dose APOSEC and from control animals

LVEDD left ventricular end-diastolic diameter, *LVESD* left ventricular end-systolic diameter, *LVSV* left ventricular stroke volume, *LVEF* left ventricular ejection fraction, *HR* heart rate, *CI* cardiac index, CO cardiac output, *ns* no significance versus control

* $p < 0.05$ versus control

** $p < 0.01$ versus control

*** $p < 0.001$ versus control

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signalling pathways known to be associated with cardiac conditioning or cytoprotection [19, 21].

The co-incubation of human cardiac myocytes with APOSEC led to a rapid phosphorylation of several important survival factors such as AKT, Erk1/2, p38 MAPK (all part of the ischaemia reperfusion injury salvage kinase pathway, RISK), c-JUN, cAMP-response element binding protein (CREB) and Hsp27 [5, 8, 19, 40]. Moreover, Bcl-2 and BAG1, known anti-apoptotic effectors, were massively enhanced within 24 h.

To identify key factors mediating the cytoprotective effect of APOSEC, we tried to inhibit the activity of some factors found in APOSEC at higher concentrations (IL-8, VEGF, ENA-78, MMP9) by utilizing neutralizing antibodies. As shown in Fig. 4e, the neutralization of these selected factors alone or in combination thereof was not sufficient in attenuating the phosphorylation of CREB/Hsp27 and Bcl-2/BAG1 expression in cultured human cardiomyocytes exposed to APOSEC. These results suggest that other or even yet unidentified factors or combinations thereof or other pleiotropic effects might be responsible for the beneficial effects seen by APOSEC treatment.

In addition, as seen in Table 1, we have identified a great variety of proteins in APOSEC defined to be relevant for homing of progenitor cells to sites of ischaemia. We believe that enhanced presence of c-kit+ and CD68+ cells in the infarcted area is a direct consequence of early single-dose intravenous administration of APOSEC after the onset of myocardial ischaemia. Based on these findings, we suggest that APOSEC IV is causative for myocardial resistance to hypoxia-induced cell damage in AMI and circumvented inflammation.

According to the guidelines issued by the European Society of Cardiology and the American Heart Association (AHA), prompt restoration of coronary perfusion within the shortest time is currently considered to be the standard of cardiac care for patients suffering from ST-elevation myocardial infarction (STEMI) [3, 41]. We therefore have chosen a similar approach by utilizing a closed chest

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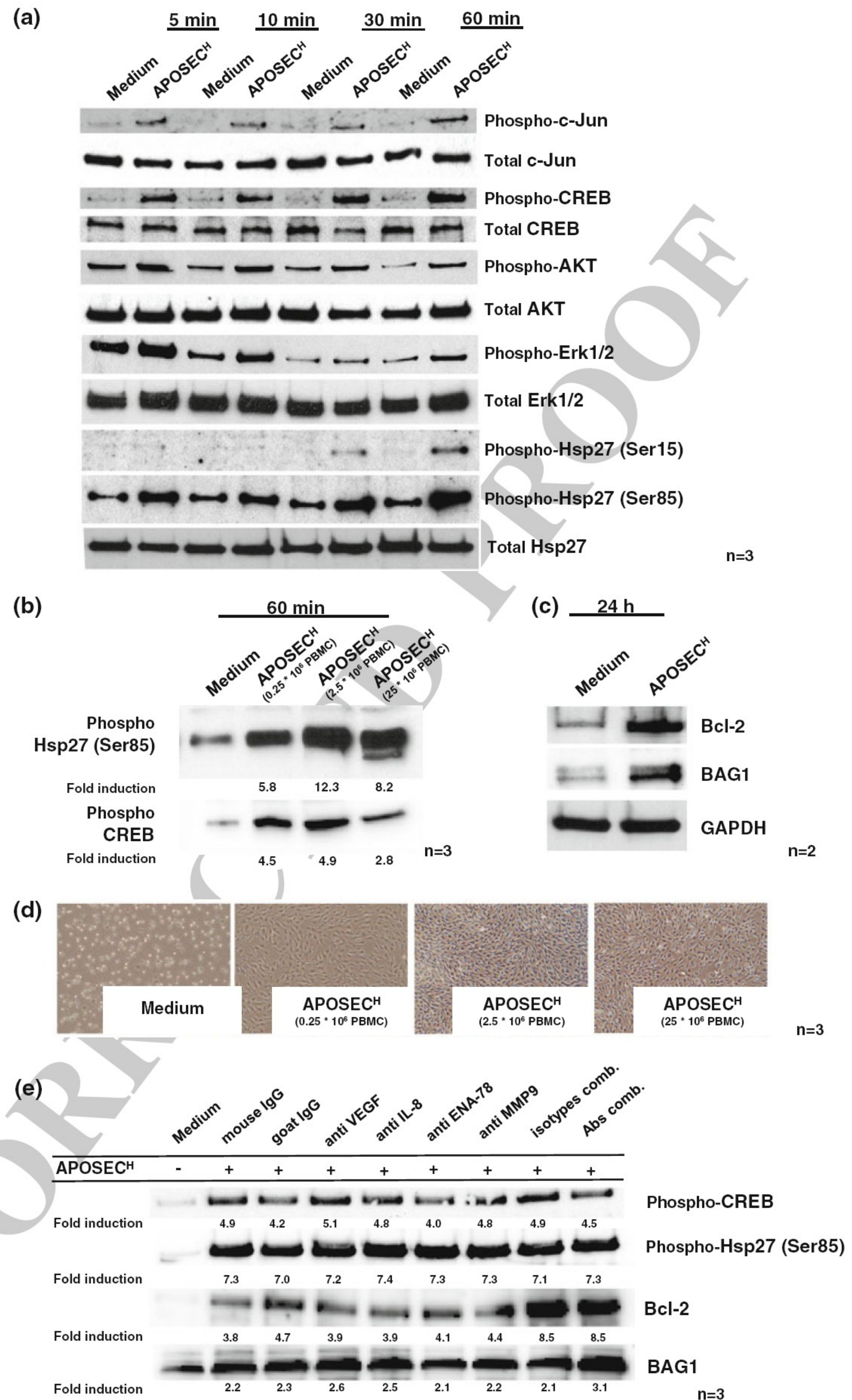

Fig. 4 APOSEC^{H} induces cytoprotective protein expression and activation. **a** Cell extracts were prepared after stimulation with APOSEC^{H} or control medium after the indicated time intervals. Western blot analysis showed activation of c-Jun, CREB, AKT, ERK1/2 and Hsp27. **b** shows the dose-dependent induction of Hsp27 and CREB in cultured cardiomyocytes. **c** 24 h after treatment expression of the anti-apoptotic proteins Bcl-2 and BAG1 was analyzed by Western blotting. Proteins were normalized to the house-keeping gene GAPDH. One representative experiment of two is shown. **d** shows a dose-dependent cytoprotective effect of APOSEC in a starvation induced cell death model in cardiac myocytes. **e** shows that neutralization of selected factors (VEGF, IL-8, ENA-78 and MMP9) does not attenuate the induction of CREB and Bcl-2 in cardiomyocytes by APOSEC co-incubation

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reperfused AMI large animal model mimicking the human AMI scenario with "primary PCI for coronary occlusion". In addition to this standardized treatment of AMI in humans, we applied high and low-dose APOSEC 40 min after starting of the LAD balloon occlusion. These time intervals were selected to accord with the clinical scenario of earliest possible intravenous therapy with approximately 30-40 min delay from symptom to first needle time. Our obtained short and long-term MRI results evidenced that one high-dose APOSEC infusion led to a highly significant improvement of cardiac function and attenuation of myocardial infarction, indicating a dose-dependent effect.

Thus, APOSEC would appear to represent a "biological" which prevents experimental myocardial infarction by causing peri-infarct conditioning and stimulation of regenerative effects in the hypoxic myocardium.

Conclusion and outlook

Beneficiary blood products such as intravenous immunoglobulin (IVIG), coagulation factors or plasma protein concentrates have confirmed their clinical value in recent decades. Unlike these derivatives, APOSEC is a product made of soluble factors secreted by irradiated PBMC. This lyophilized "biological" combines the following clinically favourable features: (a) easily obtainable raw material (PBMC) for APOSEC production from the blood of healthy donors obviating the need to process cell of diseased patients avoiding disadvantageous effects such as stem cell dysfunction or a reduced secretory capacity when using cells of diseased patients [30]; (b) minimal or no antigenicity owing to protein-only content; and (c) "off the shelf" utilization in the clinical setting of AMI diagnosis requiring only single-dose administration. We conclude from our in vitro and in vivo data that APOSEC IV exhibits the features of a highly effective peri-infarct conditioning drug compound. Since the effectiveness of APOSEC has been demonstrated in our pre-clinical studies, the use of APOSEC with human experimental subjects is warranted. With regard to the underlying mechanisms, further studies are necessary to fully understand the mode of action of APOSEC, and we strongly believe that our present study can serve as a good basis for such a study in the future.

Limitations

In all three experimental settings (rat AMI model, porcine reperfused AMI model, in vitro experiments using human cells), APOSEC was tested in a syngeneic fashion only to obviate inter-species influences. However, the composition of the supernatants and its effects on human cardiac myocytes in vitro were exclusively analyzed for APOSEC derived from human cells, any differences in the secretory capacity of rat or porcine PBMC cannot entirely be ruled out.

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Conflict of interest The study was funded by the Christian Doppler Research Association, APOSCIENCE AG and the Medical University Vienna. The authors declare competing financial interests. The medical university has claimed financial interest (patent number EP2201954, WO2010070105-A1, filed 18 Dec 2008). H.J.A. is a shareholder of APOSCIENCE AG, which owns the rights to commercialize APOSEC for therapeutic use.

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