Abstract

Clusterin (Apolipoprotein J), a plasma protein with cytoprotective and complement-inhibiting activities, localizes in the infarcted heart during myocardial infarction (MI). Recently, we have shown a protective effect of exogenous clusterin in vitro on ischaemically challenged cardiomyocytes independent of complement. We therefore hypothesized that intravenous clusterin administration would reduce myocardial infarction damage. Wistar rats undergoing experimental MI, induced by 40 min ligation of a coronary vessel, were treated with either clusterin (n=15) or vehicle (n=13) intravenously, for 3 days post-MI. After 4 weeks, hearts were analysed. The putative role of megalin, a clusterin receptor, was also studied. Administration of human clusterin significantly reduced both infarct size (with 75 ± 5%) and death of animals (23% vehicle group vs. 0% clusterin group). Importantly, histochemical analysis showed no signs of impaired wound healing in the clusterin group. In addition, significantly increased numbers of macrophages were found in the clusterin group. We also found that the clusterin receptor megalin was present on cardiomyocytes in vitro which, however, was not influenced by ischaemia. Human clusterin co-localized with this receptor in vitro, but not in the human heart. In addition, using a megalin inhibitor, we found that clusterin did not exert its protective effect on cardiomyocytes through megalin. Our results thus show that clusterin has a protective effect on cardiomyocytes after acute myocardial infarction in vivo, independent of its receptor megalin. This indicates that clusterin, or a clusterin derivate, is a potential therapeutic agent in the treatment of MI.
Chapter 3

Introduction

Heart failure resulting from acute myocardial infarction (AMI) plays a major role in morbidity and mortality in the Western World.[1,2] Current AMI treatment focuses on reperfusion of the ischaemic myocardium. This reperfusion, however, results in the induction of acute inflammation in the heart, which has been shown to inflict additional damage to the myocardium.[3,4] Ischaemia, results not only in areas of necrotic cardiomyocytes, but also in areas of reversibly damaged cardiomyocytes that surround these necrotic areas.[3] Inflammation also targets these cardiomyocytes, which results in increased infarct size.[3] One of the inflammatory mediators that deposits in the heart after AMI is clusterin. Clusterin, a secreted protein, is increased in several cardiovascular diseases including AMI.[8] After AMI, clusterin was found on cardiomyocytes in the necrotic areas, where it co-localized with,[5,9] and inhibited several factors of the complement system.[10–12] In addition, we and others have previously shown clusterin on complement negative cardiomyocytes in the border zones in the infarcted heart.[5,13] We also found that clusterin protected cardiomyocytes from ischaemia-induced cell death in vitro, in the absence of complement.[5] This suggests an additional cardioprotective role for clusterin independent of the complement system.[5] These cytoprotective effects of clusterin therefore may serve to protect cardiomyocytes during AMI. Hence, clusterin administration after AMI may be a new therapeutic strategy to limit myocardial damage. In this study, we therefore analysed the effect of intravenous administration of human clusterin during the acute inflammatory episode on an AMI model in the rat. We also investigated the role of the clusterin receptor megalin herein, as it has been shown, in other cells than cardiomyocytes, that clusterin can exert its protective effect through this receptor.[14,15]

Materials and Methods

Clusterin purification

Clusterin was purified from human serum by affinity chromatography, as described previously.[16] SDS-PAGE of the purified clusterin showed a single protein band at 70 kD (Figure 1A). Moreover, Western blot analysis confirmed that this protein indeed was clusterin (Figure 1B). Finally, we measured the circulating levels of human clusterin in the rat after injection, using a clusterin-specific ELISA according to the manufacturer’s protocol (BioVendor, Modrice, Czech Republic).

Rat acute myocardial infarction

This study was approved by the VU animal ethics and welfare committee, and conforms to the Guide for care and use of laboratory animals published by the US National Institutes of Health. Myocardial infarction was induced in female Wistar rats (Harlan Laboratories, Horst, The Netherlands) (240–280 g), by 40 min ligation of the left anterior descending coronary artery as described previously.[17] First, a pilot study was performed in which rats received a single bolus
Clusterin reduces infarct size after AMI

Figure 1: Clusterin purification. A) SDS-PAGE protein staining of clusterin extract, showing a single band, indicating purity of clusterin protein (in duplo). B) Western blot analysis of clusterin extract, indicating that the extracted protein indeed is clusterin (in triple, left to right in decreasing concentrations).

of clusterin (control and clusterin n=7). Subsequently, an experiment was performed in which animals were treated with an intravenous (tail vein) bolus of clusterin for three consecutive days, starting within 10 min after reperfusion (control: NaCl 0.9% w/v, n=13, clusterin: 300 µg per rat per day, n=15, sham, n=4). The clusterin dose was based on in vitro experiments, in which a dose of 20 µg/mL was found to be most optimal. Translating this to the rat, a rat of 250 g has approximately 15 mL of blood, resulting in 15 mL x 20 µg/mL = 300 µg per rat. Rats were killed 28 days after AMI, when remodelling of the infarcted heart is completed theoretically.

Histological staining

Phosphotungstic acid-haematoxylin staining was performed as described previously.[17] Infarct size was quantified in the lower three slides (Figure 2A) using Q-PRODIT analysis (Leica microsystems, Cambridge, UK). It was found that sham operation resulted in a fibrosis/granulation tissue area lower than 0.01% of the total left ventricle area. Therefore, rats in which AMI was induced, albeit with an infarct size <0.01%, were excluded from the analysis (two control and two clusterin rats).

Immunohistochemistry

To investigate the effect of elevated clusterin levels on the infiltration of inflammatory cells and complement deposition, we performed immunohistochemistry on paraffin sections of the rat heart using antibodies for: myeloperoxidase (MPO) specific for neutrophilic granulocytes (1:500; DakoCytomation, Eindhoven, The Netherlands), CD68 (macrolialin) specific for macrophages
(1:100; MCA341R Serotec, UK), C3d (1:200; DakoCytomation) and C-reactive protein (CRP, 1:50; DakoCytomation). To investigate the presence of clusterin and megalin in the infarcted human heart, we performed immunohistochemistry on frozen sections with AMI infarction (1–4 days old), using a mouse anti-human clusterin antibody (1:750, mAb G7, gift of Dr. Murphy, Australia), and a rabbit anti-megalin antibody (1:10, h-245, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For this purpose, myocardial tissue from autopsied patients died due to AMI was obtained from the Department of Pathology, with approval of the ethics committee of the VU Medical Center, Amsterdam, and complied with the principles of the Declaration of Helsinki. The use of leftover material after the pathological examination is completed, as such is part of the standard patient contract in our hospital. Heart tissue was sampled as soon as possible, within 24h of death. Antigen retrieval was performed with 0.1% (w/v) pepsin (30 min, 37°C) for CD68, or by heat activation in citrate buffer (0.01 M, pH 6.0) for MPO, CRP and C3d. Slides were incubated with primary antibodies for 60 min, and then with anti-rabbit/anti-mouse EnVision-HRP (DakoCytomation) for 30 min (MPO, CD68, megalin, clusterin) or with swine anti-rabbit antibody (DakoCytomation, 1:300) for 30 min, followed by 60 min incubation with streptavidin-biotin-horseradish peroxidase complex (1:200; DakoCytomation, CRP, C3d). Control sections were incubated with PBS instead of the primary antibody, and staining showed negative results. Numbers of extravascular inflammatory cells were quantified in the infarcted areas and the residual entire left ventricle.

**Cell culture**

H9c2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (all Gibco, Invitrogen, Carlsbad, CA, USA), in a humidified atmosphere of 5% CO2 at 37°C.

**Immunofluorescence**

Ischaemia/reperfusion was mimicked by metabolic inhibition. For this purpose, cells were incubated for 2h in a metabolic inhibition buffer, containing 0.9 mM CaCl2, H2O, 20 mM 2-deoxy-D-glucose (Sigma, St. Louis, MO, USA), 106 mM NaCl, 5 mM NaCN, 3.8 mM NaHCO3, 4.4 mM KCl and 1 mM MgCl2, H2O in H2O, pH 6.6,[5] followed by 2h incubation in culture medium. Treated cells were before ischaemia incubated with purified human clusterin (20 μg/mL) for 30 min. H9c2 cells were fixed in 4% paraformaldehyde, permeabilized with acetone/methanol (70/30%, v/v) and incubated with the primary antibody for 60 min (megalin [18]; 1:50, anti-rat clusterin: 1:25, Upstate Biotechnology, Waltham, MA, USA, and anti-human clusterin; 1:25, mAb G7, gift of Dr. Murphy, Australia). Secondary antibodies used were goat anti-rabbit (Cy-5), rabbit anti-mouse (FITC) (both 1:40, Molecular Probes, Eugene, OR, USA) and WGA (FITC) for membrane staining (wheat germ agglutinin, 1:40, Molecular Probes). Nuclei were visualized with DAPI (H1200, Vectashield; Vector Laboratories, Burlingame, CA, USA). Three-dimensional images were acquired using a ZEISS Axi-overt 200M MarianasTM inverted microscope (I.I.I) equipped with a motorized
stage (stepper-motor z-axis increments: 0.2 micron). Images were taken using a 40x oil immersion lens (Carl-Zeiss). A cooled CCD camera (C1280 1024 pixels; Cooke Sensicam, Cooke, Tonawanda, NY, USA), recorded images with true 16-bit capability. The camera is linear over its full dynamic range (up to intensities of over 4000), while dark/background currents (estimated by the intensity outside the cells) are typically <100. The microscope, camera, data viewing/processing were controlled/conducted by SlideBookTM. Images shown are optical sections from 3D image stacks.

**Viability assay: FACS analysis**

Ischaemia was mimicked by metabolic inhibition for 2h. Purified clusterin (20 µg/mL) and/or receptor-associated protein (RAP, an inhibitor that prohibits binding of proteins to megalin, 0.5 µM; Progen, Heidelberg, Germany) were added 30 min before ischaemia and during ischaemia. Cell viability was analysed using flow cytometry. For this purpose, cells (attached as well as detached cells) were collected and stained with annexin V-FITC (1:40, Bender Med Systems, Vienna, Austria) for 30 min at 37°C in serum-free medium. Annexin V was used to assess flip-flop of the cell membrane. Propidium iodide was added immediately prior to measurement (PI; 1:40, Bender Med Systems) and was used to assess membrane permeability, and thus cell death. Cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**Western blot analysis**

Ischaemia/reperfusion was mimicked by metabolic inhibition. For this purpose, cells were incubated for 2h in a metabolic inhibition buffer followed by 2h incubation in the medium. Western blot analysis was performed as described previously.[17] Primary antibodies used were rabbit anti-rat megalin (1:250 [18]), mouse anti-rat clusterin (1:250, Upstate Biotechnology), mouse anti-human clusterin (1:500, mAb G7) and mouse anti-alpha-tubulin (1:1000, ABCAM). Secondary antibodies used were rabbit anti-mouse or swine anti-rabbit antibody HRP conjugated (DakoCytomation, 1:1000).

**Statistical analysis**

GRAPHPAD Prism (GraphPad Software, La Jolla, CA, USA) 4 software was used for statistical analysis. Data are expressed as mean ± standard error. A t-test or ANOVA was used when parameters were normally distributed, and a Mann–Whitney or Kruskall–Wallis test was used for parameters that were not distributed normally. A Pearson chi-square test was used for analysis of discrete values. A P-value <0.05 was considered significant.
Results

Clusterin administration reduces infarct size and mortality in a rat model of AMI

In sham-operated rats, epicardial superficial wounds were induced (Figure 2B). In contrast, in rats with AMI, additional intramyocardial areas of fibrosis and granulation tissue were found indicative for myocardial infarction (Figure 2C). At 28 days post-AMI, no depositions of complement C3D and CRP were detected in the heart (not shown), underlining that the acute inflammatory response was completed.[19,20]

In a pilot study, we investigated the effect of a single bolus of clusterin directly after the start of reperfusion. This pilot study did result in infarct reduction of 15%; however, this was not significantly different from the vehicle-treated group. In addition, no circulating levels of human clusterin could be detected in the blood from 24 h after injection (not shown). For this reason, we prolonged the clusterin administration to 3 days post-AMI, covering the episode of acute inflammation. In this part of the study, all rats treated with clusterin (n=15) survived the whole experiment. In contrast, in control rats, a mortality rate of 23% (3/13 rats died, two on day 1, one on day 2 after AMI) was found during the acute inflammation period (P<0.05). In rats treated with clusterin, mean infarct size was reduced by 75 ± 5% compared with vehicle-treated rats (5.6% to 1.4%, P<0.05, Figure 2D). We did not find significant differences in wall thickness between groups receiving clusterin, vehicle or sham-operated rats. Moreover, histological analysis (microscopically) of the scar tissue did not show differences in scar tissue formation between clusterin and vehicle-treated groups (not shown).

Heart function was analysed using echocardiography just before the rats were killed (ProSound SSD-4000, Aloka, Japan). No differences were found in anterior wall thickness and fractional shortening between vehicle and clusterin-treated rats (Table 1). Cardiac output was increased in the clusterin group to 45 mL/min (control level of 46 mL/min [21]); however, the increase was not significant. Taken together, elevating the blood levels of clusterin through intravenous injection of purified human clusterin after AMI in rats significantly reduced infarction size and improved survival in vivo.

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Figure 2: Clusterin reduces infarct size in the rat acute myocardial infarction (AMI). A) For histochemical analysis, microscopic slides were cut from the upper surface of each slide. The lower three slides (A–C) were used for infarct area quantification. Slides cut from above the ligature were not analysed, as in none of the animals, in the upper slide, signs of infarction were detected. PTAH staining of the anterior wall of sham-operated (B) and vehicle-treated (C) rats (vital myocardium is stained blue, fibrosis red). B) Epicardial scar tissue with granulation tissue due to suture placement is visible in sham-operated rat heart (arrows). Magnification 100x. C) Infarcted myocardium contains sites of fibrosis and granulation tissue (arrows infarcted area). Magnification 25x. D) Infarct size in clusterin and in the vehicle group. Mean infarct size was expressed as percentage of the total left ventricle area. Note a 75 ± 5% in reduction of infarct size in rats receiving clusterin (P<0.05, one-sided t-test), compared with vehicle-treated control animals.
**Clusterin administration does not affect neutrophilic granulocyte number, but increases macrophage number after AMI**

To investigate a putative effect of clusterin on wound healing, we analysed neutrophilic granulocytes and macrophages numbers in the heart (Figure 3). Neutrophilic granulocytes were predominantly found in infarcted areas of the heart (approximately 20-fold higher than non-infarcted myocardium). In addition, total neutrophilic granulocyte number in the viable areas of vehicle-treated AMI rats was significantly higher than in the sham-operated controls (P<0.01), however, the total number of these cells was still very low. This is in agreement with our previous study, in which low numbers of neutrophilic granulocytes were found 28 days after infarction.[17] The number of neutrophilic granulocytes in the viable areas of clusterin treated animals, however, did not differ significantly from sham-operated animals, or the vehicle-treated group (Figure 3A). No differences was found in granulocyte number in the infarcted areas between the vehicle and clusterin-treated rats (Figure 3A,B).

As with neutrophilic granulocytes, macrophages were predominantly seen in infarcted areas of the heart (approximately 60-fold higher compared with viable myocardium). In the viable myocardium, no significant differences in macrophage numbers were found between sham-, vehicle- and clusterin-treated groups (Figure 3C). However, the number of macrophages was significantly increased after treatment with clusterin compared with vehicle-treated rats, in the infarcted areas (P<0.05, Figure 3D). Notably, in the viable myocardium, macrophages were diffusely solitarily present within the tissue, whereas in the infarcted myocardium, the macrophages were predominantly found as large conglomerates.

**Human clusterin co-localizes with the clusterin receptor megalin in vitro, but does not exert its protective effect through megalin**

It has been suggested that clusterin exerts its protective effect through megalin. However, the presence of megalin has never been investigated in cardiomyocytes. Therefore, we studied the putative presence of megalin on rat cardiomyoblasts (H9c2 cells), and the effect of ischaemia on its expression. We found megalin in a dotted pattern on H9c2 cells that were not subjected to ischaemia or human clusterin (Figure 4A-C, arrows). Added exogenous purified human clusterin co-localized with megalin, suggesting that human clusterin can bind to megalin in/on rat cardiomyocytes (Figure 4A-C). Most importantly, human clusterin was not detected independent of megalin. Although we found a slight increase in megalin expression after human clusterin addition (106 ± 7%), this was not statistically significant when compared with control cells (P=0.60).

We next analysed the effect of ischaemia and reperfusion (2h ischaemia, 2h reperfusion) on megalin expression in H9c2 cells. It was found that the distribution of megalin was similar in untreated cells and in ischaemia/reperfusion cells. Further ischaemia/reperfusion did not significantly affect the amount of megalin present on the H9c2 cells. Total intensity of megalin expression in ischaemia/reperfusion was 94 ± 7% compared with control (P=0.60).
Clusterin reduces infarct size after AMI

We next analysed the subcellular localization of megalin in H9c2 cells, using a membrane marker (WGA) and a nuclear marker (DAPI). Megalin was found throughout the cytoplasm (Figure 4D), partly showing (peri)nuclear staining. However, megalin did not co-localize with the plasma membrane marker WGA, as depicted in Figure 4E,F. Co-localization of megalin neither with two types of specific endocytosis vesicles (clathrin and caveolin-1) nor with the Golgi apparatus was found (not shown).

We further investigated whether endogeneous rat clusterin co-localized with megalin (Figure 4G). Immunofluorescent staining for rat clusterin revealed that H9c2 cells indeed

Figure 3: Effects of clusterin on neutrophilic granulocyte and macrophage infiltration. Number of neutrophilic granulocytes (A+B) and macrophages (C+D) in the heart 28 days after MI in rats treated with or without clusterin. A) Number of neutrophilic granulocytes in the non-infarcted areas of the left ventricle. Significantly more neutrophilic granulocytes were found in animals with acute myocardial infarction (AMI), compared with sham-operated animals. B) Number of neutrophilic granulocytes in the infarcted area of the left ventricle. No significant differences were found between clusterin-treated and untreated animals. C) Number of macrophages in the non-infarcted areas of the left ventricle. No significant differences were found between sham-operated, clusterin-treated and untreated animals. D) Number of macrophages in the infarcted area of the left ventricle. Significantly more macrophages were found in clusterin-treated animals, compared with vehicle-treated animals. * P<0.05, ** P<0.01
expressed clusterin, but this clusterin did not co-localize with megalin. Ischaemia and reperfusion did not affect the localization of rat clusterin in the cell.

We also studied megalin and endogenous clusterin expression in H9c2 cells at baseline and following ischaemia and reperfusion using Western blot. Both the proteins were detected in Western blot experiments (Figure 5). As negative control, human endothelial cells (HUVECs) were used, which did not show the expression of clusterin and megalin (not shown). When analysing the blots, after correcting for tubulin expression, again no statistically significant differences were found in expression between untreated control cells and ischaemia/reperfusion-treated cells (n=3).

![Western blot analysis of clusterin and megalin expression in H9c2 cells](image)

**Figure 5: Western blot analysis of clusterin and megalin expression in H9c2 cells.** Megalin (A), Clusterin (B) and alpha- tubulin (C, constitutively expressed control) expression in non-ischaemic control H9c2 cells (c) and in H9c2 cells subjected to ischaemia (I). Megalin and clusterin expression were not affected by ischaemia.

To investigate the role of megalin further, we performed immunohistochemistry for megalin and clusterin on consecutive slides of the human heart, in patients died subsequent to 1–4 days of myocardial infarction. We have previously found that clusterin is abundantly present in the human heart, in this particular infarct phase.[5] In this study, we also found that clusterin localized to necrotic cardiomyocytes (Figure 6A). In contrast, megalin expression was found diffusely in the heart. As depicted in Figure 6B (asterisk), megalin staining was not only found in cardiomyocytes but, in fact was also most prominently positive in the vascular wall. In cardiomyocytes, staining of megalin was mainly found in the cytoplasm, while plasma membrane expression of megalin was limited (Figure 6C,D, arrows). Even more, the pattern of megalin staining of cardiomyocytes was comparable between clusterin-negative (Figure 6C) and -positive areas (Figure 6D). As such, clusterin positivity of cardiomyocytes was found independent of megalin. This concurs with the in vitro study, in which we found that megalin expression was not affected by ischaemia (Figure 5). These results thus suggest that clusterin probably exerts its protective effect independent of megalin.
To investigate this further, we have studied the effect of an inhibitor of megalin, namely RAP [22], in H9c2 cells under ischaemic conditions with and without addition of human clusterin, in a viability assay. We then found that ischaemia alone reduced the number of viable (negative for both Annexin V and PI) cells by $8 \pm 3\%$. After adding purified human clusterin, significantly more viable cells were found, when compared to ischaemia alone ($P<0.01$, Figure 7). However, inhibition of megalin using RAP did not affect the protective effect of clusterin, as similar numbers of viable cells were found after treatment with clusterin and clusterin with RAP (clusterin: $97 \pm 3\%$, RAP $98 \pm 2\%$, when compared with control, $P = 0.41$). These results thus suggest that clusterin does not exert its protective effect through megalin.

**Figure 6: Megalin and clusterin expression in the human heart.** Immunohistochemical staining of megalin and clusterin in slides of the human heart with acute myocardial infarction (1–4 days old). A) Clusterin expression. B) Overview of megalin expression in a consecutive slide from A. Megalin expression was found throughout in both clusterin-positive and -negative areas, and was most prominent in the vascular wall (asterisk). C,D) Megalin expression in the clusterin-negative area (C) and positive area (D) of A in more detail. Megalin expression was found in some cardiomyocytes, and then mainly in the cytoplasm, and only a low number of cells showed clear megalin expression on the plasma membrane (arrows). The megalin staining pattern, however, did not differ between clusterin-negative and -positive areas. Magnification A,B: 100x, C,D: 400x.
Figure 7: Inhibition of megalin does not affect the protective effect of clusterin on ischaemically challenged cells. Percentage of viable cells (Annexin V and PI negative) as measured using FACS analysis, compared with untreated control cells. Isch: ischaemia. ** P<0.01.

Discussion

In this study, we analysed the effect of clusterin administration on infarct size, mortality, heart function and wound healing in a rat model of AMI. We further studied the role of the putative clusterin receptor megalin herein. We found that clusterin administration during the acute inflammation period significantly reduced infarct size and mortality, without affecting normal wound healing. In addition, we showed the expression of the clusterin receptor megalin on cardiomyocytes. However, the protective effect of clusterin could not be blocked in vitro by the megalin inhibitor RAP, indicating that clusterin exerts its protective effect on cardiomyocytes independent of megalin.

Interestingly we found that application of a single bolus of clusterin directly after reperfusion did not result in significant infarct reduction, while clusterin administration during 3 days after infarction significantly reduced infarct size by 75%. This effect was not the result of a higher plasma level reached by the three injections, as we also found that already 24h after administration, clusterin could not be detected in the plasma anymore. These results suggest that indeed the acute inflammation period after AMI plays a major role in cell damage after AMI, in addition to ischaemia itself. Therefore, clusterin must be applied during the whole acute inflammation period after AMI.

How clusterin exactly exerts this protective effect after AMI in vivo remains unknown. Earlier studies have shown that clusterin inhibits several factors of the complement system.[10–12] However, in a previous study, we have also shown that clusterin affords cardioprotection independent of complement.[5] Indeed, several other cytoprotective roles for clusterin have
been described. It has been suggested that clusterin plays a role in cytoprotection at fluid–tissue boundaries, membrane recycling in response to injury, and regulation of the membrane attack complex of complement, in cardiomyocytes, among others, after ischaemia.[5,9,12,23] It has also been proposed that clusterin in general can act as a form of secreted heat-shock protein or a chaperone molecule.[23–25]

Interestingly, next to these direct protective effects of clusterin, several cytoprotective effects of clusterin have been related to its receptor megalin. For instance, it was found that extracellular clusterin can promote uptake and clearance of toxic molecules, by binding to these molecules, through endocytosis via megalin.[14] In addition, it has been shown that clusterin mediates the uptake of cellular debris via megalin in fibroblasts and epithelial cells in vitro.[15] Finally, binding of exogenous clusterin to megalin may also activate pro-survival transduction pathways, as described in prostatic cells, where clusterin protected these cells from TNF-alpha and actinomycin D-induced cell death.[26] However, megalin expression has not been studied earlier in cardiomyocytes. We now found that megalin is expressed not only on H9c2 cells, rat cardiomyoblast cells, but also in cardiomyocytes of the human heart (Figure 6B). Although we did find co-localization of megalin and exogenous added human clusterin on H9c2 cells, remarkably, ischaemia did not have any effect on megalin expression itself (Figure 6C). In line with this finding, in the human heart, we found diffuse megalin positivity in cardiomyocytes, independent of clusterin, both inside and outside the infarcted area. In addition, we did find clusterin staining in cardiomyocytes in case megalin staining was negative. As in vitro blocking of megalin did not influence the protective effect of clusterin, these results thus suggest that clusterin does not exert its effect through megalin. The exact mechanism of how clusterin exerts its protective effect on cardiomyocytes, is subject of further research.

The inflammatory response in the heart after AMI is essential for myocardial remodelling and wound healing. Inhibition of this response may have detrimental effects, as impaired wound healing can have severe consequences such as wall rupture.[27–31] Importantly, in our study, clusterin administration did not result in reduced ventricular wall thickness, nor did it improve abnormal scar tissue formation and aneurysmatic infarcts, at 28 days after AMI. These results suggest that wound healing and scar tissue formation were not affected by the administration of clusterin. In addition, we found that clusterin treatment did not affect the neutrophilic granulocyte cell number in the infarction area at 28 days post-AMI, suggesting that granulocyte infiltration was not delayed. Interestingly, in clusterin-treated rats, the number of macrophages in the infarcted areas was significantly higher than those in control rats 28 days after AMI. Several roles have been ascribed to macrophages in infarct healing after AMI,[32,33] such as in wound healing by digestion of damaged tissue, promotion of angiogenesis, production of anti-inflammatory mediators and regulation of extracellular matrix metabolism through synthesis of matrix metalloproteinases and their inhibitors. In addition, Nahrendorf et al.[34] have described that after myocardial infarction, early macrophages digest damaged tissue, while late macrophages promote healing. We have previously shown that the macrophages in the rat heart 28 days after MI were of late cell type, by staining with an ED2 antibody.[17] These data suggest that the increased number of macrophages 28 days after AMI after clusterin treatment might improve wound healing.
In this study, we found a trend towards increased cardiac output in the clusterin-treated group, compared with the vehicle-treated group, which was borderline significant. It has been described that cardiac output is only significantly decreased in large infarcts.[35,36] In our study, the infarct size was non-aneurysmatic, which could explain the small effect of infarction on heart function. We, however, deliberately induced non-aneurysmatic infarcts, as in patients most infarcts are non-aneurysmatic, because of widespread use of revascularization therapy. Taken together, it was found that clusterin administration after start of reperfusion significantly reduced myocardial infarct size (75%) and mortality, without impairing wound healing. Furthermore, we have shown the presence of the clusterin receptor megalin on cardiomyocytes. However, our study indicates that the clusterin effect is independent of megalin. Reperfusion of ischaemic myocardium has been optimized over the last decades and can significantly reduce infarction size in patients suffering from AMI. Further reduction of myocardial damage after AMI is therefore expected to require new therapeutic approaches.[1] Our results indicate that administration of clusterin constitutes a new therapeutic approach to afford cardioprotection during AMI.

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Chapter 3

References
