Stem cell-mediated regeneration of the infarcted heart
Velde, Susanne van der

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2006

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Increased inflammatory response and neovascularization in reperfused versus non-reperfused murine myocardial infarction

Vandervelde, Susanne ¹MSc, van Amerongen Machteld J. ¹MSc, Tio, Rene A. ²MD, PhD, Petersen, Arjen H. ¹BSc, van Luyn, Marja J.A. ¹PhD, Harmsen, Martin C. ¹PhD

Departments of Pathology & Laboratory Medicine¹ and Cardiology², University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands

Cardiovascular Pathology 15 (2006) 83-90
Abstract

Introduction. Fundamental knowledge of the inflammatory response after myocardial infarction is indispensable for intervention towards cardiac regeneration. Although reperfusion is preferred as clinical therapy, for basic research also permanent ligation myocardial infarction models are widely used.

Methods. In this report, we pathohistologically compared the kinetics of the inflammatory and angiogenic response after myocardial infarction induced by permanent ligation or ligation followed by reperfusion of the left anterior descending coronary artery in mice.

Results. Permanent ligation resulted in a higher mortality rate accompanied by increased left ventricular dilatation and more progressive wall thinning. However, reperfused infarcts showed higher inflammatory cell influx. Neutrophil numbers were higher after reperfusion post-MI, although their presence was prolonged after ligation. Also the number of macrophages after reperfusion was continuously higher, but the course of macrophage influx was comparable in both models. The number of lymphocytes was low in both models. Only the peak in myofibroblast numbers at 7 days was higher after ligation than after reperfusion. Moreover, cardiomyocyte remnants were cleared faster and collagen deposition started earlier after reperfusion. In addition, reperfusion resulted in an increased angiogenic response, as was reflected in increased numbers of medium-sized and large vessels at 7 and 14 days post-MI.

Conclusion. We show less adverse remodeling together with a higher presence of inflammatory cells and enhanced neovascularization in reperfused myocardial infarction. These differences between non-reperfused and reperfused myocardial infarction should be taken into consideration for experimental use of MI models.
**Introduction**

Myocardial infarction (MI) is a great threat to life in the western world, and therefore a lot of research is focused on development of new therapies. Experimental animal research is performed in various models of myocardial infarction [1-3], of which permanent ligation of the coronary artery and ligation followed by reperfusion are the most widely used models.

To date, reperfusion is the preferred clinical therapy for myocardial infarction. It reduces mortality, even at later stages, when injured myocardial tissue can not be salvaged anymore [4;5]. Nevertheless, in basic science the ligation MI model is also often used.

Experimental models of myocardial infarction are developed in various animal species [6;7]. Development of transgenic mice, which offer a good opportunity for systematic study of the function of various genes and their products in MI [8], have enlarged the need for a thorough understanding of the pathohistologic response following MI in mice.

Moreover, the inflammatory response following myocardial infarction is determinative for tissue healing [9]. The specific inflammatory response of the MI model used is often disregarded in both timing of interventions and interpretations of outcomes. Therefore, accurate knowledge of this early transient, but substantial process of the MI model applied is fundamental for experimental use. Here, we present a qualitative and quantitative histopathological comparison of the inflammatory process, of the left ventricular remodeling and of the angiogenic response at various time points up to one month after myocardial infarction between the two most widely used murine models of MI, i.e. permanent ligation or ligation followed by reperfusion of the left anterior descending coronary artery.

**Materials and Methods**

**Animals.** Nine to 12 week old C57BL/6JolaHsd male mice (22-25 g) were obtained from Harlan Nederland (Horst, the Netherlands). Mice were housed individually prior to and after surgery. All procedures performed on mice were approved by the local committee for care and use of laboratory animals, and were performed according to strict governmental and international guidelines on animal experimentation. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Myocardial infarction.** Mice were anesthetized, orally intubated and mechanically ventilated (respiratory rate 150 strokes/min, volume 200 μl) (Hugo Sacks Elektronik, March-Hugstetten, Germany) with isoflurane (2.5%), N₂ (2%), O₂ (2%) and fixed with their left side up. Thirty minutes prior to surgery, mice received analgesic drug temgesic (0,03 mg/kg) intramuscularly. A left thoracotomy was performed via the third intercostal space, and muscles and pericardium were carefully dissected. The Left Anterior Descending artery (LAD) was localized using a dissecting microscope, and ligated with a 6-0 non-absorbable Prolene suture just proximal of the bifurcation of the LAD. In the reperfusion model, the ligation was removed after 30 min. Sham operated mice underwent the same procedure except for arterial occlusion. The intercostal space was closed with a 6-0 non-absorbable Prolene suture and the skin with a 5-0 absorbable Safil suture. Mice received 100% oxygen until wakening after which the endotracheal tube was withdrawn. Animals were given 100% oxygen via nasal cone until full recovery of consciousness. During recovery mice were kept warm with a heat lamp. All animals received, drinking water containing 1mg/ml 5-bromo-2-deoxyuridin (BrdU) (Sigma, St. Louis, USA), 3 d prior to killing.
At 2, 4, 7, 14, and 28 days post-MI mice were killed. The hearts were dissected carefully and snap-frozen in liquid nitrogen and stored at -80°C (n= 4 per time point) or fixed in 2% glutaraldehyde (GA, MERCK, Darmstadt, Germany) in 0.1 M phosphate buffer (PB) for at least 24 h (n=3 per time point) or in 2% paraformaldehyde (MERCK, Darmstadt, Germany) in 0.1 M PB for 3 h (n=1 per time point). Hearts fixed in GA were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany), and hearts fixed in PF were embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany), according to manufacturer’s instructions.

**Histological stainings.** Hearts fixed in liquid nitrogen were cut transversally in the middle third between base and apex in sections of 5 μm and stained immunohistochemically with the following antibodies: goat anti-mouse Troponin T-C for cardiomyocytes (Santa Cruz Biotechnology, Santa Cruz, USA), rat anti-mouse CD31 for endothelial cells (Pharmingen, San Diego, USA), rat anti-mouse F4/80 for macrophages (Serotec, Oxford, UK), rat anti-mouse Neutrophil for neutrophils (Serotec, Oxford, UK), mouse anti-BrdU for proliferating cells (Sigma, St. Louis, USA), hamster anti-mouse CD3ε for pan-lymphocytes (BD Pharmingen, San Diego, USA) and mouse anti-human α-smooth muscle actin (α-SMA) for myofibroblasts (Sigma, St. Louis, MO). Endogenous peroxidase was blocked by incubation with 0.1% H₂O₂ in PBS for 10 min. Endogenous biotin and avidin were blocked with avidin/biotin blocking kit according to manufacturer’s instructions (Vector Laboratories, Burlingame, USA). A Mouse on Mouse kit (Vector Laboratories, Burlingame, CA) was used for α-SMA staining. Color development was performed with 3-amino-9-ethyl-carbazole (AEC) according to prescription (Sigma, Steinheim, Germany) and sections were counterstained using Mayer’s hematoxilin (Fluka Chemie, Buchs, Switzerland). Masson Trichrome (collagen blue, myocardial cells red) was performed according to standard protocol [10] on cryo sections of 6-8 mm, fixated first in 4% formaldehyde for 1 hour room temperature followed by Bouin fixative at 60 °C for 5 min.

Blood vessels and mast cells were counted in Technovit 7100 embedded heart sections of 2 μm thickness stained with toluidin blue (Fluka Chemie, Buchs, Switzerland). BrdU staining was performed in 8100 embedded heart section of 2 μm thickness and color development was performed with 3.3-diaminobenzidine-tetrahydrochloride (DAB; Sigma, St. Louis, USA).

**Quantitative Analysis.** All quantitative analysis was performed by at least two independent investigators on blinded specimens. Infarct size, lumen surface of left ventricle (LV) and diameter of LV wall at the thinnest point were measured in images made with 1.25x objective of Masson Trichrome stained sections (Leica Qwin 4.0) with customized image processing software for surface and length measurement (Leica Microsystems, USA). The infarct size was calculated as follows: surface infarction / (surface of total LV – surface of LV lumen) * 100%. Small vessels (containing 1 endothelial cell nucleus), middle sized blood vessels (containing 2-6 endothelial cell nuclei) and large blood vessels (containing >6 endothelial cell nuclei) were counted on six images made with 40x objective (Leica Qwin 4.0) taken randomly at the epicardial site of the infarct. Neutrophils, macrophages and lymphocytes were counted on specifically immunohistochemically stained sections and were recognized as red stained cells (AEC) with blue stained nuclei (hematoxilin) on six equally divided images made with 40x objective of the myocardial infarction (Leica Qwin 4.0). Fraction of SMA-positive cells per area with α-SMA was measured in three images.
made with 40x objective using customized image processing software for staining intensity in which vessels were excluded (Leica Microsystems, USA).

**Statistical analyses** Results are presented as mean ± SEM. Inter-rater agreement was evaluated by paired samples t-testing. Statistical analysis of the data was performed using two-way ANOVA as appropriate, followed by Student’s t-test or Bonferroni test. Chi square test was performed for differences in group mortality. Values of $P<0.05$ were considered statistically significant.

**Results**

**Mortality and Morphology.** We observed a significantly lower total mortality rate after reperfusion than after ligation (5.8% vs. 35.6% respectively in the 28-day time period, $P<0.001$). The highest mortality rate was within the first 24 h after permanent occlusion (lig vs. rep: 20% vs. 2.9%, $P<0.01$). From day 2 to 7 the mortality rates were 6.7% in the ligation group and 2.9% in the reperfusion group ($P>0.05$). After one week the mortality rate in the reperfusion group was zero, while in the ligation group the mortality rates were still high (8.9%, $P<0.05$).

Adverse remodeling, measured by LV dilatation and LV wall thinning, was significantly stronger after permanent ligation. Dilatation of the LV, measured as the surface of the LV lumen, was significantly increased after ligation compared to reperfusion as of day 4 ($P<0.01$) (Fig.1a). Also the LV wall was significantly thinner after ligation compared to reperfusion again as of day 4 ($P<0.01$) (Fig. 1b). The increased adverse remodeling in ligated MI compared to reperfused MI can be seen in exemplary left ventricular Masson Trichrome stainings (Fig 2).

![Figure 1.](image)

**Figure 1.** Left ventricular remodeling in time. a. Surface of LV lumen defined as surface of lumen compared to whole LV surface. b. Thickness of LV wall measured at thinnest point in the anterior LV wall. Open bars represent the ligation model, closed bars the reperfusion model. All measurements are performed on pictures of Masson Trichrome and Troponin T-C stained transversal heart sections at a magnitude of 12.5x. *$p<0.05$, **$p<0.01$, ***$p<0.001$. $P$-value calculated for differences between the two models at one time point. Bars represent mean and SEM.
**Figure 2.** Left ventricular remodeling after myocardial infarction. Representative micrographs of left ventricular cross sections of Masson Trichrome staining at 2 (a, f), 4 (b, g), 7 (c, h), 14 (d, i) and 28 (e, j) days post-MI after ligation (2a-e) and reperfusion (2 f-j). Left pictures show a morphologic overview (original magnification 12.5x), right pictures show in more detail the infarcted left ventricular wall (original magnification 200x). Collagen stains blue, myocardium stains red.

**Inflammatory response**
Granulation tissue was of greatest cellular content in the first seven days post-MI. Of the infiltrated inflammatory cells, macrophages were present in highest numbers and for longest period in the infarcted region of both models (Fig 3). Neutrophil influx (Fig 3a) was rapid and was highest at 2 and 4 days post-MI in both models. At 4 days post-MI the number of neutrophils
was significantly higher in reperfused MI (P< 0.05), but decreased rapidly thereafter and were virtually absent at 7 days post-MI. A more gradual decline was observed in ligated MI, where neutrophils were present in significantly higher numbers at 7 days post-MI compared to reperfused MI (P<0.05). Neutrophils could only sporadically be observed at 14 days post-MI in both models.

**Figure 3.** Inflammatory cell infiltration in MI. Number of neutrophils (a.), macrophages (b.) per mm², lymphocytes per high power filed (40x objective) (c) and percentage staining of myofibroblasts (d) are shown in graphs. On the right side, exemplary pictures of specific stainings are shown of ligated and reperfused MI respectively.

Open bars represent the ligation model, closed bars the reperfusion model. *p<0.05, **p<0.01. P-value calculated for differences between the two models at one time point. Bars represent mean and SEM.

The number of macrophages (Fig 3b) peaked in both models at 4 days post-MI. Although
(P<0.01), they tended to be higher throughout 14 days post-MI in reperfused MI compared to ligated MI (P>0.05). Compared to controls the number of macrophages was significantly higher at all time points in both models (P<0.001). Numbers of lymphocytes were low in the infarcts in both models throughout the first 14 days post-MI, i.e. 10 to 100 fold lower than macrophage and neutrophil numbers at same time points (fig. 3c). At 28 days post-MI no lymphocytes were present in the infarct zone of both models. Although not significantly different between the 2 models, numbers of lymphocytes tended to be higher in reperfused MI (P>0.05). Also, there were no significant differences in lymphocyte number in time within one model (P>0.05). Mast cells were only sporadically observed in controls and in both models post-MI. Myofibroblasts (fig. 3d) were observed as spindle shaped red-stained cells in the infarcted area. Myofibroblasts were transiently present from 4 to 14 days in ligated MI and from 4 to 7 days in reperfused MI. The peak in surface area of myofibroblasts on 7 days post-MI was significantly higher in ligated MI (P< 0.05).

**Neovascularization.** We measured numbers of small, medium-sized and large vessels, defined by number of endothelial cells per vessel (1, 2 to 6 and more than 6 endothelial cells respectively). The number of small vessels declined rapidly after MI in both models (P<0.001, Fig 4a). Representative toluidine blue stained 2 μm sections, which show a decreased number of small vessels 2 days after ligation compared to healthy controls are shown in figure 5 a and b. After 2 days post-MI no further significant differences in number of small vessels appeared within the models (P>0.05). Until 7 days post-MI, the number of small vessels in reperfused MI appeared to be higher than in ligated MI (P>0.05), albeit still very low compared to number of small vessels in control animals. The number of medium-sized vessels was increased at least three-fold in both models at 4 days post-MI (Fig 4b). Whereas the number of medium-sized vessels in ligated MI remained constant in the period following 4 days post-MI, in reperfused MI the number of medium-sized vessels increased to approximately five-fold until even six-fold compared to shams at 7 and 14 days post-MI respectively (lig vs. rep P<0.05, control vs. rep P<0.001). The number of large vessels peaked at 7 days post-MI in the reperfused-MI (P<0.05), whereas number of large vessels in ligated MI remained similar post-MI (P>0.05), but a trend towards a maximum at day 14 was visible (Fig 5c). Although the number of large vessels tended to decrease after 14 days, the number of endothelial cells per vessel still increased resulting in even larger vessels (data not shown). The formation of medium-sized and large vessels was the result of active proliferation of endothelial cells, as was confirmed by the incorporation of BrdU in their cell nucleus. (Fig 5 c and d). In thin sections (2 μm) we observed BrdU-positive flat cells lining the vessels (endothelial cells). In addition non-endothelial cells adherent to the vessel wall that were positive for proliferation marker BrdU were detected (Fig 5c). The presence of red blood cells in the lumen of the vessels indicated that the vessels were functional (Fig 5d).
**Chapter 4**

**Figure 4** Time course of number of vessels in MI. Small, medium-sized and large vessels were defined by number of endothelial cells per vessel (1, 2-6 and more than 6 endothelial cells respectively). Open bars represent the ligation model, closed bars the reperfusion model. *p<0.05, **p<0.01, ***p<0.001. P-value calculated for differences between the two models at one time point. Bars represent mean and SEM.

**Figure 5.** Small vessels and proliferation of larger vessels. In the toluidine blue stained 2 μm thick Technovit 7100 sections of sham operated mice (a) and 2d after ligation (b) small vessels, some with erythrocyte inside, can be observed (arrows). In c and d endothelial cell proliferation is made visible by BrdU (brown) staining on 2 μm thick Technovit 8100 sections. Arrows: proliferating endothelial cells. Arrowheads: Adherent proliferating cells. RB: Red blood cells.

Endothelial cell proliferation, seen as flat cells bordering the vessels (endothelial cells) positive for a proliferation marker (BrdU) of two independent vessel within the epicardial site of MI can be observed. Non-endothelial cells adherent to the vessel wall that are positive for proliferation marker are also seen (c). Red blood cells in the lumen of the vessels indicates that the vessels are functional (d). Original magnification 1000x.
**Discussion**

In this comparative study between a reperfused and a non-reperfused murine model of myocardial infarction, we observed considerable pathohistological differences. After reperfusion the adverse remodeling, i.e. wall thinning and lumen dilatation was less than after permanent ligation (see fig. 6 for a schematic overview).

The reperfused infarct was infiltrated with higher numbers of inflammatory cells, in particular neutrophils and macrophages. Also, the cardiomyocyte clearance and collagen deposition was faster than after permanent ligation. Regarding the vasculature, the number of small vessels rapidly and continuously declined post-MI in both models. However, a significantly stronger angiogenic response in reperfused MI resulted in significantly higher numbers of medium sized and large vessels at 7 and 14 days post-MI compared to ligated MI. Vascular architecture in MI changed from small vessels to medium sized and large vessels in the ischemic inflammatory infarction. In conclusion, these marked pathohistological differences between the two murine MI models should be acknowledge for experimental use. The decision for a specific MI model should be tailor made, dependent on the outcome parameters. In this study, we demonstrate that outcome parameters, such as infarct remodeling or neovascularization, are influenced by the different pathohistological responses following ligation and reperfusion. Although the reperfusion model is clinically more relevant, the inflammatory response in the infarct is much stronger than after ligation. The inflammatory cells can interfere with an applied interventional experimental therapy. For example, intramyocardial delivered therapeutics will probably suffer more phagocytosis by the higher amount of macrophages. Moreover, after reperfusion the process of cardiomyocyte remnant clearance is quicker and collagen deposition is started earlier. Together with the earlier evanescence of neutrophils after reperfusion, the inflammatory process is accelerated after reperfusion compared to ligation, which should be acknowledge for timing of an experimental intervention. Also important in timing of the intervention, is the higher number of medium-sized and large vessels after 7 and 14 days post-MI, which provides a better perfusion from the second week on after reperfusion. Another clear

---

**Figure 6.** This figure shows a schematic overview of left ventricular remodeling after permanent occlusion of the coronary artery (ligated MI) and occlusion followed by reperfusion (reperfused MI). RV = Right Ventricle, LV = Left Ventricle, MI = Myocardial Infarction. Especially the transmural extent of the infarct after ligation with concomitant LV wall thinning and lumen dilatation versus the epicardial and endocardial sparing with less LV remodeling after reperfusion is apparent.
advantage of the reperfusion model is the lower mortality post-MI. However, since adverse remodeling is less pronounced, it will be more difficult to obtain strong differences in infarct remodeling between an interventional and control group, which may lead to the need of larger experimental groups.

After ligation, the response is mainly dependent on the effect of ischemia on the heart, whereas after reperfusion the response is more complex because of the additional effect of ‘reperfusion injury’ [11;12]. In conclusion, knowledge of the pathohistological process in the different models of myocardial infarction, as presented here, is fundamental for experimental use.

Acknowledgements
We thank Greetje Groen for her technical support. We are very grateful for the statistical analysis of all data by dr. Ilja Nolte, PhD.

References