

Molecular mapping of the regenerative niche in a murine model of myocardial infarction

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Abstract. Adult stem cells are distributed through the whole organism, and present a great potential for the therapy of different types of disease. For the design of efficient therapeutic strategies, it is important to have a more detailed understanding of their basic biological characteristics, as well as of the signals produced by damaged tissues and to which they respond. Myocardial infarction (MI), a disease caused by a lack of blood flow supply in the heart, represents the most common cause of morbidity and mortality in the Western world. Stem cell therapy arises as a promising alternative to conventional treatments, which are often ineffective in preventing loss of cardiomyocytes and fibrosis. Cell therapy protocols must take into account the molecular events that occur in the regenerative niche of MI. In the present study, we investigated the expression profile of ten genes coding for chemokines or cytokines in a murine model of MI, aiming at the characterization of the regenerative niche. MI was induced in adult C57BL/6 mice and heart samples were collected after 24 h and 30 days, as well as from control animals, for quantitative RT-PCR. Expression of the chemokine genes CCL2, CCL3, CCL4, CCL7, CXCL2 and CXCL10 was significantly increased 24 h after infarction, returning to baseline levels on day 30. Expression of the CCL8 gene significantly increased only on day 30, whereas gene expression of CXCL12 and CX3CL1 were not significantly increased in either ischemic period. Finally, expression of the IL-6 gene increased 24 h after infarction and was maintained at a significantly higher level than control samples 30 days later. These results contribute to the better knowledge of the regenerative niche in MI, allowing a more efficient selection or genetic manipulation of cells in therapeutic protocols.

Introduction

The main function of adult stem cells is to maintain tissue homeostasis by replacing worn-out cells. Adult stem cells are also activated in response to signals of inflammation and tissue damage, and have an important role in the repair of tissues or organs (1). All adult tissues and organs have their own compartment of stem cells. Hematopoietic stem cells have been used for the therapy of hematologic diseases for over 40 years, and more recently adult stem cells have been intensively studied for their therapeutic potential in a great number of other diseases (2). Mesenchymal stem cells (MSC), which are distributed through the whole organism (3), have attracted attention due to their great therapeutic potential.

For the successful therapeutic use of stem cells, a more detailed understanding is necessary not only of their basic biological characteristics (4) but also of the regenerative niche. This will allow the design of therapeutic studies with cells able to respond appropriately to signs and to resist the lesion microenvironment, which is often unfavorable to them (5,6).

Myocardial infarction (MI) is caused by a lack of blood supply to the heart muscle resulting in necrosis and triggering a cascade of events, involving several cell types and signaling molecules. This leads to inflammation and scarring with consequent tissue remodelling, as well as mechanical dysfunction and electrical conduction pathway abnormalities (7). MI is listed among the acute coronary syndromes that represent the most common cause of morbidity and mortality in the Western world. Currently, pharmacological, percutaneous and surgical treatments of ischemic cardiomyopathy are unable to prevent the loss of cardiomyocytes and fibrosis that is established (8). When ischemic disease is very advanced and diffuse, a condition affecting up to 12% of patients with ischemic heart disease, the available methods are not effective (9).

Cell therapy appears as a promising treatment for ischemic heart disease. The design of efficient therapeutic strategies must consider the molecular events which are present at the MI regenerative niche, so that the most appropriate cells can be selected to minimize the loss of cardiomyocytes and promote

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the return of normal function of the myocardium, through revascularization of ischemic zones and production of new cardiomyocytes (8).

Cytokines and chemokines play an important role in the recruitment of stem cells to the site of damage and in inducing their therapeutic responses (10). Soon after the lesion is produced, leukocytes are attracted and release inflammatory products that cause further tissue damage. The role of a number of them in the inflammatory condition that characterizes cardiovascular diseases such as atherosclerosis and MI has been described, but variable results are still seen in experimental and clinical studies (11,12).

The molecular investigation of signalling molecules involved in intercellular interactions contributes to a better characterization of the regenerative niche in MI (13,14). Although the mechanisms of action are not completely understood, the mapping of genes expressed in the ischemic heart over different periods is well advanced (15), and further exploration of the molecular profile of the regenerative niche is necessary as a first step in the design of protocols for cell therapy.

Materials and methods

Animals. Adult (8-10-week-old) male C57BL/6 mice, weighing around 25 g, were maintained under standard conditions and treated in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. All procedures were approved by a local Research Ethics Committee. This study was approved by the Research Ethics Committee of Universidade Luterana do Brasil.

Myocardial infarction. Myocardial infarction was induced in anaesthetized (0.05 mg/100 g ketamine and 0.01 mg/100 g xylazine, intraperitoneally) mice by ligation of the left coronary artery with 7.0 prolene suture, as previously described (16) (Fig. 1A). Control mice were not operated, as previous studies showed no significant differences between non-operated and sham-operated animals (13).

Echocardiographic evaluation. Echocardiographic measurements were obtained under the recommendations of the American Society of Echocardiography 24 h after MI induction. The transthoracic echocardiogram was performed by using a Sequoia 512 (Acuson Corporation, Mountain View, CA), which offers a 10-13 MHz multifrequency linear transducer. All measurements were based on the average of three consecutive cardiac cycles. Mice were anesthetized as described above. The ejection fraction [EF (%) = (final diastolic volume-final systolic volume/final diastolic volume) x 100%] was obtained by a modified Simpson's method, by the formula:

$$V = \left[\frac{\pi}{4} \right] \sum_{i=1}^{20} A_i B_i \times \left[\frac{L}{20} \right]$$

where L = LV length divided into 20 discs (i=1 to i=20) from basis to apex, with the diameter of each disc being determined in 2 apical views (denoted as A and B).

The MI akinetic area was delimited taking into account the movement of LV walls, by the observation of longitudinal,

apical and transversal views of LV. Regions with systolic thickness under normal or absent, as well as, portions with paradoxal movement were considered as infarcted. Thus, the MI akinetic area (%) was measured by the ratio of these regions by the total area of LV walls, as previously described by us and others (17,18). Only animals with an EF ≤50% and infarcted area ≥30% were used in the experiments.

Molecular evaluation. A group of six mice were sacrificed 24 h after infarction, and a second group of 6 animals was sacrificed after 30 days. A third group of 6 healthy animals was used as control. The heart was arrested in diastole by perfusion with saline and collected. Samples from the left ventricle were collected, macerated, added into 1 ml of TRIzol reagent (Invitrogen, Sao Paulo, SP, Brazil) and kept at -20°C until RNA extraction, performed according to the supplier's instructions. After extraction, RNA was quantified using NanoDrop ND-1000 spectrophotometer and purity was determined by absorbance at 260/280 nm.

cDNA synthesis was performed from 1 µg of RNA, with oligo(dt) primers and the M-MLV Reverse Transcriptase kit (Invitrogen) using the Applied Biosystems Thermal Cycler Veriti. The expression of ten genes was assessed by quantitative real-time RT-PCR (qRT-PCR): interleukin 6 (IL-6), chemokine CXC motif ligand 2 (CXCL2), 10 (CXCL10) and 12 (CXCL12), chemokine CC motif ligand 2 (CCL2), 3 (CCL3), 4 (CCL4), 7 (CCL7), and 8 (CCL8), and chemokine CX3C motif ligand 1 (CX3CL1). The hypoxanthine-guanine phosphorybosyltransferase (HPRT) gene was used as an endogenous reference gene. The primers used were described in previous studies (Table I), except for CCL7 and HPRT, which were designed with FastPCR software. The CXCL12 isoform searched was the β variant, the same which is present in kidney, but other isoforms can exist in heart.

The 25 µl total volume PCR reaction mixture contained 12.5 µl of 100-fold diluted cDNA and the following reagents obtained from Invitrogen, in the following final concentrations: 1X PCR buffer, 3 mM MgCl₂, 100 µM dNTPs, 200 nM of forward and reverse primers, 0.16X SYBR®-Green, 0.25 units of Platinum Taq polymerase and 3.45 µl of H₂O. The conditions for thermocycling were 1 cycle of 94°C for 5 min, 40 cycles of 94°C for 15 sec, 60°C for 10 sec, 72°C for 15 sec and 60°C for 35 sec, a cycle of dissociation of 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec and were performed in 7300 Real-Time PCR System from Applied Biosystems (Foster City, CA).

Data analysis. Transcript levels were obtained from qRT-PCR analysis using the 2^{-ΔCT} method (19), and were presented as relative expression of the gene of interest and the reference gene. Data were expressed as mean of 2^{-ΔCT} ± standard deviation. For statistical analysis the paired Student's t-test was used, with the GraphPad Prism 5.0 software. A P-value <0.05 was considered statistically significant.

Results

Echocardiographic examination showed infarction of the left ventricular anterior and lateral walls in experimental mice group (Fig. 1B), validating the model used in this study.

Table I. Primer sequences used in this study.

Gene	Alternative name	Primer	Reference
CXCL12	SDF-1	Forward: TGAGGCCAGGGAAGAGTGAG Reverse: GACACATGGCGATGAATGGA	17
IL-6		Forward: TTCCATCCAGTTGCCTTC Reverse: TTCTCATTTCCACGATTTC	18
CCL7	MCP-3	Forward: CTCATAGCCGCTGCTTTCAG Reverse: ACAGCTTCCCAGGACACCGA	
CXCL2	MIP-2	Forward: CGCCCAGACAGAAGTCATAG Reverse: TCCTCCTTTCCAGGTCAGTTA	19
CCL2	MCP-1	Forward: TTAACGCCCCACTCACCTGCTG Reverse: GCTTCTTTGGGACACCTGCTGC	20
CCL3	MIP-1A	Forward: TGAATGCCTGAGAGTCTTGG Reverse: TTGGCAGCAAACAGCTTATC	19
CCL4	MIP-1B	Forward: TGCCCTCTCTCTCTCTTGTCT Reverse: CAGGAAGTGGGAGGGTCAGA	21
CCL8	MCP-2	Forward: GTGCTTCTTTGCCTGCTGCTCATA Reverse: AGACATACCCTGCTTGGTCTGGAA	22
CXCL10	IP-10	Forward: GAAATCATCCCTGCGAGCCT Reverse: TTGATGGTCTTAGATTCCGGATTC	23
CX3CL1	Fractalkine	Forward: ACGAAATGCGAAATCATGTGC Reverse: CTGTGTCTCTCCAGGACAA	24
HPRT		Forward: AATTATGGACAGGACTGAAAGACTTGCT Reverse: TCCAGCAGGTCAGCAAAGAATTTATAGC	

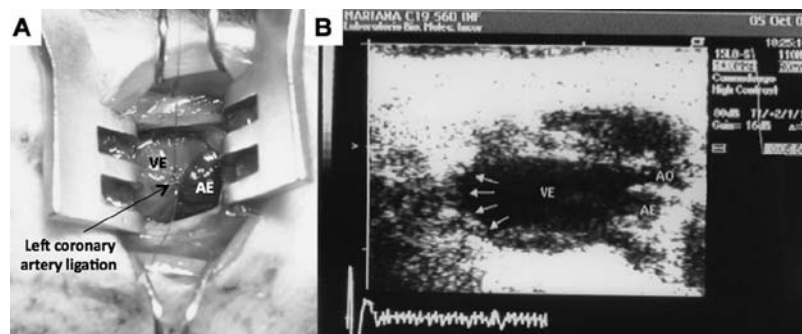


Figure 1. (A) Photography of the myocardial infarction procedure. The arrow indicates the left coronary artery ligation. (B) Echocardiogram of an infarcted mouse. Arrows indicate the myocardial infarcted area of the left ventricular wall. VE, left ventricle; AE, left atrium; AO, aorta.

Most of the chemokine CC motif ligand genes investigated had their expression markedly increased in the acute phase of infarction, after 24 h of ischemia. Expression of CCL2, CCL3 and CCL4 was increased 19-fold, 7.8-fold and 25.2-fold, respectively (Fig. 2). For CCL7, a 36-fold increase was observed (Fig. 3A). Gene expression returned to baseline levels 30 days after ischemia, without significant differences compared to non-ischemic control samples. Expression of the CCL8 gene, on the other hand, showed no significant increase after 24 h of ischemia, but was increased 4.3-fold after 30 days of MI (Fig. 3B).

The response of CXCL genes to infarction was more variable. At 24 h, a marked increase of expression (610.5-fold) was observed for the CXCL2 gene, whereas CXCL10 expression showed a more discrete increase (3.4-fold) and expression of CXCL12 was not modified (Fig. 4). In all cases, gene expression 30 days after infarction was similar to that of control samples.

Expression of the IL-6 gene was significantly increased in the acute phase (53.5-fold) as well as in the chronic phase (9.8-fold) of MI (Fig. 5A). Expression of the CX3CL1 gene was not modified by the experimental procedure (Fig. 5B).

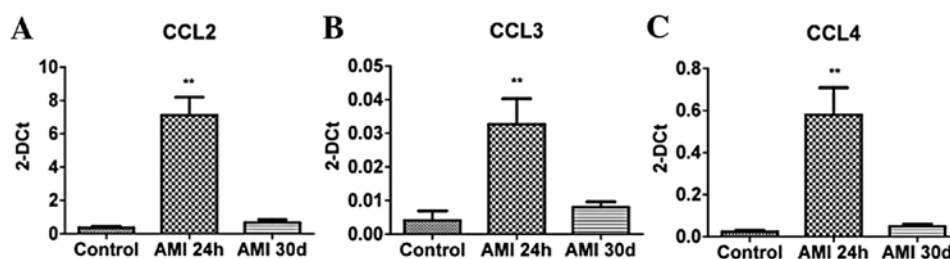


Figure 2. (A) In the acute phase of infarction (24 h), CCL2 expression was increased 19.0-fold ($P<0.01$), and 30 days later it was 1.8-fold higher than in control samples (not significant). (B) CCL3 expression was increased 7.8-fold 24 h after infarction ($P<0.01$), but on day 30 the increase (1.9-fold) was non-significant. (C) CCL4 was increased by 25.2-fold ($P<0.01$) after 24 h and returned to basal levels at 30 days (2.1-fold). AMI, acute myocardial infarction.

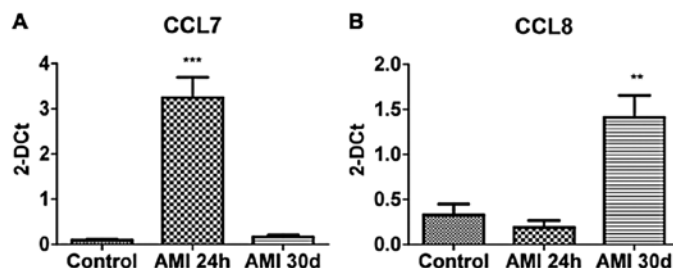


Figure 3. (A) After 24 h of cardiac ischemia, the expression of CCL7 was 36.0-fold higher than in healthy control mice ($P<0.001$). After 30 days of ischemia, the expression was 1.8-fold higher, not significantly different compared to the control. (B) After 24 h of AMI, the expression of CCL8 was similar to control samples (-1.7-fold), but after 30 days it showed a 4.3-fold significant increase ($P<0.01$). AMI, acute myocardial infarction.

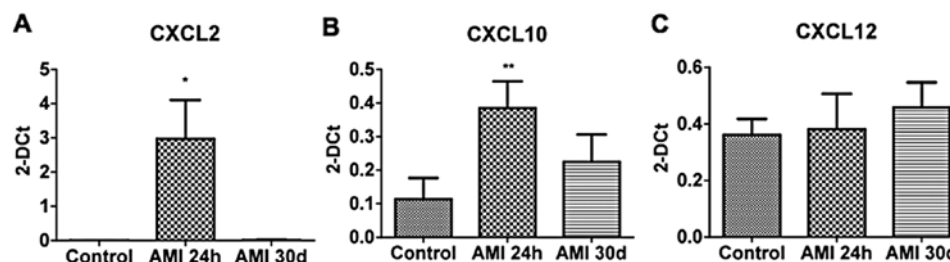


Figure 4. (A) In the acute phase of myocardial infarction, CXCL2 showed the highest elevation in expression among the genes analyzed (610.5-fold) as compared to control samples ($P<0.05$). After 30 days a non-significant increase of 3.8-fold was observed. (B) Expression of CXCL10 increased 3.4-fold after 24 h of infarction ($P<0.01$), and decreased to basal levels after 30 days (2.0-fold, not significant). (C) Expression of CXCL12 showed a non-significant increase of 1.8 and 1.5-fold after 24 h and 30 days of myocardial infarction, respectively. AMI, acute myocardial infarction.

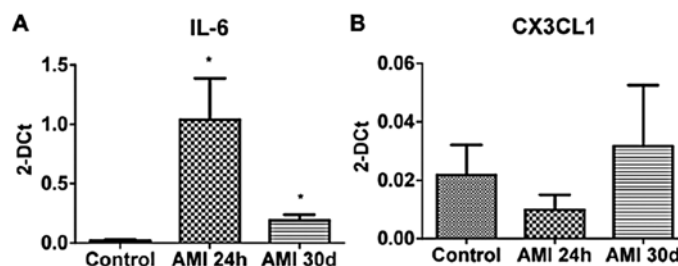


Figure 5. (A) Expression of IL-6 was increased 53.5-fold after 24 h of infarction, compared with control, $P<0.05$. After 30 days, this expression was greatly reduced, but was still significantly higher (9.8-fold) than in control samples ($P<0.05$). (B) Expression of CX3CL1 was not modified after 24 h and 30 days of myocardial infarction. AMI, acute myocardial infarction.

Discussion

In this study, we analyzed the expression of genes coding for cytokines and chemokines in the acute and chronic phases of

MI in C57BL/6 mice. Expression of 6 of the 10 genes investigated in this study (CCL2, CCL3, CCL4, CCL7, CXCL2 and CXCL10) showed significant increase in the acute phase of MI, and reverted to baseline levels after 30 days. For CCL2 and

CCL3, the increase was 19.0-fold and 7.8-fold, respectively that of control samples. Similar results have been reported by Parissis *et al* (20), who observed that plasma levels of these proteins were transiently increased in patients admitted with MI. The increased protein levels seen during the first week reverted to baseline levels in most of the patients after 14 days. Increased expression of CCL2 and CCL3 were also observed in a murine model of MI, between 2 and 7 days, and 6 h and 7 days, respectively (13). Other studies, however, have shown different results. In one, plasma levels of CCL2 were not significantly increased in MI patients (21), whereas in a second study, decreased plasma levels of CCL3 and CCL4 were observed in patients with ST-segment elevation MI followed by reperfusion, in periods of 2, 7 and 60 days (22). In terms of CCL4, whereas our results show an increased expression 24 h after infarction, in a similar study this increase was observed only 3 days later (14).

Expression of CCL7 in the acute phase of MI was 36-fold higher than in control samples. Few studies have been conducted focusing on this chemokine, which has already been shown to attract MSC in migration assays as well as induced MI models (23). Furthermore, genetic modification of MSC with the CCR1 gene (CCL7 receptor) was shown to increase the viability, recruitment and retention of those cells in the MI regenerative niche (24). Plasma levels of CCL7, however, were not increased in MI patients (25).

In 24 h samples, CXCL2 expression was 610.5-fold higher than in control samples, in agreement with a previous study which showed that expression of CXCL2 was the highest among various cytokines and chemokines investigated in a murine model of MI (13). For CXCL10, a 3.4-fold increase was observed at 24 h. Different results for the expression of this gene after MI have been reported. Husberg *et al* (14) observed a significant increase in the expression of CXCL10 only 3 days after infarction in a murine model, and a study with MI patients showed decreased plasma levels for this chemokine (22).

In the present study, chemokines CXCL12 (SDF-1) and CX3CL1 showed no increase in the expression 24 h or 30 days after MI. Controversial results have been reported on the expression of these genes in similar studies. CXCL12 is considered to play an important role in the recovery of MI (26-31), but its expression in the infarcted heart or in the plasma is not always high. In rats, CXCL12 was detected 1 day after infarction, peaking on day 7 and declining to basal levels around 14 days later (32). In mice, a slightly decreased gene expression of SDF-1, primarily located within the infarcted region, was reported (13). In humans, plasma levels of CXCL12 gradually decreased between 2, 7 and 60 days after MI (22). Although controversial, these studies do not alter the role that CXCL12 plays in the recovery of MI (26-31). In a recent study, it was observed that the overexpression of CXCR4 (SDF-1 receptor) enhanced MSC recruitment and penetration into the ischemic regenerative niche, resulting in more efficient tissue repair (33).

Expression of CX3CL1 has been shown to increase following MI. In mice, CX3CL1 was highly expressed at the gene and protein level, in experimental periods of 3, 5, 7 and 14 days after MI (14). In patients with acute coronary disease, plasma levels of CX3CL1 and its receptor CX3CR1 was increased (22,34).

Contrary to all other chemokines investigated in the present study, expression of CCL8 was not modified in the acute period of MI, but showed a significant increase 30 days later (4.3 times). Although it has already been shown to increase after 5 and 7 days of myocardial induction, returning to basal levels on day 14 (14), few studies have investigated the pattern of expression of this chemokine after MI.

Finally, the increased expression of IL-6 in the acute and chronic phases of MI when compared to healthy control is in agreement with a previous study of Deten *et al* (35), who showed the elevation of IL-6 mRNA levels in periods from 3 h to 12 weeks after induction of MI in rats. Other studies, however, have shown different kinetics for the expression of the IL-6 gene. Vandervelde *et al* (13) observed a peak expression of IL-6 6 h after coronary artery ligation, but this increase was maintained only up to 2 days. In another study, the expression of IL-6 was significantly increased only after 7 days of MI, having returned to baseline levels on day 14 (14). Interestingly, in patients with coronary artery disease the IL-6 gene does not seem to be a marker for this condition (36).

These considerations show that, despite the great number of studies aiming at the determination of the molecular profile of ischemic heart tissue, the picture is still very inconclusive. The more detailed knowledge of the regenerative niche in MI is important for the identification of sub-populations of cells with greater potential to respond to these signals and exert a more efficient therapeutic role. Alternatively, genetic modification of these cells may be used to increase their therapeutic potential (37). These studies will allow the design of more efficient cell therapy protocols for cardiac diseases.

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