Secreted Frizzled-Related Protein-1 Improves Postinfarction Scar Formation Through a Modulation of Inflammatory Response

Laurent Barandon, Frédéric Casassus, Lionel Leroux, Catherine Moreau, Cécile Allières, Jean-Marie Daniel Lamazière, Pascale Dufourcq, Thierry Couffinhal, Cécile Duplâa

Objective—The inflammatory response after myocardial infarction plays a crucial role in the healing process. Lately, there is accumulating evidence that the Wnt/Frizzled pathway may play a distinct role in inflammation. We have shown that secreted frizzled-related protein-1 (sFRP-1) overexpression reduced postinfarction scar size, and we noticed a decrease in neutrophil infiltration in the ischemic tissue. We aimed to further elucidate the role of sFRP-1 in the posts ischemic inflammatory process.

Methods and Results—We found that in vitro, sFRP-1 was able to block leukocyte activation and cytokine production. We transplanted bone marrow cells (BMCs) from transgenic mice overexpressing sFRP-1 into wild-type recipient mice and compared myocardial healing with that of mice transplanted with wild-type BMCs. These results were compared with those obtained in transgenic mice overexpressing sFRP-1 specifically in endothelial cells or in cardiomyocytes to better understand the spatiotemporal mechanism of the sFRP-1 effect. Our findings indicate that when overexpressed in the BMCs, but not in endothelial cells or cardiomyocytes, sFRP-1 was able to reduce neutrophil infiltration after ischemia, by switching the balance of pro- and antiinflammatory cytokine expression, leading to a reduction in scar formation and better cardiac hemodynamic parameters.

Conclusion—sFRP-1 impaired the loop of cytokine amplification and decreased neutrophil activation and recruitment into the scar, without altering the neutrophil properties. These data support the notion that sFRP-1 may be a novel antiinflammatory factor protecting the heart from damage after myocardial infarction. (Arterioscler Thromb Vasc Biol. 2011;31:e80-e87.)

Key Words: acute coronary syndromes ■ immune system ■ leukocytes ■ vascular biology

Leukocyte recruitment is involved in the autoimmune reaction necessary for host defense against infection, and it is also required for wound healing. In the event of coronary artery occlusion and subsequent myocardial infarction (MI), the ischemic tissue endures an inflammatory reaction, which is a prerequisite for healing and scar formation.1,2 After cardiomyocyte death, the ischemic myocardium is characterized by a neutrophil infiltration, which facilitates the elimination of dead cells, followed by a myofibroblast proliferation improving tensile strength and an angiogenic response increasing tissue perfusion and cell survival.3 Infarcted myocardial size is an important determinant of patient prognosis and depends, in part, on the complex healing processes. Modulation of the cellular and molecular mechanisms regulating the inflammatory response following MI could lead to a reduction of the deleterious scar evolution, which is the leading cause of heart failure and death.4

There is accumulating evidence that the Wnt/Frizzled (Wnt/Fzd) pathway plays a distinct role in inflammation and immunity.5,6 It has been demonstrated that the Wnt/Fzd pathway plays a crucial role in a wide range of developmental processes, from stem cell maintenance to mammalian hemopoiesis and lymphopoiesis.7–9 Studies on the role of WntD in Drosophila have elucidated a potential pivotal role of Wnt in the cascade of inflammation.10 In humans, it has been shown that Wnt5A is secreted by activated antigen-presenting cells and by inflammatory synoviocytes from rheumatoid arthritis joints, through a Toll-like receptor dependent signaling via the central inflammatory regulator nuclear factor-xB,11,12 Wnt5a in turn promotes cytokine synthesis. This finding has been recently been extended, demonstrating that Wnt5A targets its receptor Fzd5 through noncanonical signaling (increased phosphorylation of CamKII), and induces expression of the proinflammatory cytokines interleukin

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e80
(IL)-1, IL-6, and IL-8. The Wnt5A signaling pathway was shown to be specifically blocked by secreted frizzled-related protein-1 (sFRP-1), a member of the sFRP family, which acts as a soluble modulator of the Wnt signaling.\textsuperscript{13,14}

We have previously demonstrated an upregulation of sFRP-1 and distinct Wnt and Fzd member expression after MI.\textsuperscript{15} In a transgenic mouse ubiquitously overexpressing sFRP-1, we used a model of MI induced by coronary artery ligation and found that sFRP-1 significantly reduces postinfarction scar size and improves cardiac hemodynamic parameters. Besides the impact of sFRP-1 on matrix remodeling and capillary growth, sFRP-1 decreased neutrophil infiltration in the ischemic tissue.\textsuperscript{15}

On the basis of our previous data and in light of recent reports on Wnt signaling and inflammation, we aimed to further elucidate the role of sFRP-1 in the postischemic inflammatory process. In the current report, we found that in vitro, sFRP-1 was able to block leukocyte activation and cytokine production. To extend these findings in vivo, we transplanted bone marrow cells (BMCs) from transgenic mice overexpressing sFRP-1 into wild-type recipient mice and compared myocardial healing with that of mice transplanted with wild-type BMCs, in a mouse model of MI. These results were compared with those obtained in transgenic mice overexpressing sFRP-1 specifically in endothelial cells (ECs) or in cardiomyocytes to better understand the spatiotemporal mechanism of the sFRP-1 effect. Our findings indicate that when overexpressed in the bone marrow (ie, leukocytes), but not in ECs or cardiomyocytes, sFRP-1 was able to reduce neutrophil infiltration after ischemia, by switching the balance of pro- and antiinflammatory cytokine expression, leading to a reduction in scar formation and better cardiac hemodynamic parameters.

**Materials and Methods**

**In Vitro Assays**

**Cell Culture**

HL-60 (a human myeloid leukemia cell line) and human umbilical vein endothelial cells were maintained in RPMI 1640 (Sigma Chemical Co) supplemented with 10% (v/v) heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. For assays, confluent human umbilical vein ECs were switched for 24 hours from medium with serum to serum-free medium.\textsuperscript{16–18}

**Neutrophil Isolation**

Human neutrophils were isolated from whole blood obtained from normal human volunteers following a protocol described previously.\textsuperscript{19} This technique allows for rapid isolation of functionally active neutrophils with >90% purity as demonstrated by flow cytometry analysis with CD 16 staining.

**sFRP-1 Assays**

Because the recombinant bovine FrzA protein used is almost identical to its homologous murine sFRP-1 protein (98% identity), in this report we refer to it as recombinant sFRP-1 protein (rsFRP-1). In all experiments, cells were activated with 10 nmol/L rsFRP-1 protein.\textsuperscript{20}

**Cytokine Expression and Polymerase Chain Reaction Analysis**

Total RNA was prepared from HL-60 or human neutrophils in guanidinium thiocyanate buffer, and reverse transcription-polymerase chain reaction was performed as previously described. Negative controls without reverse transcription were prepared in parallel for each RNA sample. Semiquantitative polymerase chain reaction for IL-1, IL-6, IL-8, IL-10, tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1, and β-actin was performed as previously described.\textsuperscript{21} All experiments were done in triplicate.

**Inflammatory Cell Proliferation and Apoptosis**

HL-60 and human neutrophils were incubated for 48 hours in the absence or presence of rsFRP-1. Cell proliferation was performed manually using a hemocytometer determining cell density. For apoptosis, inflammatory cells were incubated with TNF-α for 20 hours in the absence or presence of rsFRP-1. Cells were labeled with annexin V–fluorescein isothiocyanate (Dako) and CD 16– annexin V – by flow cytometry analysis (fluorescence-activated cell sorting analyzer ODAM-ATC 3000). Each experiment was performed in triplicate.

**Neutrophil F-Actin Polarization**

HL-60 and human neutrophils were activated with TNF-α in the absence or presence of rsFRP-1. The cells were analyzed by flow cytometry for CD18, CD11a, and CD11b expression using fluorescein isothiocyanate–conjugated antibodies (BD Pharmingen).\textsuperscript{23}

**BMC Transplantation**

This study was conducted in accordance with both institutional and European Community guidelines for experimental animal use (L358–86/609/EEC). To overexpress sFRP-1 specifically in leukocytes, we transplanted wild-type mice with bone marrow cells overexpressing sFRP-1. We have previously demonstrated that in this model, sFRP-1 transgene is expressed in bone marrow cells (BMCs) and circulatory mononuclear cells (line CMV/sFRP-1).\textsuperscript{15} To further examine the engraftment of BMCs, we backcrossed the CMV/sFRP-1 or CMV/littermates (CMV/Litt) onto ROSA 26 mice (Jackson Laboratory) ubiquitously expressing β-galactosidase gene reporter,\textsuperscript{24} generating ROSA-CMV/sFRP-1 mice or ROSA-CMV/Litt (≥6 generations), which were used as BMC donors. As analyzed by flow cytometry using 18-fluorodeoxyglucose staining, chimerism ranged from 45% to 85%, similar to results from previous hematopoietic studies using this model of BMC transplantation.\textsuperscript{25} These mice are referred as BMC ROSA-CMV/sFRP-1 or BMC ROSA-CMV/Litt and were subjected to MI by ligation 2 months after irradiation as previously described.\textsuperscript{26}

**Transgenic Mouse Lines**

Transgenic mice were previously constructed using the FrzA sequence (the bovine homolog of murine sFRP-1 gene). To simplify the text, we refer to FrzA as sFRP-1.

Two different transgenic mouse lines, previously described, were used in this study. In the first one, the sFRP-1 transgene was specifically overexpressed in ECs under a Tie-2 promoter (line Tie2-tTA/TRE-sFRP-1).\textsuperscript{24} In the second one, the sFRP-1 transgene was specifically overexpressed in cardiomyocytes under α-myosin heavy chain (MHC) promoter (line α-MHC-tTA/TRE-sFRP-1).\textsuperscript{21} Transgenic mice overexpressing sFRP-1 in ECs and their corresponding littermates are called Tie-2/sFRP-1 and Tie-2/Litt mice. Transgenic mice overexpressing sFRP-1 in cardiomyocytes and their
corresponding littermates are called MHC/sFRP-1 and MHC/Litt mice.

In Vivo Models
The mouse model of MI, hemodynamic studies, necropsy examination, and data analysis were based on that previously described15 (see supplemental material, available online at http://atvb.ahajournals.org).

Statistical Analysis
Results are expressed as mean±SD. Comparisons of continuous variables between 2 groups were performed by a 1-way ANOVA and subsequently, if statistical significance was observed, by a 2-sided paired *t* test (Statview 5-1, Abacus). A value of *P*<0.05 was considered significant.

Results
Based on our previous data that mice overexpressing sFRP-1 have a decrease in neutrophil infiltration in ischemic tissue during the first week after MI, we aimed to study the impact of sFRP-1 on neutrophil properties in vitro, as well as the subsequent in vivo consequence of sFRP-1 overexpression in leukocytes, on the modulation of the inflammatory response after MI.

rsFRP-1 Does Not Impair Neutrophil Proliferation or Apoptosis
As shown in Figure 1A, HL-60 granulocyte cell line proliferation was not affected by rsFRP-1. rsFRP-1 did not induce apoptosis as demonstrated by annexin V staining in flow cytometry analysis (Figure 1B, *P*=not significant).

rsFRP-1 Impeded Neutrophil Activation
IL-1β, TNF-α, and IL-8 mRNA expressions were all significantly upregulated after TNF-α stimulation, in freshly iso-

![Figure 1. A, Cell proliferation: effect of recombinant secreted frizzled-related protein-1 (rsFRP-1) on proliferation of HL-60 (a human myeloid leukemic cell line). B, Apoptosis: flow cytometry analysis with annexin V and CD-16 labeling under control conditions and with rsFRP-1 adjunction. C, Neutrophil polarization: neutrophil F-actin polarization stained with phalloidin-rhodamine after formyl-methionyl-leucyl-phenylalanine (fMLP) incubation in the absence (control) or presence of rsFRP-1. D, Cytokine expression: relative levels of different cytokine transcripts of freshly isolated human neutrophils with or without tumor necrosis factor (TNF) induction. Each experiment was performed in triplicate (*P*<0.001).](http://atvb.ahajournals.org/)

![Figure 1. A, Cell proliferation: effect of recombinant secreted frizzled-related protein-1 (rsFRP-1) on proliferation of HL-60 (a human myeloid leukemic cell line). B, Apoptosis: flow cytometry analysis with annexin V and CD-16 labeling under control conditions and with rsFRP-1 adjunction. C, Neutrophil polarization: neutrophil F-actin polarization stained with phalloidin-rhodamine after formyl-methionyl-leucyl-phenylalanine (fMLP) incubation in the absence (control) or presence of rsFRP-1. D, Cytokine expression: relative levels of different cytokine transcripts of freshly isolated human neutrophils with or without tumor necrosis factor (TNF) induction. Each experiment was performed in triplicate (*P*<0.001).](http://atvb.ahajournals.org/)
lated human neutrophils. As demonstrated in Figure 1D, preincubation of human neutrophils with rsFRP-1 dramatically reduced TNF-α/H9251-induced TNF-α/H9251 and IL-8 mRNA up-regulation \((P < 0.001)\). In contrast, rsFRP-1 did not modify TNF-α/H9251-induced IL-1\(\beta\)/H9252 expression.

**In Vivo Overexpression of sFRP-1 in BMCs Reduced Neutrophil Infiltration in the Scar and Inversed the Balance of Pro- and Anti-Inflammatory Cytokine Expression**

The in vivo effects of sFRP-1 on MI were studied on C57Bl6/J mice transplanted with sFRP-1 overexpressing BMCs (ROSA/sFRP-1 BMCs). As a control, BMCs from littermates were also transplanted in C57Bl6/J mice (ROSA/Litt BMCs). Neutrophil infiltration, evaluated by myeloperoxidase immunostaining, was dramatically decreased in the scar 2 and 7 days after infarction in ROSA/sFRP-1 BMC transplanted mice, compared with the ROSA/Litt BMC transplanted control mice \((P < 0.001)\) (Figure 2A). No difference in macrophage and T lymphocyte infiltration was observed between ROSA/sFRP-1 BMC transplanted mice and ROSA/Litt BMC transplanted control mice during the first 2 weeks. To assess whether leukocyte infiltration reduction was due to
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mice (H11021P0.01) (Figure 2B). TNF-α-with mRNA levels obtained in ROSA/Litt BMC transplanted antiinflammatory cytokine IL-10 mRNA (at day 7) compared and, by contrast, a significant increased expression of the proinflammatory cytokine IL-6 mRNA expression (day 4) area 4 days after infarction. We observed that ROSA/sFRP-1 protein-1, IL-6, and IL-10, largely detected in the ischemic and their corresponding control littermates (Figure 2B). Conversely, no difference in cytokine expression sFRP-1 mice compared with their corresponding control mice or in neutrophil infiltration was observed in Tie2/sFRP-1 transgenic mice (Figure 3A). No difference in chemotactic protein-1 expression was not impaired in ROSA/sFRP-1 or BMC/sFRP-1 and in their corresponding control littermates. It should be noted that leukocyte counts in the blood and bone marrow were similar 4 days after ligation in each group of mice (not shown).

We then investigated whether sFRP-1 overexpression in BMCs might affect cytokine production during the MI repair process. It is widely accepted that in this model, MI results in inflammatory cell infiltration in transgenic mice overexpressing sFRP-1 exclusively in ECs under Tie2 promoter control (Tie2/sFRP-1 transgenic mice) or in cardiomyocytes under α-MHC promoter control (α-MHC/sFRP-1 transgenic mice). Note that the sFRP-1 transgene was not expressed in bone marrow mononuclear cells or in circulating white blood cells in Tie2/sFRP-1 transgenic mice (Figure 3A). No difference in neutrophil infiltration was observed in Tie2/sFRP-1 transgenic mice or in α-MHC/sFRP-1 transgenic mice, as compared with their respective littermates, 2 and 7 days after infarction. Endothelial or cardiomyocyte sFRP-1 overexpression did not alter macrophage or lymphocyte infiltration.

Exogenous sFRP-1 expression in leukocytes, we evaluated inflammatory cell infiltration in transgenic mice overexpressing sFRP-1 in ECs with mRNA levels obtained in ROSA/Litt BMC transplanted mice (Figure 2B). TNF-α, IL-1β, monocyte chemotactic protein-1, IL-6, and IL-10, largely detected in the ischemic area 4 days after infarction. We observed that ROSA/sFRP-1 BMC transplantation led to a significant reduction of the proinflammatory cytokine IL-6 mRNA expression (day 4) and, by contrast, a significant increased expression of the antiinflammatory cytokine IL-10 mRNA (at day 7) compared with mRNA levels obtained in ROSA/Litt BMC transplanted mice (P<0.01) (Figure 2B). TNF-α, IL-1β, and monocyte chemotactic protein-1 expression was not impaired in ROSA/sFRP-1 mice compared with their corresponding control littermates. Conversely, no difference in cytokine expression was observed between Tie2/sFRP-1 or HCC/sFRP-1 mice and their corresponding control littermates (Figure 2B).

Overexpression of sFRP-1 in BMCs Increased Cell Density but Not Capillary Density in the Scar

The overall cell density and index of proliferation in the scar were increased in mice grafted with BMCs overexpressing sFRP-1 (ROSA/sFRP-1) and in mice overexpressing sFRP-1 in ECs (Tie2/sFRP-1), compared with their corresponding control littermates 15 days after MI (Figure 2C, P<0.01). At this same time point, the infarcted area of Tie2/sFRP-1 mice displayed more capillary density compared with Tie2/Litt mice (Figure 2C, *P<0.01). No statistical differences were observed in capillary density between ROSA/sFRP-1 or MHC/sFRP-1 and in their corresponding control littermates.

Overexpression of sFRP-1 in BMCs Reduces Cardiac Rupture and Scar Size and Improves Hemodynamic Parameters

To evaluate the effect of sFRP-1 on postinfarction scar size, we compared myocardial evolution in 3 mouse lines overexpressing sFRP-1 (Tie-2, MHC, and BMC ROSA) to that in their corresponding littermates.

After MI, there was no difference in early mortality (within 24 hours after surgery) between the 3 groups. However, the incidence of fatal cardiac rupture of the left ventricular wall, confirmed by necropsy, was statistically significantly (P<0.01) lower in BMC ROSA/sFRP-1 mice (10.5%) compared with their corresponding control littermates (26.3%), as well as the other transgenic mouse lines and their corresponding control littermates (range 25% to 26.6%) (Table 1).

Fifteen days after MI, the percentage of left ventricular scar size was statistically significantly decreased in BMC ROSA/sFRP-1 mice compared with that in all other groups (n=6 in each group, P<0.001; Figure 3A and 3B). Evolutions in scar and septal thickness are reported in Table 1.

Changes in scar size and thickness in BMC ROSA/sFRP-1 mice were correlated with improved hemodynamic parameters as shown in Figure 4 and Table 2. Differences in maximum and minimum dp/dt were amplified in BMC ROSA/sFRP-1 after dobutamine infusion compared with their corresponding control littermates (Figure 4 and Table 2). When overexpressed in ECs (Tie2/sFRP-1), sFRP-1 improved hemodynamic parameters compared with their corresponding control littermates (Figure 4), but not when sFRP-1 was overexpressed in cardiomyocytes.

In summary, overexpression of sFRP-1 in BMCs reduced neutrophil infiltration, reduced scar size, and improved hemodynamic parameters, but it did not modify angiogenesis. In contrast, overexpression of sFRP-1 in ECs and cardiomyocytes did not reduce postinfarct scar size or leukocyte infiltration. When overexpressed in ECs (Tie2/sFRP-1),...
sFRP-1 had an angiogenic effect, which correlated with an improvement of hemodynamic parameters. sFRP-1 overexpression in cardiomyocyte had no effect on hemodynamic parameters.

Discussion

Ischemic heart disease remains the leading cause of death in Western countries. Improving our knowledge and understanding of the healing process after MI could potentially lead to new therapeutic targets to avoid heart failure after MI. To our knowledge, this is the first report of an in vivo study demonstrating that sFRP-1, a modulator of Wnt/Fzd pathway, can regulate the postischemic myocardial inflammatory process. We demonstrate that this regulation was effective in an autocrine manner, when sFRP-1 was expressed by leukocytes (ie, bone marrow cells), and that neutrophils were the specific Wnt/Fzd target in this model. These experiments provide evidence of how regulation of the inflammatory process by sFRP-1 could be beneficial for myocardial healing, through an impairment of the cytokine amplification loop.

Because of the lethal effect of most Wnt/Fzd deletions, we used a novel approach to further elucidate the role of this pathway through a Wnt/Fzd modulator, sFRP-1. The sFRP proteins are able to bind either the Wnt ligands or the Fzd receptors (Fzd4, Fzd5, and Fzd7). In previous studies, we have shown that in vitro, sFRP-1 protects ECs from apoptosis and favors EC migration and differentiation, in a glycogen synthase kinase 3 (GSK3)/H and Rac1-dependent manner. In vitro, sFRP-1 expression has been previously demonstrated to lead to robust vessel formation in different angiogenic models (eg, tumoral, plug, chorioallantoic membrane assays). Similarly, in vivo, EC-specific overexpression of sFRP-1 in transgenic mice leads to increased muscle neovascularization in ischemia-induced angiogenesis. Because of these vascular properties, we investigated the role sFRP-1 in the healing process after MI. In a transgenic mouse ubiquitously overexpressing sFRP-1, it has been demonstrated that sFRP-1 plays an important role, increasing capillary density, reducing scar size, and improving hemodynamic parameters. Studying this model, it was also noted that there was a significant reduction in leukocyte infiltration in the scar of sFRP-1 transgenic mice. Further characterization of these transgenic mice established that sFRP-1 was also overexpressed in the BMCs and circulating leukocytes.

sFRP-1 Effects In Vitro on Neutrophils

Since the first report of upregulated expression of cytokines such as interleukins IL-6, IL-8, and IL-15 by Wnt5a in rheumatoid arthritis synovial fibroblasts, there has been a surge of interest in Wnt signaling in the context of inflammation in pathogenic disorders. It has been reported that Wnt5a-Fz5 signaling promotes IL-12 synthesis and...

Figure 4. Effect of different secreted frizzled-related protein-1 (sFRP-1) overexpression levels on hemodynamic parameters after myocardial infarction. Maximum and minimum dP/dt without or with dobutamine infusion were recorded with the Millar's probe at 15 days after MI (n=6, P<0.001).

Table 2. Effect of Different sFRP-1 Overexpression Levels on Hemodynamic Parameters After MI

<table>
<thead>
<tr>
<th>Tie-2 Line</th>
<th>MHC Line</th>
<th>BMC Transplantation</th>
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<tr>
<td>Littermate</td>
<td>sFRP-1</td>
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MI indicates myocardial infarction; sFRP-1, secreted frizzled-related protein-1; MHC, myosin heavy chain. *P<0.05 compared with littersmates.
enhances the inflammation induced by microbial stimulation in macrophages.\textsuperscript{12} Recently, Pereira et al, using an in vitro model of inflammatory macrophage activation, reported that macrophages stimulated with interferon-γ and lipopolysaccharide consistently upregulate Wnt5a via Toll-like receptor activation. Wnt5a in turn upregulates expression of proinflammatory genes IL-6, IL-1β, and IL-8.\textsuperscript{13,14} Wnt5a, by increasing phosphorylation of CamKII, is capable of activating the proinflammatory Ca2+/CamKII pathways in macrophages. This effect was completely abrogated by sFRP-1 and inhibited the lipopolysaccharide/interferon-γ-induced CamKII phosphorylation.\textsuperscript{13} Others reported that DKK1 (a Wnt blocker) was sufficient to block the overexpression of proinflammatory factors in retinal inflammation.\textsuperscript{59}

To verify that the Wnt/Fzd pathway alters the inflammatory process, we attempted to evaluate in vitro how sFRP-1 could modulate inflammatory cell activation. sFRP-1 affected leukocyte proliferation but did not play a role in leukocyte apoptosis, chemotaxis, polarization, or integrin expression. However, sFRP-1 significantly impaired leukocyte activation in response to triggers. In response to TNF-α, sFRP-1 significantly reduced in vitro TNF-α and IL-8 expression, which are cytokines known to contribute to the proinflammatory response.\textsuperscript{30–34} Our results corroborated the work of Pereira et al,\textsuperscript{13,14} demonstrating the potential effect of sFRP-1 in modulating the inflammatory response.

sFRP-1 Effects In Vivo

Previous reports on Wnt signaling in the context of inflammation studied in vitro mechanisms of leukocyte activation by Wnt and occasionally confirmed with ex vivo proof-of-concept, in the context of pathogenic disorders. In the current report, we specifically studied myocardial healing using a mouse model of MI, to further elucidate the in vivo role of sFRP-1 and Wnt/Fzd in the inflammatory process.

The inflammatory response after MI is essential in the healing process.\textsuperscript{1,2} It contributes to the scar remodeling and ventricular shaping. Consequences of changes in the inflammatory response could be beneficial, leading to granulation tissue and restoring hemodynamic parameters, or harmful, leading to left ventricle dilatation and cardiac rupture.\textsuperscript{1,2,35,36} Neutrophils are activated in the bone marrow by cytokines (IL-6, IL-8, C5a, and TNF-α) and secreted by ischemic tissue. After mobilization via the blood flow, they adhere on the endothelial layer and transmigrate into the infarct area. This is the earliest cell adhesion event following tissue insult.\textsuperscript{37–40} Activated neutrophils participate in the cleaning of the scar, but they can also in some cases be deleterious, increasing superoxide production, free radicals, and proinflammatory cytokines.\textsuperscript{41}

Using bone marrow transplantation, we overexpressed sFRP-1 specifically in leukocytes and compared its effect on the inflammatory processes, to that in overexpression in ECs or in cardiomyocytes. Specific sFRP-1 overexpression in BMCs altered the inflammatory response after MI. Neutrophil infiltration in the scar was significantly reduced within 48 hours after ischemia and lasted up to 7 days, but there was no reduction in macrophage or T lymphocyte infiltration. sFRP-1 significantly reduces IL-6 expression, a major cytokine in the proinflammatory response, and sFRP-1 favored IL-10, a potent antiinflammatory cytokine.\textsuperscript{30–34} This effect was correlated with a specific reduction of post-MI cardiac rupture rate (with the caution that a limited number of mice were analyzed) and scar size and with an improvement in hemodynamic parameters.

Resolution of postinfarction inflammation is likely to involve multiple overlapping regulatory mechanisms controlling various proinflammatory pathways activated in the infarcted myocardium. In our study, in vivo, sFRP-1 not only increased IL-10 but also decreased IL-6 mRNA in a significant, early manner in mouse MI, and there was a trend toward a decrease in TNF-α and IL-1β. In vitro, rsFRP-1 dramatically reduced TNF-α and IL-8 mRNA upregulation specifically in neutrophils. So there are multiple lines of evidence that sFRP-1’s effect is mediated not only by IL-10 (or another unique cytokine) but also by an impairment of the balance of pro- and antiinflammatory cytokines. Moreover, the role of IL-10 in resolution of postinfarction inflammation remains controversial.\textsuperscript{42,43} As another example, Burchfield et al demonstrated that intramyocardial injection of bone marrow mononuclear cells mediates cardiac protection after MI and that this is, at least in part, dependent on IL-10. However, the IL-10-dependent improvement provided by transplanted cells was not caused by reduced infarct size, neutrophil infiltration, or capillary density.\textsuperscript{44}

With this approach, we also confirmed the angiogenic effect of sFRP-1 when overexpressed specifically in ECs and its impact on left ventricle function. However, sFRP-1 EC overexpression had no effect on leukocyte recruitment or cytokine expression. When overexpressed specifically in cardiomyocyte, sFRP-1 did not offer any advantage to myocardial healing. We speculate that this lack of effect may be due to the death of cardiomyocytes occurring during MI. The systematic use of corresponding littermates provided a reliable control for the sFRP-1 transgene effect.

Conclusions

In vivo modulation of the inflammatory response is difficult to monitor, and results of therapeutic alteration of the inflammatory response have often been controversial. Here, we demonstrated that sFRP-1, by switching the balance between proinflammatory and antiinflammatory cytokines, impaired the loop of amplification and decreased neutrophil activation and recruitment into the scar, without altering the neutrophil properties. These data support the notion that sFRP-1 may be a novel antiinflammatory factor protecting the heart from damage after MI.

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Disclosures

None.

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19. Barandon et al sFRP-1 and Inflammatory Response in MI e87


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Supplement Material

Materiel and Methods

In vitro Assays

Cell culture: U-937 (human leukemic monocyte lymphoma cell line), EL-4 (murine T-lymphoma cell line), HL-60 (human myeloid leukemic cells) and HUVEC (Human Umbilical Vein Endothelial Cells) were maintained in RPMI 1640 (Sigma Chemical Co.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco) and 100 U/mL–1 penicillin/100 gmL–1 streptomycin (Sigma–Aldrich) in a humidified atmosphere of 5% CO2 at 37°C. For assays with HUVEC, once cells had reached confluence, they were switched for 24 hours to medium with serum or serum-free medium containing BSA (1%), insulin (1 µmol/L), transferrin (200 µg/mL), ascorbate (0.2 mmol/L), and sodium selenite (6.25 ng/mL) (all from Sigma). 1-3

Neutrophil isolation: Human neutrophils were isolated from whole blood obtained from normal human volunteers following a protocol described previously 4. Briefly, neutrophils were separated from other blood cells by dextran sedimentation (2% dextran in 0.9% NaCl) and ficoll-percoll (Pharmacia) density centrifugation followed by hypertonic lysis of residual red blood cells. Purified neutrophil were then washed once and resuspended in HBSS devoid of Ca2+ and Mg2+ and used within 4 h. This technique allows for rapid isolation of functionally active neutrophils with > 90% purity as demonstrated by FACS analysis with CD 16 staining.

sFRP-1 adjunction assays: Because the recombinant bovine FrzA protein used is almost identical to its homologous murine sFRP-1 protein (98% identity), in this report we refer to it as recombinant sFRP-1 protein (rsFRP-1). In all experiments, cells were activated with 10 nmol/L estimated rsFRP-1 protein 5.

Cytokines expression and PCR analysis: Total RNA was prepared from HL 60 or human neutrophils in guanidinium thiocyanate buffer, and RT-PCR was performed as previously described6. Negative controls without RT were prepared in parallel for each RNA sample. Semi-quantitative PCR was performed as previously described7.
For quantitative analysis of cytokines expression *in vivo*, 500 ng of total RNA from mouse infarcted tissues were reverse-transcribed; PCR was done using IQ SYBR Green supermix (Bio-Rad). An MJ Research Opticon and the following parameters were used for real-time PCR: 95°C for 5 minutes followed by 35 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 15 seconds. Negative controls without RT were prepared in parallel for each RNA sample. All experiments were done in triplicate. The following sense and antisense primers were used, respectively: 

**IL-1**, 5'- AGA CCA ACC AGT GCT GCT GAA G -3' and 5'- CTT AGT GCC GTG AGT TTC CCA G -3', **IL-6** 5'- AAC AAG TTC GGC TTC CAA TGG - 3' and 5'- CGT TTT GCC CGT CTC TGG AT -3', **IL-8**, 5'- AAG AGC CAG GAA GAA ACC ACC G - 3' and 5'- ACC CTAA CAA CAG ACC ACC ACA ATA C -3', **IL-10**, 5'- TGG GGT TTC TCT CTT AGC C-3' and 5'- GCT TTG CTT TGG TCA TCC ACC-3', **TNF-α**, 5'- AAG AGC CAG GAA GAA ACC ACC G - 3' and 5'- ACG CTC GGT GCC CAC CAC AAT -3', **MCP-1** 5'- CAG GTC TCT GTC ACG CT TCT-3' and 5'- AGT ATT CAT GGA AGG GAA TAG-3' and **β-Actin** 5'- GGA GGA GCT GGA AGC AGC C -3' and 5'- GCT GTG CTA CGT CGC CCT G -3'.

**Inflammatory cell proliferation and apoptosis:** U 937, HL 60 and human neutrophils were incubated in medium culture for 48 H in the absence or presence rsFRP-1. Cell proliferation was performed manually using a hemocytometer determining cell density. For apoptosis, inflammatory cells were incubated with TNF-α for 20hrs in the absence or presence rsFRP-1. Cells were labelled with Annexin V-FITC (Dako) and CD 16-PC 5 fluorochrom (Immunotech). The rate of apoptosis was determined by the ratio with apoptotic cells (CD 16- Annexin V +) and viable cells (CD 16 + Annexin V -) by FACS analysis (FACS analyzer ODAM-ATC 3000). Each experiment was performed in triplicate.

**Neutrophil @-2 integrin expression:** HL 60 and human neutrophils were activated with TNF-α in the absence or presence rsFRP-1. The cells were analyzed on FACS (FACS analyzer ODAM-ATC 3000) for CD18, CD11a, and CD11b expression using fluorescein isothiocyanate (FITC)-conjugated antibodies (BD Pharmingen).

**Neutrophil F-actin polarization, qualitative (confocal microscopy) and quantitative (FACS) assay:** For qualitative analysis, cell polarization was determined by F-actin staining with phalloidin-rhodamine
(Molecular Probes). HL60 and Human neutrophil cells were plated on fibronectin-coated slides, and after incubation (1 h, 37°C) were stimulated with fMLP (100 nM, 3 min, 37°C) in the absence or presence of rsFRP-1. After washing, plates were fixed with 2% paraformaldehyde (10 min, 20°C) in PBS, permeabilized with 0.02% Triton X-100 (2 min, 20°C), and incubated (1 h, 25°C) with phalloidin-rhodamine. Fluorescence was examined with a confocal microscope (Nikon PCM 2000) as described\textsuperscript{10}. For quantitative analysis, F-actin polymerization was evaluated by FACS analysis (FACS analyzer ODAM-ATC 3000) using Phalloidine-FITC staining (1/100 Sigma) as described previously\textsuperscript{10}.

**Bone Marrow Cells Transplantation**

This study was conducted in accordance with both institutional and European Community guidelines for experimental animal use (L358–86/609/EEC). In order to over-express sFRP-1 specifically in leukocytes, we transplanted wild type mice with bone marrow cells over expressing sFRP-1. We have previously demonstrated that in this model, sFRP-1 transgene is expressed in bone marrow cells (BMC) and circulatory mononuclear cells (Line CMV/sFRP-1)\textsuperscript{11}. To further examine the engraftment of BMC, we backcrossed the CMV/sFRP-1 or CMV/Litt onto ROSA 26 mice (Jackson Laboratory, USA) ubiquitously expressing beta-galactosidase (β-Gal) gene reporter\textsuperscript{12}, generating ROSA-CMV/sFRP-1 mice or ROSA-CMV/Litt (>6 generations), which were used as BMC donors.

BMC of donor mice (ROSA-CMV/sFRP-1 and control ROSA-CMV/Litt mice) were extracted from femur bones and purified in ficoll paque\textsuperscript{8}. Recipient C57BL/6J mice (RM) were lethally irradiated (9 grays), and transplanted 24 hours later with $5 \times 10^5$ ROSA-CMV/sFRP-1 or ROSA-CMV/Litt BMC, by sub-claviar venous injection under sterile conditions, to create chimeric mice. Engraftment (chimerism) in RM was determined by peripheral blood and BMC analysis using 18-fluorodeoxyglucose staining followed by fluorescence-activated cell sorter analysis as previously described\textsuperscript{13}. Several organs, such as the heart, lungs, spleen and liver, were harvested for immunostaining with β-Gal antibody. Chimera ranged from 45% to 85%, similar to results from previous hematopoietic studies using this model of
BMC transplantation. These mice were referred as BMC ROSA-CMV/sFRP-1 or BMC ROSA-CMV/Litt and were subjected to MI by ligation two months after irradiation as previously described\textsuperscript{13}.

**Transgenic mouse lines**

Transgenic mice were previously constructed using FrzA sequence (bovine homolog of murine sFRP-1 gene). To simplify the reading of the text, we will refer to FrzA as sFRP-1.

Two different transgenic mouse lines, previously described, were used in this study. In the first one, sFRP-1 transgene was specifically over-expressed in endothelial cells under a Tie-2 promoter (Line Tie2-tTA/TRE-sFRP-1)\textsuperscript{10}. In the second transgenic mouse line, sFRP-1 transgene was specifically over-expressed in cardiomyocytes under α-MHC promoter (Line α-MHC-tTA/TRE-sFRP-1)\textsuperscript{7}.

Characterization of these mouse lines has been previously reported. All mice were viable and did not appear to have any organic abnormalities. Transgenic mice over-expressing sFRP-1 in endothelial cells and their corresponding littermates were called Tie-2/sFRP-1 and Tie-2/Litt mice, transgenic mice over-expressing sFRP-1 in cardiomyocytes and their corresponding littermates were called MHC/sFRP-1 and MHC/Litt mice.

**In vivo Models**

**Myocardial Infarction Model:** The mouse model of myocardial infarction (MI) was based on that previously described\textsuperscript{11}. After a left thoracotomy, a 7/0 polypropylene suture was placed around the left anterior descending coronary artery near the origin of the pulmonary artery. MI was attested by the blanching of the apex, by the dyskinetic function of the free wall ventricle and by the ST segment elevation of the ECG.

**Hemodynamics Studies:** Mice were anesthetized with a solution of tribromoethanol (12 μL/g of 2.5% solution) fifteen days after MI. The right carotid artery was cannulated by a 1.4 Fr Millar microtipped transducer (Millar). Aortic blood pressure was recorded, and the catheter was pushed into the left ventricle: hemodynamic parameters including dp/dt min and dp/dt max with or without dobutamine
infusion (60µg, intra-peritoneally) were recorded in a closed chest preparation as previously described\textsuperscript{11}.

**Necropsy examination:** For morphometric studies, the heart was arrested in diastole by KCL injection and was fixed during 20 min under pressure (diastolic arterial pressure) in 4% paraformaldehyde (PFA). The heart was excised and stored for 3 hours in 4% paraformaldehyde at 4°C, then cut in three sections (base, middle and apex), embedded in paraffin and each section was sliced. Slides were stained with Masson’s trichrome to visualize the area of necrosis. The images of each section were captured and the percentage of infarcted LV was then calculated as the ratio of scar area to scar plus non-infarcted LV area (planimetry software). The result is the mean of the 3 sections\textsuperscript{11}. For immunochemistry procedures, hearts were arrested in diastole and fixed in 100% methanol before paraffin embedding. Immunohistochemistry was performed as previously described\textsuperscript{11}. The following antibodies were used to detect myeloperoxydases (Myeloperoxydases, Dako), T lymphocytes (CD-3, Serotec), macrophages (F4/80, Caltag), endothelial cells (CD-31, Pharmingen).

**Data analysis:** Percentage of infarcted LV was calculated as the ratio infarcted area / infarcted + non-infarcted LV area. For inflammatory cells count, a minimum of 30 randomized pictures was recorded at 40X magnification for each animal (n ≥ 5) in the scar area with a camera connected to a PC. Positive cells were manually counted on captured pictures with the help of Sigma Plot software. Leukocytes infiltration and capillarity density were reported as the number of positive cells per mm\textsuperscript{2}.

**Statistical analysis**

Results were expressed as mean ± SD. Comparisons of continuous variables between 2 groups were performed by a one-way ANOVA and subsequently, if statistical significance was observed by a two-sided paired t test (Statview 5-1, Abacus). A value of p <0.05 was considered significant.


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