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Decreased pool of mesenchymal stem cells is associated with altered chemokines serum levels in atrophic nonunion fractures

Myrielle Mathieu, DVM, PhD\textsuperscript{a} - Sabrina Rigutto PhD\textsuperscript{a}, Aude Ingels, MSc\textsuperscript{a}, Delphine Spruyt, BIR\textsuperscript{a}, Nadia Stricwant, MSc\textsuperscript{a}, Ilham Kharroubi, PhD\textsuperscript{a}, Valentina Albarani, PhD\textsuperscript{b}, Marc Jayankura, MD\textsuperscript{c}, Joanne Rasschaert, PhD\textsuperscript{a}, Enrico Bastianelli, MD\textsuperscript{b}, Valérie Gangji, MD, PhD\textsuperscript{a,d}

M. Mathieu and S. Rigutto contributed equally to this work.

\textsuperscript{a} Laboratory of Bone and Metabolic Biochemistry, Université Libre de Bruxelles, 808 Route de Lennik, 1070 Brussels, Belgium.

e-mail: myrielle.mathieu@ulb.ac.be, Sabrina.Rigutto@ulb.ac.be, aude.ingels@live.be, dspruyt@ulb.ac.be, nadia.stricwant@ulb.ac.be, iokharrou@ulb.ac.be, jrasscha@ulb.ac.be

\textsuperscript{b} Bone Therapeutics SA, 8 Rue Adrienne Bolland, 6041 Gosselies, Belgium

e-mail: valentina.albarani@bonetherapeutics.com, enrico.bastianelli@bonetherapeutics.com

\textsuperscript{c} Department of Orthopedics and Traumatology, Erasme Hospital, Université Libre de Bruxelles, 808 Route de Lennik, 1070 Brussels, Belgium

\textsuperscript{d} Department of Rheumatology and Physical Medicine, Erasme Hospital, Université Libre de Bruxelles, 808 Route de Lennik, 1070 Brussels, Belgium

e-mail: Valerie.Gangji@erasme.ulb.ac.be

CORRESPONDING AUTHOR

Myrielle Mathieu
Laboratoire de Biochimie Osseuse et Métabolique, Université Libre de Bruxelles, 808 Route de Lennik, 1070 Brussels, Belgium

E-mail: myrielle.mathieu@ulb.ac.be

Phone: +3225556208

Fax: +3225556230
ABSTRACT

Nonunion fractures can cause severe dysfunction and are often difficult to treat mainly due to a poor understanding of their physiopathology. Although many aspects of impaired fracture healing have been extensively studied, little is known about the cellular and molecular mechanisms leading to atrophic nonunion. Therefore, the aim of the present study was to assess the pools and biological functions of bone marrow-derived mesenchymal stem cells (hMSCs) and circulating endothelial progenitor cells (EPCs) in atrophic nonunion patients compared to healthy subjects, and the systemic levels of growth factors involved in the recruitment, proliferation and differentiation of these cells. In nonunions, the pool of hMSCs was decreased and their proliferation delayed. However, once committed, hMSCs from nonunions were able to proliferate, differentiate into osteoblastic cells and mineralize in vitro as efficiently as hMSCs from healthy subjects. In parallel, we found altered serum levels of chemokines and growth factors involved in the chemotaxis and proliferation of hMSCs such as leptin, interleukin-6 (IL-6) and its soluble receptor, platelet-derived growth factor-BB (PDGF-BB), stem cell factor (SCF) and insulin-like growth factor-1 (IGF-1). Moreover, we showed that the number of EPCs and their regulating growth factors were not affected in nonunion patients. If nonunion is generally attributed to a vascular defect, our results also support a role for a systemic mesenchymal and osteogenic cell pool defect that might be related to alterations in systemic levels of factors implicated in their chemotaxis and proliferation.

KEYWORDS:
Bone healing; nonunion; mesenchymal stem cells; endothelial progenitor cells; chemokines;
1 INTRODUCTION

Atrophic nonunions are defined as fractures that have not made any progression toward healing six months after the fracture-related injury [1,2]. Although the majority of the fractures unite successfully, 5 to 10% result in nonunions [3]. Some risk factors will predispose to the development of a nonunion such as type and site of fracture, presence of a chronic illness, bone loss, fracture comminution with bone and soft tissue devascularization, instability, infection and tobacco use [2]. Yet, if these risk factors may predispose to develop a nonunion, the underlying physiopathology is still unclear.

The importance of osteogenesis and angiogenesis in fracture healing is well established. However, the specific roles of human mesenchymal stem cells (hMSCs), endothelial progenitor cells (EPCs) and their related growth factors and chemokines are still under debate. During fracture repair, recruited hMSCs proliferate and differentiate in chondroblasts and osteoblasts to promote woven bone formation for the constitution of the bony callus [4,5]. A reduced pool and proliferation capacity of hMSCs have already been shown and could explain the altered bone healing in nonunion [6,7]. But, to the best of our knowledge, little is known about the differentiation potential and function of the hMSCs in NU patients.

During the repair process, EPCs differentiate in endothelial cells to allow revascularization of the fracture site. Although it has been shown that neovascularisation was present within the fracture gap of nonunions [8,9] and that EPCs transplantation improves bone healing [10,11], the pool and biological activities of EPCs have never been assessed in atrophic nonunions.

During fracture repair, the recruitment, proliferation and differentiation of hMSCs and EPCs are induced by cytokines, chemokines and growth factors secreted at the fracture site and released in the peripheral circulation [12]. It has already been demonstrated that the level of
some growth factors such as TGF-β1, PDGF-AB and bFGF is altered either at the fracture site or in the systemic circulation after trauma in nonunion patients [13-17]. Nevertheless the role of growth factors or chemokines involved in the recruitment, proliferation and differentiation of hMSCs and EPCs in established atrophic nonunion has been little studied.

As nonunion treatment is still very challenging [18,19], there is a need for a better understanding of these cellular and molecular mechanisms underlying impaired fracture healing [4,12]. Therefore, in order to elucidate the cellular and molecular mechanisms underlying atrophic nonunions, we studied the pools and biological functions of bone marrow-derived hMSCs, circulating EPCs and the growth factors or chemokines involved in their recruitment, proliferation and differentiation in NU patients compared to healthy subjects.
2 MATERIAL AND METHODS

2.1 Subjects and clinical characteristics

Atrophic nonunion patients were enrolled in the study and compared to control patients (healthy volunteers). An atrophic nonunion was defined as a fracture that had failed to demonstrate any radiographic improvement for three consecutive months. Nonunion was associated with clinical findings consistent with a fracture nonunion such as inability to bear weight on the affected extremity and pain on palpation [1,13]. Exclusion criteria included septic nonunion, head injury, diseases that might interfere with bone metabolism such as renal insufficiency, alcohol abuse, liver diseases, inflammatory rheumatic diseases, systemic inflammation (C reactive protein > 0.5 mg/dl), endocrine diseases (osteoporosis, diabetes, hyperthyroidism or hypothyroidism) and malabsorption. The control group consisted of healthy volunteers without any medical history. The local institutional ethical committee approved the study and all subjects gave their written informed consent.

The characteristics of nonunion patients and healthy volunteers are shown in Table 1. Twenty-four atrophic nonunion patients and 85 healthy controls were enrolled in the study. Nonunion and control populations were matched for age. The two groups were comparable for body mass index (BMI) but not for tobacco use. The bone sites of nonunion were: 3 humerus, 3 radius, 1 ulna, 3 femur, 7 tibia, and 1 fibula, 3 metatarsus, 1 calcaneum and 2 clavicles. The mean time from the fracture related injury was 10.6 ± 1.3 months. None of the control patients sustained a fracture except three who had a fracture of a small bone one year prior to the inclusion in the study. All patients in the nonunion groups were on non-steroid anti-inflammatory drugs and analgesics such paracetamol (at a dose < 4g/day) or tramadol (at a dose < 150 mg/day). Half of the patients were taking anxiolytics such as alprazolam (at a dose < 1mg/day). Healthy volunteers were not taking any treatment.
Two skilled physicians performed the bone marrow aspiration from the posterior iliac crest following a standardized procedure. For each patient or control, about 40 ml of bone marrow was harvested. Bone marrow was obtained from 16 nonunion patients (42.3 ± 12.4 years) and 23 healthy controls (34.7 ± 10.4 years). Blood was collected from 23 nonunion patients (39.9 ± 13.0 years) and from 75 healthy controls (37.8 ± 10.2 years).

2.2 Culture, isolation and characterization of human mesenchymal stem cells

Bone marrow was diluted 1:0.5 with phosphate buffered saline (PBS), overlaid on Ficoll®-Hypaque (GE Healthcare, Healthcare Bio-Sciences AB, Sweden UK) and centrifuged to isolate mononuclear cells (MNCs) following a standardized procedure. Isolated MNCs were washed with PBS, resuspended in Dulbecco’s Modified Eagle Medium (DMEM) and plated in DMEM with low glucose concentration (DMEM low glucose, Lonza, Verviers, Belgium) containing 10% fetal bovine serum (FBS, Lonza, Verviers, Belgium), 100 U/ml penicillin (Lonza, Verviers, Belgium), 100 µg/ml streptomycin (Lonza, Verviers, Belgium) and 2 mM glutamine (Lonza, Verviers, Belgium) at a density of 5.7 x 10^4 cells/cm². Human MSCs were detached by enzymatic treatment (TrypLE™ Select, Invitrogen, Gent, Belgium) at day 14 (defined as passage 0) and then replated and cultured until day 21 (defined as passage 1).

For mesenchymal phenotypic characterization, hMSCs were analyzed at passage 1 by flow cytometry. Cells were stained with the following antibodies: CD34-PE (BD Biosciences, San Jose, USA), CD45-FITC (BD Biosciences, San Jose, USA), CD73-PE (BD Biosciences, San Jose, USA) and CD90-APC (R&D Systems, Abingdon, United Kingdom) [20,21]. Fluorescence was measured on a FACSCanto II flow cytometer (BD Biosciences, San Jose, USA) and data were processed using Diva software v5.0. At least 5,000 events were acquired for each sample. The data are shown as logarithmic histograms of a representative single experiment.
2.3 Mesenchymal stem cell studies

2.3.1 Fibroblastic colony forming unit (CFU-F) assays

To compare the pool of hMSCs in nonunions and controls, the number of fibroblastic colony-forming units (CFU-Fs) was counted in the two groups. For this purpose, MNCs were plated at a density of $5.7 \times 10^4$ cells/cm$^2$ in 25 cm$^2$ plastic culture flasks (Sigma Aldrich, Bornem, Belgium) in DMEM-FBS-10% medium. After ten days, the medium was removed and cells were stained with Diff Quik solution (Siemens, Tarrytown, USA). CFU-Fs were then counted using a standard inverted microscope. The number of CFU-Fs was expressed per million of seeded MNCs.

2.3.2 Human MSCs proliferation

To study hMSCs proliferation in atrophic nonunions and controls, the area and density of CFU-Fs were measured. For this purpose, flasks were divided into 14 equal areas and a central colony was selected in each sector to determine size and density. The area of the colonies was determined using the Motic Images Plus Version2.0ML (Motic, VWR, Belgium) and results were expressed per µm$^2$. The colonies densities were semi-quantitatively scored from 1, when cells were all individualized, to 5 when cells were tightly packed [7]. Results are presented as the mean of the 14 colonies selected, or less if the number of colonies was not reached. Human MSCs culture yields were also measured. The primary culture yield was defined as the ratio between the number of MNCs seeded and the number of hMSCs harvested at passage 0. The secondary culture yield was defined as the ratio between the number of hMSCs seeded and the number of cells obtained at passage 1.

2.3.3 Osteoblastic differentiation and mineralization of hMSCs

In order to assess the osteogenic activity, expression of ALP and mineralization were measured and scored after three weeks of culture in an osteogenic medium. For this purpose,
after passage 1, cells were seeded at a density of $5.7 \times 10^4$ cells/cm$^2$ in α-MEM (Lonza, Verviers, Belgium) containing 15% FBS100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM β-glycerophosphate (Sigma-Aldrich, Bornem, Belgium), $10^{-8}$ M dexamethasone (Sigma-Aldricht, Bornem, Belgium) and 50 µg/ml ascorbic acid (Sigma-Aldricht, Bornem, Belgium). Human MSCs seeded in parallel at similar density in α-MEM-FBS-15% in the absence of the osteogenic agents were used as negative controls. Media were refreshed every week. After 21 days, media were withdrawn and cells were washed with PBS. For ALP staining, cells were fixed for 30 seconds with a solution containing 60% acetone and 40% citrate and rinsed 45 seconds with water. Cells were then incubated 30 minutes in the dark with fresh staining solution, composed of Fast Blue RR Salt and Naphotol AS-MX phosphate. For mineralization assessment, cells were fixed for 15 minutes in 0.4% formaldehyde (Klinipath, Olen, Belgium), rinsed with PBS and rinsed twice with deionized water. Cells were then stained for 10 minutes with alizarin red solution (Sigma-Aldricht, Bornem, Belgium) to visualize calcium crystals present in the matrix. Osteoblast differentiation and function, measured by the intensity of ALP staining and alizarin red staining, were scored from 0 (no staining) to 2.5 (intense staining). Results are presented as the difference between the score obtained in osteogenic medium and negative control medium.

2.4 Endothelial progenitor cell studies

In order to investigate the pool and biological functions of EPCs, peripheral blood-derived mononuclear cells (PBMCs) were cultured in endothelial medium and their clonogenic counterpart were counted and characterized.

2.4.1 Early outgrowth endothelial progenitor cell

Blood was collected in heparin tubes and PBMCs were obtained after isolation by Ficoll®-Hypaque procedure. $5 \times 10^6$ PBMCs were plated in 6-well fibronectin-coated plate. After two
days, the non-adherent cells were harvested and plated in a 24-well fibronectin-coated plate (1 x 10^6 cells per well), in duplicate. The quantification of early outgrowth endothelial progenitor cells (CFU-Hill) colonies was assessed at day 5. Wells containing CFU-Hill colonies were stained with Diff-Quik (Siemens, Tarrytown, USA) and each colony was scored between 1 to 4, depending on its size and morphology. Presented results are the sum of these scores [22].

2.4.2 Late outgrowth endothelial progenitor cells

To obtain late outgrowth EPCs, PBMCs were plated onto 3 separated wells, on a 6-well fibronectin-coated plate, in EGM2 medium (Lonza, Saint-Beauzire, France) composed of endothelial cell basal medium-2 (EBM2), 5% fetal bovine serum (FBS) and growth factors. At 48 hours, non-adherent cells were discarded. Colonies of EPCs appeared between 7 and 30 days of culture and consisted of well-circumscribed cobblestone monolayers (Fig. 5). Colonies were counted with an inverted microscope at 40x magnification. The colonies were then trypsinized, harvested and replated in 25 cm² flasks for further studies. If the required number of cells was reached after proliferation in subcultures of colonies, the EPCs phenotype was confirmed by FACS with the following markers: CD31-FITC (BD Biosciences, San Jose, USA), CD34-PE (BD Biosciences, San Jose, USA), and CD144-PE (BD Biosciences, San Jose, USA) [23]. Fluorescence was measured on a FACSCanto II flow cytometer and data were processed using Diva software v5.0. At least 5,000 events were acquired for each sample.

2.5 Measurements of serum growth factors and chemokines implicated in hMSCs and EPCs function

Serum and plasma were collected in dry and EDTA tubes respectively, centrifuged, aliquoted and frozen at -20°C until use. The serum and plasma levels of growth factors and chemokines
were measured by enzyme-linked immunosorbent assays (ELISA). The following factors were measured in the serum: platelet-derived growth factor (PDGF-BB), insulin-like growth factor-1 (IGF-1), transforming growth factor β-1 (TGF-β1), stem cell factor (SCF), leptin, interleukin-6 (IL-6), soluble IL-6 receptor (sIL-6R), interleukin-8 (IL-8), vascular-endothelial growth factor (VEGF), Dickkopf-related protein 1 (DKK-1), metalloproteinase 2 (MMP2), angiopoietin-1 and angiopoietin-2 (Quantikine™, R&D systems, Abingdon, United Kingdom). Stromal-derived factor-1 (SDF-1) was measured in plasma (Duoset™, R&D systems, Abingdon, United Kingdom).

2.6 Statistical analysis

All values are expressed as mean ± SEM. All reported p values are 2-sided, and statistical significance is assessed at the 5% level. The normality of distribution was tested with a Kolmogorov-Smirnov test. For parametric data, differences between groups were tested by a Student t-test. For non-parametric data, differences between groups were analyzed by using a Mann-Whitney test. Linear regressions were used to investigate the correlations between serum levels of growth factors and CFU-F count, density and size.

3 RESULTS

3.1 Phenotypic characterization of hMSCs

Phenotypic characterization of hMSCs was assessed at passage 1 (day 21). The phenotypic characterization of hMSCs obtained from nonunion patients and controls were similar. In both groups, hMSCs presented a typical mesenchymal profile [21]: positive for CD73 and CD90 and negative for CD34 and CD45 (Fig. 1).
3.2 Mesenchymal stem cells function in atrophic nonunion

3.2.1 Fibroblastic colony forming units (CFU-Fs) assays

In order to assess the pool of hMSCs in the two groups, the number of colonies was counted after ten days of culture. As shown in figures 2A and 2B, the number of CFU-Fs was lower in nonunion patients compared to controls. In the nonunion group, 20.0 ± 5.9 CFU-F (ranging from 0.2 to 65.7, with a median value of 5.94 CFU-F; n = 14) were counted against 48.6 ± 6.6 in the control group (ranging from 16.5 to 96.0, with a median value of 49.7 CFU-F ; n = 14; \( p = 0.005 \)). The morphology of hMSCs was however similar in both groups, presenting a typical fibroblast-like shape (Fig. 2A). In the nonunion group, the CFU-Fs number, size and density were not different between smokers and non-smokers (data not shown).

3.2.2 Human MSCs proliferation

As shown in figure 2C and 2D, cell density per colony was significantly smaller in the nonunion group compared to the control group. Indeed, the density scored at 1.6 ± 0.1 in the nonunion group (n = 14) and 2.6 ± 0.3 in the control group (n = 14) (\( p = 0.009 \)). The size of the CFU-Fs measured as an area, was 3.5 ± 0.3 mm² in the nonunion group (n = 14) and 5.1 ± 0.3 mm² in the control group (n = 14) (\( p = 0.005 \)). There was no difference in terms of number, density and size of the colonies between male and female within each group (data not shown). The primary culture yield, corresponding uncommitted hMSCs, was significantly lower in the nonunion group than in the control group (Fig. 3A). The mean yield was 4.6 ± 0.8 % and 14.7 ± 2.8 % in the nonunion group (n = 15) and in the control group (n = 16) respectively (\( p = 0.002 \)). No difference was observed between male and female within the same group (data not shown). As shown in figure 3B, the secondary culture yield, which corresponds to the proliferation of mature hMSCs, was comparable in the two groups. Altogether, these data suggest that the pool and the proliferation of hMSCs is decreased in
nonunion till they undergo commitment. Once committed, cells recover their capacity to proliferate in vitro.

3.2.3 Osteoblastic differentiation and mineralization of hMSCs

To study the mineralization capacity, hMSCs at passage 1 were cultured for 3 weeks in an osteogenic medium. ALP and ARS staining were similar in the two groups (Fig. 4A and 4B). These results show that hMSCs isolated from nonunion and control groups have a similar capacity to differentiate in osteoblasts and to mineralize.

3.3 Endothelial progenitor cells function in atrophic nonunion

3.3.1 Early outgrowth endothelial progenitor cells

Early outgrowth EPCs are derived from the myeloid lineage (CD34\(^-\), CD14\(^+\) cells) of hematopoietic stem cells and contribute to angiogenesis by a paracrine action. These cells are marker of vascular function [22]. The morphology (Fig. 5A) and score of CFU-Hill colonies (Fig. 5B), a measurement of the pool of early outgrowth EPCs, were similar in the two groups. In the nonunion group, the colonies were scored at 55.4 ± 16.7 (ranging from 0 to 179.0, with a median value of 31.2; n = 14) against 37.8 ± 13.0 in the control group (ranging from 0 to 265.0 with a median value of 21.5; n = 21; p = 0.241).

3.3.2 Late outgrowth endothelial progenitor cells

Late outgrowth EPCs present extensive proliferative capacity, are able to form a vascular network in vitro and to incorporate themselves in situ in developing vessels [23]. The reported frequency of late outgrowth EPC colonies observed in healthy donors is 39% [24]. In this study we observed colonies in 4 out of 10 nonunion cultures and in 3 out of 11 healthy donors. As shown in figure 5C, the colonies presented the characteristic cobblestone aspect in the two groups. Nonunion and control late outgrowth EPCs stained positive for the typical
endothelial markers CD31, CD34, and CD144 (data not shown). Altogether, these results show that the pool of early and late outgrowth EPCs is similar in nonunion as in healthy donors.

3.4 Serum levels of growth factors and chemokines implicated in hMSCs and EPCs recruitment, proliferation and differentiation

We measured chemokines and growth factors involved in hMSCs and EPC recruitment and proliferation such as SCF, PDGF-BB, MMP2, IL-8, IL-6 and its soluble receptor sIL-6R. We also measured growth factors implicated in hMSC and EPC differentiation. For angiogenesis VEGF, Ang-1 and Ang-2 were measured and for osteogenesis IGF-1, TGF-β1 and DKK-1 were assessed.

As shown in figure 6, the serum levels of SCF, PDGF-BB, MMP2 and IGF-1 were significantly decreased in nonunions compared to controls. Serum leptin level was increased (p = 0.042) as well as IL-6 serum level while the soluble receptor sIL-6R was decreased (respectively p = 0.002 and p = 0.006) (Fig. 6). The serum levels of the chemokines SDF-1 and IL-8, and the growth factors VEGF, Ang-1, Ang-2, TGF-β1 and DKK-1 were similar in both groups (Table 2).

SCF was correlated to CFU-F count (r = 0.733, n = 12, p = 0.007), PDGF-BB was correlated to CFU-F density (r = 0.795, n = 7, p = 0.033), leptin and IL-6 were correlated to BMI (r = 0.859, n = 15, p < 0.001 for leptin; r = 0.544, n = 15, p =0.023 for IL-6), and leptin was inversely correlated to CFU-F size (r = 0.771, n = 7, p = 0.042). Moreover, there was a trend towards an inverse correlation between IL-6 and CFU-Fs size, but the sample size was too small (r = 0.584, n = 5, p = 0.185).

In the nonunion group, the systemic level of growth factors and chemokines were not different between smokers and non-smokers (data not shown).
4 DISCUSSION

The importance of angiogenesis and osteogenesis in fracture healing is well established. However, the pathophysiology of nonunion remains still unclear; in particular, the specific roles of MSCs, EPCs and their related growth factors and chemokines are still under debate. In the present study, although we confirmed a lower pool and proliferation of hMSCs, we showed for the first time a normal osteogenic potential of nonunion hMSCs. Indeed, after the first replating, proliferation of NU hMSCs was still reduced as compared to control hMSCs but the difference was no more statistically significant. Moreover, the ability of hMSCs to differentiate into osteoblastic cells and mineralize was similar in nonunions and controls. In parallel, we found altered serum levels of growth factors involved in chemotaxis and proliferation of hMSCs such as IL-6 and its soluble receptor sIL-6R, leptin, PDGF-BB, SCF and IGF-1. Moreover, we showed for the first time that the pool of EPCs and their regulating growth factors were not affected in nonunion patients.

Angiogenesis plays a pivotal role in fracture healing to promote revascularization of the injured site. New vessels are needed for the mobilization of osteoprogenitor cells and growth factors implicated in osteogenesis. We showed for the first time that the number of early and late outgrowth EPCs and the systemic level of growth factors and chemokines implicated in their function were similar in both studied group suggesting that nonunions have the potential for revascularization through both de novo vessels formation and a favorable pro-angiogenic paracrine action. Reed et al. also demonstrated that the vascularity of biopsies taken from the fracture gap of atrophic nonunions and healing fractures were similar [9]. Comparable results have been shown in animal models of bone defect [8,25].

If the present study gives arguments in favor of a good potential for revascularization, it also suggests that atrophic nonunion could be related to an insufficient number of hMSCs capable to form new bone. Indeed, if the pool of bone marrow-derived hMSCs was decreased and
proliferation delayed, once committed, cells were able to differentiate and mineralize in vitro. A decreased pool and an altered proliferation of bone marrow-derived hMSCs have already been described at the fracture site and in the iliac crest of nonunions [6,7], but the present study is the first to show a normal osteogenic potential of nonunion hMSCs in vitro.

Analgesics and anxiolytics are not known risk factors for atrophic nonunion fractures. In the literature, although, there is conflicting evidence about the effect of NSAIDs on fracture repair, NSAIDs seem to inhibit endochondral bone formation in humans [2,26]. Moreover, NSAIDs were found to suppress proliferation and differentiation of MSCs in human and mice [27,28]. As all patients in the nonunion group were on NSAIDs and as healthy volunteers were not taking any treatment, this could explain, in part, the lower proliferation of hMSCs in nonunions.

Reduction of the hMSCs pool could be responsible for an insufficient development of bone cells and an insufficient production of growth factors and chemokines implicated in the recruitment and differentiation of hMSCs and EPCs. In this situation, the paracrine environment could be unsuitable to initiate a bone healing, favoring the development of nonunion. One hypothesis could be that the decreased number of hMSCs observed in nonunion could be due to an imbalance between recruitment and renewal of cells. Indeed in nonunion, hMSCs could be recruited for a longer time at a higher rate leading to an increased number of circulating osteogenic progenitor cells and lower number of hMSCs in the bone marrow [3,29]. The decreased pool of hMSCs could also be related to an altered capacity of these cells to adhere in in vitro culture. The present study was not designed to assess the number of circulating osteoprogenitor cells. Therefore, in an attempt to explain why the hMSCs pool was decreased in nonunions, we measured the peripheral blood levels of signaling molecules involved in normal fracture repair and particularly, the molecules involved in the recruitment, renewal and differentiation of hMSCs.
We showed a significant increase of IL-6 and a decrease of sIL-6R in nonunion patients. IL-6 is a potent chemokine secreted at the injury site of bone healing by endothelial cells, adipocytes, monocytes/macrophages, T-cells, mast cells, fibroblasts and stromal cells [30,31]. IL-6 requires the presence of its soluble receptor, sIL-6R, to modulate MSCs and osteoblasts recruitment, proliferation and differentiation [32], essential for bone repair. During fracture healing, IL-6 has been shown to play a role in initiating the repair cascade by recruiting MSCs, EPCs and inflammatory cells, but also enhancing extracellular matrix synthesis, and stimulating angiogenesis [31]. In a model of distraction osteogenesis, IL-6 appeared to be one of the key cytokines involved in the complex network of signal cascades that differentially affect immature and mature osteoblastic lineage cells [33]. However, little is known about the exact role of IL-6 and its soluble receptor in the pathophysiology of nonunion. On one hand, the increased level of IL-6 in the serum might have been favorable for the recruitment of hMSCs in the circulation and cause a decrease number of hMSCs in the bone marrow. On the other hand, high levels of IL-6 might negatively affect hMSCs proliferation in the bone marrow. Indeed, a negative proliferative effect of IL-6 was shown in vitro and in vivo in a model of distraction osteogenesis [33]. Moreover, the low level of sIL-6R could impair hMSCs differentiation [34]. Finally, the altered levels of IL-6 and its soluble receptor could affect osteogenesis. Indeed, this cytokine is described as a double edge sword actor of osteogenesis because, if it usually sustains bone formation, IL-6 may also drive bone loss in various osteolytic pathologies [31]. If the role of IL-6 is intrinsically linked to its soluble receptor, its role is also linked to factors such as leptin that activate the same STAT signaling pathway [32]. Leptin and IL-6 are part of a group of biologically active polypeptides, the adipokines, produced by adipocytes or adipose tissue. Both adipokines were increased in nonunions and their levels were correlated to the BMI. Leptin, like IL-6, is known to directly affect osteoblast proliferation and/or differentiation [35]. Leptin inhibits
bone formation through the sympathetic nervous system by stimulating the beta-adrenergic receptors on osteoblasts [36]. However, as for IL-6, the effect of leptin on bone is complex and negative and positive effects on bone formation have been reported in humans [35]. As IL-6/sIL-6R and leptin are actors of multiple processes in MSCs and osteoblasts recruitment, proliferation and differentiation, their altered serum levels in nonunions are difficult to interpret and should be further investigated. Nevertheless, in the present study, we showed that leptin and IL-6 were correlated to the BMI, and that leptin was correlated to the size of CFU-Fs, suggesting a relationship between BMI, adipokines and altered proliferation of hMSCs in nonunion patients.

In the present study, we also observed decreased levels of PDGF-BB and MMP2 in atrophic nonunion patients. PDGF is an angiogenic growth factor secreted by the alpha granules of platelets as well as endothelial cells, vascular smooth muscle cells, and macrophages. It also participates actively in all phases of fracture healing by promoting chemotaxis and proliferation of MSCs and osteoblasts [15,37]. Moreover, PDGF induces MMP2 expression and activation, leading to the migration of osteogenic progenitor cells during bone formation [38]. PDGF-AB was shown to be decreased both locally and in the systemic circulation of patients developing nonunions, between 2 and 4 weeks after the fracture [39,40] and local administration of human recombinant PDGF-BB successfully enhanced healing of bone defects [41]. Here we showed that PDGF-BB and MMP2 were also decreased in chronic nonunion patients and that there was a correlation between the serum level of PDGF-BB and the density of CFU-Fs.

Our results also demonstrated for the first time a decreased level of SCF in atrophic nonunion patients. SCF-ckit signaling is a key niche component; it maintains hematopoietic stem cells, EPCs and hMSCs proliferation and differentiation, thereby promoting angiogenesis and osteogenesis and enhancing fracture healing [42]. In the present study, decreased serum level
of SCF was correlated to a lower number of CFU-F colonies. As the peri-vascular stromal cells are one of the major sources of SCF in the stem cells niche [43], low CFU-F numbers could explain the lower levels of SCF and thereby the decreased osteogenesis in nonunion patients.

Finally, IGF-1 is an important actor in fracture healing [2,12,39,44] involved in MSCs proliferation and migration [45] and was shown to be decreased in nonunions. Nevertheless, its implication in nonunion is still not well understood. The transplantation of IGF-1 over-expressing MSCs improved fracture healing in a mice model of nonunion and was suggested as a new therapy for nonunions [46]. Our results strengthen the idea that IGF-1 is an important actor of atrophic nonunions.

On the other hand, we showed similar levels of Ang-1, Ang-2, SDF-1, IL-8, VEGF, TGF-β1 and DKK-1 between chronic nonunions and controls. These signaling molecules participate in bone healing through various action such as; (1) the recruitment of progenitor cells such as hematopoietic stem cells, EPCs, and, to a small degree MSCs [47,48]; (2) angiogenesis [15,49]; (3) and osteogenesis [15,49-51]. Some of these factors were showed to be altered in the early stages of atrophic nonunions. For example, the serum level of TGF-β1 was decreased in developing nonunions and was proposed as an early diagnostic biomarker for atrophic nonunion fractures [14,17]. In the present study, we measured the serum level of chemokines and growth factors 10.6 ± 1.3 months after the fracture injury. At this stage of the disease, we showed altered levels of growth factors and chemokines implicated in the chemoattraction and proliferation of hMSCs and these results were associated with a decreased pool of hMSCs. However, at this chronic stage of the disease, the growth factors involved in angiogenesis and osteogenesis were comparable in nonunions and controls, coinciding with similar pools of EPCs and a similar potential of hMSCs to differentiate in osteoblastic cells and to mineralize.
In conclusion, although nonunion is generally attributed to a vascular defect, our results support the idea that a defect of the systemic mesenchymal/osteogenic cell pool, probably related to alterations of growth factors implicated in their chemoattraction and proliferation, could play a role in the disease. Yet, we demonstrated that the osteogenic potential of nonunion hMSCs was preserved. These data are novels and add to the knowledge of chronic atrophic nonunion fracture, when the disease is diagnosed and when novel therapeutic approaches are foreseen.

5 AKNOWLEDGMENT

This work was supported by the « Fonds Européen de Développement Régional ». We thank Professor Jean-Philippe Hauzeur for blood and bone-marrow samples harvesting.

6 REFERENCES


7 FIGURES CAPTION

Figure 1 – Phenotypic characterization of hMSCs from nonunions and controls at passage 1. Representative images of flow cytometric analysis of hMSCs stained with antibodies directed against CD34, CD45, CD73, and CD90 cells in control subjects (n = 12) (A-D) and nonunion patients (n = 10) (F-I).

Figure 2 – CFU-F assays. Colonies were stained with Diff-Quik ten days after initial seeding. (A) Representative images of CFU-F colonies in nonunions and controls at day 10 (macroscopic view, magnification x10 and x40). (B) Total number of colonies measured in control and nonunion groups at day 10 (n = 14). (C) CFU-F density of colonies from control and nonunion groups (n = 14) estimated with a score ranging from 1 to 5. (D) Colonies areas measured at day 10 in control and nonunion groups (n = 14). All results are expressed as means ± SEM; * p < 0.01).

Figure 3 – Human MSCs proliferation. (A) Primary culture yield. Cells from control (n = 16) and nonunion (n = 15) groups were counted at passage 0 (day 14) and the primary yield was expressed as the ratio of cells obtained and seeded (*p < 0.01). (B) Secondary culture yield. Cells from control and nonunion groups were counted at passage 1 (day 21) and the secondary yield was expressed as the ratio of cells obtained and seeded (n = 12).

Figure 4 – Osteogenic differentiation and mineralization of hMSCs. (A) Differentiation of hMSCs. Mineralization was assessed by ARS (controls, n = 10; nonunions, n = 9) and ALP staining (controls, n = 8; nonunions, n = 9) of hMSCs after passage 1 and cultured in osteogenic medium for 21 days. Dark grey boxes represent control hMSCs and light gray boxes represent hMSCs from nonunions.

Figure 5 – Endothelial progenitor cell function in nonunions. (A) CFU-Hill colonies obtained at day 5 were stained with Diff-Quik staining and scored depending on their size. Dark grey box represents CFU-Hill colonies from controls (n = 21) and light grey box represents CFU-
Hill colonies from nonunions (n = 14). (B) Representative picture of a colony of early outgrowth EPCs colonies at day 5 in controls and nonunions (original magnification x40).

**Figure 6** – Serum levels of growth factors and chemokines.

Levels of (A) PDGF-BB (nonunions n = 18; controls, n = 29), (B) MMP2 (nonunions n = 11; controls, n = 26), (C) SCF (nonunions, n = 15; controls, n = 24), (D) IGF-1 (nonunions, n = 16; controls, n = 27), (E) IL-6 (nonunions, n = 17; controls, n = 24), (F) sIL-6R (nonunions, n = 14; controls, n = 24) and (G) leptin (nonunions, n = 16; controls, n = 16). Levels were measured in serum from control (dark grey boxes) and nonunion patients (light grey boxes).

*p < 0.05; **p < 0.01.

**Table 1: Subjects characteristics**

<table>
<thead>
<tr>
<th>Bone-marrow donors</th>
<th>Controls</th>
<th>Nonunions</th>
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<tbody>
<tr>
<td>Number of subjects</td>
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<td>16</td>
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<tr>
<td>Age, years (mean ± SD)</td>
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<td>42,3 ± 12,4</td>
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<td>Male/female</td>
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<td>11/5</td>
<td>NS</td>
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<td>Smokers (%)</td>
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<tr>
<td>BMI</td>
<td>26,3 ± 3,9</td>
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<table>
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<td>Male/female</td>
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<tr>
<td>Smokers (%)</td>
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<td>50</td>
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<tr>
<td>BMI</td>
<td>26,3 ± 4,2</td>
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p values are two-sided; NS, not significant; BMI, Body Mass Index.
Table 2: Serum and plasma levels of growth factors and chemokines.

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<th></th>
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<tr>
<td></td>
<td>Mean ± SEM n</td>
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<tr>
<td>TGF-β1, ng/ml</td>
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<td>29.8 ± 1.4 14</td>
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<td>DKK-1, pg/ml</td>
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<td>1.71 ± 0.15 5</td>
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<tr>
<td>SDF-1, pg/ml</td>
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<td>144 ± 33 12</td>
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<tr>
<td>Il-8, pg/ml</td>
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<td>22.3 ± 5.2 15</td>
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<td>VEGF, pg/ml</td>
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<td>Ang-1, ng/ml</td>
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<td>Ang-2, ng/ml</td>
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TGF-β1, Transforming growth factor β-1; DKK-1, Dickkopf-related protein 1; SDF-1, Stromal-derived factor-1; IL-8, Interleukin-8; VEGF, Vascular-endothelial growth factor; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; two sided p values.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
HIGHLIGHTS

The pool of bone marrow-derived mesenchymal cells is decreased in nonunions.
The osteogenic potential of mesenchymal cells is preserved in nonunions.
Serum levels of proliferative growth factors and chemokines are altered in nonunions.
The pool of endothelial progenitor cells is preserved in atrophic nonunions.
Serum levels of angiogenic growth factors are preserved in atrophic nonunions.