CXCR4 Antagonism to Treat Delayed Fracture Healing

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Abstract (200)

A significant number of fractures develop non-union. Stem cell homing is regulated through SDF-1 and its receptor CXCR4. Stem/progenitor cell populations can be endogenously mobilised by administering growth factors with a pharmacological antagonist of CXCR4, AMD3100, which may be a means to improve fracture healing.

Methods: A 1.5mm femoral osteotomy in Wistar rats was stabilised with an external fixator. Rats were pre-treated with PBS(P), VEGF(V), IGF-1(I) or GCSF(G) prior to AMD3100. A control group (C) did not receive growth factors or AMD3100. Bone formation after five weeks was analysed.

Results: Group P had a significant increase in total bone volume (p=0.01) and group I in % bone in the fracture gap (p=0.035). Group G showed a decrease in bone volume. All treated groups had an increase in trabecular thickness. Histology showed decreased cartilage tissue associated with increased bone in groups with improved healing, and increased fibrous tissue in poorly performing groups.

Conclusion: Antagonism of SDF1-CXCR4 axis can boost impaired fracture healing. AMD3100 given alone was the most effective means to boost healing whilst pre-treatment with GCSF reduced healing. AMD3100 is likely mobilizing stem cells into the blood stream that home to the fracture site enhancing healing.

IMPACT STATEMENT

Currently around 10% of fractures progress to delayed or non-union with significant morbidity and economic impact. Endogenous mobilisation of stem cells by pharmacological antagonism of their homing and migration receptor CXCR4 with AMD3100 experimentally reduced delayed union development. Endogenous mobilisation may therefore translate as a low risk means to boost healing and could potentially be given as a prophylaxis to patients with fractures at risk of delayed healing or non-union. These patients may include fragility fractures, comminuted tibial fractures, or when treating established non-unions. This approach could have promise for other conditions that may benefit from stem cell treatments.
Introduction

A significant number of bone defects and fractures do not heal (1), with estimates of around 100,000 fractures per year developing non-union in the USA(2). In order to achieve bone union, there is a need to recruit a range of cells including inflammatory cells, endothelial cells and stem cells, from a range of tissue sources including muscle, bone marrow, adipose tissue and periosteum (3). At rest there are low basal levels of peripherally circulating skeletal progenitors; approximately 1 per $10^6$-8 blood mononuclear cells(4-6), whilst the numbers of levels of Mesenchymal Stem Cells (MSCs)(7), and Endothelial Progenitor Cells (EPCs)(8) in the blood steam increase post bone fracture. The chemokine stromal cell derived factor 1 (SDF1, also known as CXCL12), and its receptor CXCR4, have a key role in stem cell migration from the bone marrow stroma into the circulation and are believed to be important for homing of stem cells to a fracture site (9). Local increases in SDF1 expression have been measured in distraction osteogenesis, stress fractures and segmental defects (10) (11) (12). Parabiotic studies have demonstrated labelled stem cell mobilisation is from the bone marrow through the peripheral circulation and these cells are able to then contribute to the fracture healing process(13,14).

Mobilisation of haematopoietic stem cells is a mainstay of clinical bone marrow transplantation to treat a range of blood related malignancies, and GCSF was the first growth factor used for this purpose(15). A highly selective, high affinity competitive antagonist of the CXCR4 receptor, AMD3100, (16) is commercially known as Moziblitm, and rapidly mobilises high numbers of haematopoietic stem cells by blocking their interaction with SDF1 in the marrow niche (17-20). It has been shown in mice that when AMD3100 is given after pre-treatment with VEGF rather than GCSF, there is preferential mobilisation of a population of MSCs and EPCs relative to haematopoietic stem cells (21,22).

To date, a few groups have started to investigate mobilisation of stem cells to augment bone healing. Critical sized calvarial defects have shown enhanced healing with 15 daily injections of AMD3100 in mice(21,23), and a single dose of AMD3100 was also show to improve intramedullary trabecular bone re-formation (23,24). For evaluation of diaphyseal long bone healing, Kumar et al. mobilised MSCs by pre-treatment with IGF1 followed by
AMD3100, in a coapted mouse tibial segmental fracture, and showed a significant increase in bone mineral density (24,25). AMD3100 has also been evaluated after creating an ‘Einhorn style’ mouse femoral fracture stabilised with a single intramedullary pin, and fracture healing was accelerated (22,24,25).

None of the above studies allow for direct comparison of the different protocols, and neither do they test the effect of endogenous mobilisation in a translationally relevant delayed union model. Therefore, based on current literature, this study aimed to compare different mobilisation protocols (AMD3100 alone, or after pre-treatment with GCSF, IGF1 or VEGF) in a biomechanically controlled, delayed-union fracture model, of a rat femoral osteotomy stabilised using an external fixator (24). The hypothesis was that antagonism of the SDF1/CXCR4 axis using AMD3100 would improve bone healing in a delayed union fracture model, and pre-treatment with IGF1 or VEGF prior to AMD3100 would have the greatest efficacy due to their preferential mesenchymal stem cell mobilisation.

**Methodology**

**Growth factor and AMD3100 preparation**

AMD3100 octahydrochloride hydrate (Sigma-Aldrich A5602), stock solution was prepared by dissolving 5mg lyophilized product in 0.5mls sterile water, then added to 4.5mls PBS to produce a 1mg/ml injection solution, which was aliquoted and stored at -20°C until needed. Rat Vascular Endothelial Growth Factor 165 (VEGF) (PeproTech, USA, 400-31) was prepared by dissolving the lyophilized product in sterile water to make a 0.1mg/ml stock solution and then 1ml of stock solution was added to 4mls of sterile Phosphate buffered saline (PBS) + 0.1% Bovine Serum Albumin (BSA) (Sigma-Aldrich A9418), to achieve 100ug/ml injectable solution which was aliquoted and stored at -20°C until needed. Recombinant human Insulin-like Growth Factor-1 (IGF1) (PeproTech, USA, 100-11) and murine Granulocyte Colony Stimulating Factor (GCSF) (PeproTech, USA 250-05) were prepared in the same manner. Finally, PBS + 0.1% BSA, ‘sham growth factor’, to determine the effects of AMD3100 alone was also prepared.
Fracture Model

Twelve to fourteen week old female wistar rats (230-300g), were randomly assigned to one of five groups: PBS + AMD3100 (n=6) (PBS-AMD), VEGF + AMD3100 (n=8) (VEGF-AMD), IGF1 + AMD3100 (n=6) (IGF1-AMD), and GCSF + AMD3100 (n=6) (GCSF-AMD). A linear Type 1a micro-external fixator, with titanium blocks and carbon-fiber bars was placed on the left cranial lateral femur following a lateral surgical approach (26). Using a precision guide, four bicortical 1.4mm diameter end-threaded self-tapping stainless steel pins were placed in predrilled 1.0mm holes. Consistent proximodistal positioning was based on the distal extent of the greater trochanter. Pins were exited through separate skin incisions and the custom variable spacing fixator was attached(26). A mid-diaphyseal femoral osteotomy, with no periosteal stripping was made using a diamond tipped hand-saw, whilst applying sterile saline coolant/lubricant. A precision spacer ensured a fixed distance between the cis cortex and connecting blocks of 9mm. The fixator was then used to distract the osteotomy gap to 1.5mm using a second precision spacer. The biceps femoris was closed over the osteotomy with a single horizontal mattress suture (1.5M PDS II, Ethicon, UK), and then the skin was closed with intradermal continuous suture (1.5M monocryl, Ethicon, UK). Activity was unrestricted post surgery. In two rats the surgical wounds failed to heal and were removed from the study, leaving n=5 for both the GCSF-AMD and PBS-AMD groups.

Twenty-four hours post surgery, rats were given a single i.p. injection of either VEGF, IGF1, GCSF, or PBS once daily for four days at 100ug/kg(21-25). On day five, they were given a single injection of AMD3100 at 5mg/kg(21-25). All i.p. injections including AMD3100 and sham PBS were administered at a volume of 0.5mls/100g bodyweight based on the day 0 pre-surgical weight. Rats were sacrificed at five weeks post-operatively. All procedures were carried out with the local Animal Welfare and Ethical Review Body approval under personal and project licenses issues by the UK Home Office under the 1986 Animal Scientific Procedures Act.
MicroCT and Radiographic analysis

The left femur with the fixator in place was retrieved. In order to reduce microCT beam-hardening artifact generated from the interaction of the X-ray beam and the metallic implant, a radiolucent PEEK fixator block was connected externally to the fixator pins after careful removal of the skin with surrounding soft-tissues, and then without disturbing the fracture callus the titanium block fixator was then removed. Samples were fixed in 10% buffered formaldehyde for up to three days. The formalin fixed samples were wrapped in cling film to prevent dehydration and mounted into a sample holder for microCT scanning. Samples were scanned using a Bruker Skyscan 1172 microtomograph machine (Bruker, Belgium), at 60KV, 167uA with a 0.5mm aluminum filter. A rotation step of 0.5 degrees, without frame averaging, and an image pixel size of 4.89um was used. A single image capture image was taken with the image intensification ‘scout’ prior to scanning, for 2D radiographic assessment of the osteotomy union. Radiographic scouts were randomised and blinded to score the general impression of healing according to the AO-ASIF recommendations for long bone fractures; united, not united or uncertain (27) as follows: ununited where there was no mineralized tissue bridging between the ends of the osteotomy; uncertain where there was new bone formation, however a radiolucent line remained between the proximal and distal segments, and united where no gap between bone ends was visible.

MicroCT scans were reconstructed using NRecon (Bruker, Belgium) with smoothing=2, ring artifact reduction=12% and beam hardening artifact=41%. Analysis was performed with CTAn (Bruker, Belgium). Using the measuring tool, the centre point of the osteotomy was determined and the transverse slice at that point was selected as the reference slice. The central 60% of the osteotomy gap. i.e. only new bone formation within the osteotomy was analysed. The callus was isolated using a 2D ROI shrink wrap stretching over holes <40 pixels, despeckled <150 voxels and then 3D analysis was performed.

Histology preparation

Bones were decalcified in a 12.5% solution of ethylenediaminetetraacetic acid and sequentially dehydrated for 24 hours, followed by de-fatting with chloroform for 48 hours.
and then embedded into wax, with the fixator pins orthogonal to the facing surface of the block. Fixator blocks and pins were removed once the wax had set and a microtome (ThermoFisher Scientific, UK) was used to make 5μm thick slices. The alignment of the blocks within the microtome was altered as necessary to ensure a central sagittal slice through the femur. The position of a mid-sagittal section through the fracture gap was assessed using the fixator pin tract holes. Wax slices were mounted onto positively charged glass slides (X-tra, Leica biosystems, UK), de-waxed and then hydrated. Samples were then stained with Haematoxylin (Sigma-Aldrich, UK) nuclear stain for five minutes. Excess stain was removed by gentle washing with water for five minutes. Slides were counterstained in 1% Eosin (Sigma-Aldrich, UK) for four minutes and then washed and dehydrated in increasing concentrations of alcohol. Slides were cleaned in xylene and mounted under 40mm coverslips using Pertex Mounting Medium (CellPath plc, UK).

**Histomorphometric analysis**

Slides were observed under a light microscope (KS-300 Zeiss, UK). Histomorphometric analysis using the 2.5x objective was performed on the most central slice, using a line-intercept method with a grid scaled to the graticule and drawn using powerpoint (Microsoft, USA). The grid covered the entire visual field from top to bottom (lateral to medial cortex) and was centered over the osteotomy; its width was equivalent to the original 1.5mm osteotomy. Grid ‘density’ was 120 intersections and grid squares were 160μm in both directions. Intersections were then scored as bone, cartilage, fibrous tissue, vascular (red blood cells seen not within tissue matrix) or void.

**Statistical Analysis**

As the data was non-parametric, analyses included the Mann-Whitney U (MWU) and Kruskal-Wallis (KW) as appropriate. Significance was set a p<0.05 and tests were analysed with SPSS version 24 (IBM, Chicago, USA).
Results

Influence of mobilization on fracture healing

Radiographic score showed a reduction in ununited (non-union) and increases in uncertain and united (union) for all groups compared with the GCSF-AMD group. Radiographically united fractures occurred in 4/5 animals in the PBS-AMD group whereas this was reduced for the VEGF, IGF, GCSF pre-treated groups with control animals only showing a united rate in two animals out of seven (Table 1). MicroCT analysis showed that all groups other than GCSF-AMD had improved healing over the controls (Table 2). PBS-AMD had twice the bone volume within the osteotomy (8.9±2.2 μm^3, p=0.01), compared with the untreated control (4.3±3.1μm^3). Not only was the BV increased, but the overall callus volume (TV) was increased compared with controls (15.3±3.6 vs 9.2±6.1 μm^3) (Table 2, Figure 1). The percentage bone volume was not significantly increased owing to a relative proportional increase in bone and non-mineralised callus tissue. Additionally, the bone structure was different in the PBS-AMD group compared with controls. Animals in the PBS-AMD group showed a significant increase in trabecular thickness, 0.061±0.002μm compared with those in the control group, which had a thickness of 0.042±0.003μm (P=0.03) (Table 2, Figure 2).

The VEGF-AMD group did not show any significant differences from the control group. Interestingly, animals in the GCSF-AMD group had a significant increase in percentage BV (BV/TV) 63.1±7.3% vs 53.8±20.8% (p=0.048), but the actual volume of the callus (TV 4.3±4.7 vs 9.2±6.1μm^3) and BV (2.5±2.6 vs 4.3±3.1μm^3) was reduced compared with controls. However, trabecular thickness was significantly higher 0.069±0.03 vs 0.042±0.008μm (p=0.048) (Figure 2), but total porosity was significantly lower 36.9±7.3% vs 46.2±20.8% (p=0.048), indicating that although GCSF-AMD group had less overall total woven bone, the bone formed was less porous and the size of each bone forming region within the fracture gap was larger than in controls.

IGF1-AMD group also had an increase in bone volume (5.1±4.2μm^3), compared with controls. Percentage BV (BV/TV) was significantly increased (p=0.035), and the overall callus size was the same as controls (TV 9.1±7.6 vs controls 9.2±6.1μm^3). There was also a
significant increase in trabecular thickness $0.062\pm 0.008\mu m$ ($p=0.01$) (Figure 2). Total porosity was significantly lower within the fracture gap of animals treated with IGF1-AMD, compared with controls; $40.8\pm 5.6\%$ vs $46.2\pm 20.8\%$ ($p=0.035$). The spread of data was not significantly different.

When comparing between all groups, there were significant differences in bone volume (BV) ($p=0.033$) (Figure 1), trabecular thickness (Tb.Th) ($p=0.003$) (Figure 2), total porosity (TotPor) ($p=0.043$) and percentage bone volume (BV/TV) ($p=0.043$). All treated groups had greater bone formation than control, other than GCSF-AMD, which had a negative impact on healing. However, only PBS-AMD reached statistical significance for increased overall bone volume (BV) and IGF1-AMD for % bone (BV/TV) within the callus. Notably, all groups had significant increases in trabecular thickness other than VEGF-AMD. Three-dimensional reconstructed images of the representative groups are shown in figure 3. For full microCT quantitative morphology results see table 2.

**Histomorphometric analysis**

The 2.5x histomorphologic analysis corroborated the microCT data, however the differences were not statistically significant for percentage cartilage ($p=0.053$) and percentage fibrous tissue ($p=0.059$) for the PBS-AMD compared with controls. Patterns of increased bone formation were associated with decreased cartilage in groups with improved healing, whilst the worse performing groups had an increase in fibrous tissue with decreasing bone and cartilage formation (Figure 4). Vascularisation of the tissues was not significantly different, although animals in the GCSF-AMD group had the lowest levels of vascularisation, whilst groups with more bone formation had higher levels of vascularisation. However, the method of assessment was relatively non-specific, and immunohistochemistry staining for CD31, alpha-SMA or other endothelial markers would be a means to make a more comprehensive assessment of vascularisation.

Although not quantified, the cartilage tissue present in the GCSF-AMD group was observed to have fewer hypertrophic chondrocytes than the other groups (Figure 5), suggesting reduced or slowed endochondral ossification. When comparing the controls to the best and worst performing groups, the control groups had a large area of cartilage in the
central region, whereas the AMD3100 treated group had increased woven bone, but the GCSF group had a predominance of a highly cellular granulation type tissue (Figure 5). The PBS-AMD group, which had the highest levels of bone and vascular tissue, had reduced cartilage and no fibrous tissue (Figure 4). However, in other groups, cartilage formation was increased, suggesting conversion to bone by endochondral ossification (Figure 4). The next highest bone formation was seen in VEGF-AMD, which also showed a low level of fibrous tissue and higher level of vascular tissue on histomorphometric analysis.

Discussion

This study was the first to evaluate the potential effects of stem-progenitor mobilisation in compromised fracture healing in rats, and demonstrated that AMD3100 antagonism in the early inflammatory phase of fracture healing has a beneficial influence on bone formation. Other studies have shown similar benefits in mice but critically, this study allowed for direct comparison of different pre-treatment protocols, and for the first time demonstrated efficacy of endogenous mobilisation in a mechanically standardised delayed union model. In this model of delayed union, there were significant increases in bone content within the fracture and a reduction in uncertain and un-united radiographic categories. This confirms that this strategy can improve compromised fracture healing, however because the animals were terminated after 5 weeks, it is unclear whether this strategy could avoid a non-union forming. Nonetheless, there may be translational benefit for treating at risk groups of non-union, such as tibial, humeral or clavicular fractures(28).

All strategies tested other than GCSF-AMD did improve fracture healing. AMD3100 without growth factor pre-treatment gave significant increases in bone formation as measured on microCT, with a bigger (proportionally mineralized) callus compared to controls. This is similar to the findings of Toupadakis et al, who gave AMD3100 only, but over three sequential days, rather than as a single dose(25,29). Although not performed here due to the high complexity, parabiotic studies with recapitulated labelled bone marrow(13,14) would offer a mechanistic understanding of the exact processes leading to improved fracture healing.
Prior mobilisation studies have shown in mice that pre-treatment with VEGF or IGF1 preferentially increased the numbers of MSCs mobilised into the peripheral circulation (22,24,30). Based on these studies, we hypothesized that these growth factors would have a greater influence on fracture healing than giving AMD3100 alone, which comparatively mobilises lower levels of MSCs, endothelial progenitors and haematopoietic stem cells. The results of this study suggest that AMD3100 is the most effective protocol to improve fracture healing, bringing into question whether total numbers of different cells types, or their relative combinations are more important. This study shows a conclusive benefit of AMD3100, which has been comprehensively shown to exert its main interaction at the CXCR4 receptor(31), which would lead to the release of cells that could enhance repair. However, it is impossible to totally exclude other mechanisms that may improve healing not due to stem/progenitor mobilisation.

An issue with measuring circulating stem/progenitor cells is that they provides only a 'snap-shot' of the circulating pool of cells at the predicted peak elution time of one hour post administration of AMD3100(21,23,25,29). The duration and character of the profile of cell elution into the circulatory system is probably more important and so the true kinetics of the mobilisation is unknown, and hence, although VEGF or IGF1 pretreatment prior to AMD3100 mobilised more MSCs at one hour post administration, this does not mean the total number is greater. It is also clear that mobilised cells home back to the bone marrow, or indeed other tissues such as liver, spleen, or lungs and therefore mobilisation has to be considered as a highly dynamic and complicated process(22,30,32). Therefore, the only accurate evaluation of the potential of endogenous mobilisation for fracture healing is with an in vivo fracture model.

Three mouse studies have also shown AMD3100 alone to improve bone formation(21,23,25,29), and this work in a rat model of delayed union, further identifies AMD3100 along as more effective than combining with growth factor pre-treatment. This may be due to physiological elevation of growth factors after fracture, including VEGF and IGF1(12,32,33), or that the mobilisation profile including cell types, timing of mobilisation and total numbers of cells are most beneficial with AMD3100 alone. Toupadakis et al. gave three sequential doses of AMD3100 after fracture and studies on the elution and
pharmacokinetics of AMD3100 show that serial administration will induce a peak mobilisation at one hour post treatment to the same level each time, suggesting that receptor/system desensitization does not occur (24,29). This may mean that the number of stem/progenitor cells available to home to the fracture site could be further increased, but this remains to be demonstrated as beneficial over a single dose. Another consideration that has not yet been addressed is the time of delivery of AMD3100, and presumably this has to be associated with SDF1 release at the fracture site and the maturity of tissue in the fracture gap. Consideration of the effect of CXCR4 blockade at the recipient fracture site also needs thought, as protracted treatment with AMD3100 throughout fracture healing or during distraction osteogenesis reduces healing(12,33-35). AMD3100’s short half-life of 0.9 hours in rodents (19) likely underlies the benefit of short lived blockade early in fracture healing, as it does not persist and inhibit ongoing migration of cells into the fracture site. AMD3100 therapy deserves further evaluation as the route to clinical translation is relatively simple, being already licensed for haematopoietic stem cell mobilisation.

IGF1 pretreated groups showed the development of a relatively more mineralized callus with a significant increase in % bone. Kumar et al. evaluated IGF1 with AMD3100 in a mouse model and showed a significant increase in fracture bone mineral density on DXA scan, similar to the % bone volume in this study. They also showed that IGF1 alone gave a moderate improvement in bone density, whereas AMD3100 alone did not(24,36). This is in contrast with this study and the differences may relate to peculiarities of their model and species. As the only other group with a significant increase in bone formation, IGF1 and AMD3100 combined may also warrant further investigation.

As VEGF preceding AMD3100 has previously been demonstrated in mouse models to release the largest number of MSCs, it was the hypothesized that maximal mobilisation of these cells would be facilitated by the administration of VEGF and this would lead to the greatest bone healing. However, pretreatment with this growth factor did not show a significant increase in healing unlike AMD3100 alone or pretreatment with IGF1. This would suggest that the differential mobilisation from this combination was less beneficial than the mobilisation profile from AMD3100 alone. As hypoxia and subsequent
vascularisation of tissues within the fracture site plays a crucial role in progressive fracture healing and VEGF is a potent angiogenesis promoter with a role in endochondral and intramembranous bone formation\(^{(22,34,35)}\), there was an expectation that VEGF would have beneficial effects. Indeed, local delivery of VEGF in rabbit mandibular defects showed increased density of bone formation, although not the quantity\(^{(36-38)}\). Histomorphometric assessment of vascularisation was not the objective in this study, but notably, the AMD3100 group had the highest number of blood vessels, although, significant differences were not detected between groups. The VEGF pre-treated group had the second highest percentage vascularised tissue, but the significance of that is difficult to know. In any case, there appears no significant advantage over AMD3100 for improving bone formation.

Pretreatment with GCSF prior to AMD3100 reduced fracture healing, which has not been previously shown. Interestingly, this group had a significant increase in % bone, which was indicative of a much smaller overall callus that proportionally had a higher bone volume component compared with controls. This bone region also had increased trabecular thickness and reduced porosity. All treatment groups, including GCSF-AMD had increased trabecular thickness indicating thicker woven bone formation, but in the GCSF-AMD group the smaller callus had bone present that was structurally more dense. The reduced bone volume may relate to the less mature chondrocytes seen histologically, indicative of delayed endochondral ossification, which may in turn be due to excessive inflammation from mobilised inflammatory cells. Increased presence of haematopoietic lineage osteoclast precursors, leading to bone reduction rather than deposition is also possible. Histologically, GCSF-AMD had the lowest level of bone, cartilage and vascular tissue, and the highest level of fibrous tissue, suggesting a pattern of reduced endochondral ossification, reduced blood supply and fibrous tissue development. Pitchford et al. showed that GCSF-AMD induced mild mobilisation of MSCs and EPCs, but was principally a very effective mobiliser of haematopoietic stem cells and neutrophils\(^{(22,39)}\). It is possible that the increased influx of neutrophils may have affected the progression of inflammation at the fracture site, preventing healing. CD34+ cells, which are a particularly well-represented population when mobilisation is performed with GSCF ± AMD3100, are considered a
population enriched in endothelial progenitors and haematopoietic stem cells. Transplantation of these cells, has shown improved healing in several studies\(^\text{(37,38,40)}\). However, this selected CD34+ population are a subset, which may explain the differences in healing seen, compared with mixed mobilised populations that include CD34+ cells. This has been borne out by studies showing a mixed GCSF mobilised mononuclear cell fraction being less efficacious than a sub-selected CD34+ population\(^\text{(39)}\) and excessive inflammation associated with the mononuclear cell population was suggested to be the cause. One study however, has shown improved fracture healing with GCSF treatment alone, given on five consecutive days. Interestingly, their study lasted 200 days and significant differences weren’t seen until at least 20-30 days, with a reduction in the osteotomy gap distance. Bone volume was significantly increased from around 30 days, but all rats went on to non-union\(^\text{(40)}\).

In conclusion, AMD3100 significantly increased fracture healing in a delayed union femoral model and was superior to protocols with growth factor pre-treatment. This would suggest that peak MSC mobilisation protocols previously identified are not solely beneficial for fracture healing, however, further work is required. In contrast, pre-treatment with GCSF, which preferentially mobilises haematopoietic stem cells and neutrophils had a negative effect on fracture healing and should be avoided. Further evaluation of the timing, dose, and frequency of administration of AMD3100 is warranted as it potentially offers a rapid route to clinical translation.

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References

1. Rodriguez-Merchan EC, Rodriguez-Merchan EC, Gomez-Castresana F, Gomez-Castresana F. Internal Fixation of Nonunions. Clin. Orthop. Relat. Res. 419, 13, 2004.

2. Nandra R, Grover L, Porter K. Fracture non-union epidemiology and treatment. Trauma. 7 ed. 18(1), 3, 2015.

3. Marsell R, Einhorn TA. The biology of fracture healing. Injury. 42(6), 551, 2011.

4. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating Skeletal Stem Cells. The Journal of Cell Biology. 153(5), 1133, 2001.

5. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res. 2(6), 477, 2000.

6. He Q, Wan C, Li G. THE OSTEOGENIC POTENTIAL OF A RAT PERIPHERAL BLOOD MONONUCLEAR CELL SUBSET. Journal of Bone & Joint Surgery, British Volume. 88-B(SUPP III), 392, 2006.

7. Alm JJ, Koivu HMA, Heino TJ, Hentunen TA, Laitinen S, Aro HT. Circulating plastic adherent mesenchymal stem cells in aged hip fracture patients. Journal of Orthopaedic Research. 28(12), 1634, 2010.

8. Ma X-L, Sun X-L, Wan C-Y, Ma J-X, Tian P. Significance of circulating endothelial progenitor cells in patients with fracture healing process. J Orthop Res. 30(11), 1860, 2012.

9. Peled A. Dependence of Human Stem Cell Engraftment and Repopulation of NOD/SCID Mice on CXCR4. Science. American Association for the Advancement of Science; 283(5403), 845, 1999.
10. Lee DY, Cho T-J, Lee HR, Park MS, Yoo WJ, Chung CY, et al. Distraction osteogenesis induces endothelial progenitor cell mobilization without inflammatory response in man. Bone. 46(3), 673, 2010.

11. Kidd LJ, Stephens AS, Kuliwaba JS, Fazzalari NL, Wu ACK, Forwood MR. Temporal pattern of gene expression and histology of stress fracture healing. Bone. Elsevier Inc; 46(2), 369, 2010.

12. Toupadakis CA, Wong A, Genetos DC, Chung D-J, Murugesh D, Anderson MJ, et al. Long-term administration of AMD3100, an antagonist of SDF-1/CXCR4 signaling, alters fracture repair. Journal of Orthopaedic Research. 30(11), 1853, 2012.

13. Kumagai K, Vasanji A, Drazba JA, Butler RS, Muschler GF. Circulating cells with osteogenic potential are physiologically mobilized into the fracture healing site in the parabiotic mice model. Journal of Orthopaedic Research. 26(2), 165, 2008.

14. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Circulating Bone Marrow-Derived Osteoblast Progenitor Cells Are Recruited to the Bone-Forming Site by the CXCR4/Stromal Cell-Derived Factor-1 Pathway. Stem Cells. John Wiley & Sons, Ltd; 26(1), 223, 2008.

15. Bendall LJ, Bradstock KF. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. Cytokine & Growth Factor Reviews. 2014.

16. Rosenkilde MM, Gerlach L-O, Jakobsen JS, Skerlj RT, Bridger GJ, Schwartz TW. Molecular Mechanism of AMD3100 Antagonism in the CXCR4 Receptor. Journal of Biological Chemistry. 279(4), 3033, 2004.

17. Wan Y. Bone marrow mesenchymal stem cells: Fat on and blast off by FGF21. International Journal of Biochemistry and Cell Biology. Elsevier Ltd; 45(3), 546, 2013.

18. Lévesque J-P, Hendy J, Takamatsu Y, Simmons PJ, Bendall LJ. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. J. Clin. Invest. 111(2), 187, 2003.
19. Hendrix CW, Flexner C, MacFarland RT, Giandomenico C, Fuchs EJ, Redpath E, et al. Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers. Antimicrob. Agents Chemother. 44(6), 1667, 2000.

20. Calandra G, McCarty J, McGuirk J, Tricot G, Crocker S-A, Badel K, et al. AMD3100 plus G-CSF can successfully mobilize CD34+ cells from non-Hodgkin’s lymphoma, Hodgkin’s disease and multiple myeloma patients previously failing mobilization with chemotherapy and/or cytokine treatment: compassionate use data. Bone Marrow Transplant. 41(4), 331, 2008.

21. Wang XX, Allen RJ, Tutela JP, Sailon A, Allori AC, Davidson EH, et al. Progenitor cell mobilization enhances bone healing by means of improved neovascularization and osteogenesis. Plast. Reconstr. Surg. 128(2), 395, 2011.

22. Pitchford SC, Furze RC, Jones CP, Wengner AM, Rankin SM. Differential mobilization of subsets of progenitor cells from the bone marrow. Cell Stem Cell. 4(1), 62, 2009.

23. McNulty MA, Virdi AS, Christopherson KW, Sena K, Frank RR, Sumner DR. Adult stem cell mobilization enhances intramembranous bone regeneration: a pilot study. Clin. Orthop. Relat. Res. 470(9), 2503, 2012.

24. Kumar S, Ponnazhagan S. Mobilization of bone marrow mesenchymal stem cells in vivo augments bone healing in a mouse model of segmental bone defect. Bone. 50(4), 1012, 2012.

25. Toupadakis CA, Granick JL, Sagy M, Wong A, Ghassemi E, Chung D-J, et al. Mobilization of endogenous stem cell populations enhances fracture healing in a murine femoral fracture model. Cytotherapy. 15(9), 1136, 2013.

26. Harrison LJ, Cunningham JL, Strömberg L, Goodship AE. Controlled induction of a pseudarthrosis: a study using a rodent model. J Orthop Trauma. 17(1), 11, 2003.
27. Müller ME, Allgöwer M, Schneider R, Willenegger H. Manual of Internal Fixation. springer.com. Berlin, Heidelberg: Springer Berlin Heidelberg; 1979.

28. Mills LA, Aitken SA, Simpson AHRW. The risk of non-union per fracture: current myths and revised figures from a population of over 4 million adults. Acta Orthop. 1st ed. **88**(4), 434, 2017.

29. Broxmeyer HE, Orschell CM, Clapp DW, Hangoc G, Cooper S, Plett PA, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J. Exp. Med. **201**(8), 1307, 2005.

30. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. Nat. Rev. Immunol. **6**(2), 93, 2006.

31. Fricker SP. Physiology and Pharmacology of Plerixafor. Transfusion Medicine and Hemotherapy. **40**(4), 237, 2013.

32. Simpson AHRW, Mills L, Noble B. The role of growth factors and related agents in accelerating fracture healing. J Bone Joint Surg Br. **88**(6), 701, 2006.

33. Xu J, Chen Y, Liu Y, Zhang J, Kang Q, Ho K, et al. Effect of SDF-1/Cxcr4 Signaling Antagonist AMD3100 on Bone Mineralization in Distraction Osteogenesis. Calcif Tissue Int. **100**(6), 641, 2017.

34. Street J, Bao M, deGuzman L, Bunting S, Peale FV, Ferrara N, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci USA. **99**(15), 9656, 2002.

35. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med. **5**(6), 623, 1999.

36. Kleinheinz J, Stratmann U, Joos U, Wiesmann H-P. VEGF-Activated Angiogenesis During Bone Regeneration. Journal of Oral and Maxillofacial Surgery. **63**(9), 1310, 2005.
37. Kuroda R, Matsumoto T, Kawakami Y, Fukui T, Mifune Y, Kurosaka M. Clinical Impact of Circulating CD34-Positive Cells on Bone Regeneration and Healing. Tissue Eng Part B Rev. 20(3), 190, 2014.

38. Matsumoto T, Kuroda R, Mifune Y, Kawamoto A, Shoji T, Miwa M, et al. Circulating endothelial/skeletal progenitor cells for bone regeneration and healing. Bone. 43(3), 434, 2008.

39. Fukui T, Mifune Y, Matsumoto T, Shoji T, Kawakami Y, Kawamoto A, et al. Superior Potential of CD34-Positive Cells Compared to Total Mononuclear Cells for Healing of Nonunion Following Bone Fracture. cell transplant. 24(7), 1379, 2015.

40. Herrmann M, ZEITER S, Eberli U, Hildebrand M, Camenisch K, Menzel U, et al. Five Days Granulocyte Colony-Stimulating Factor Treatment Increases Bone Formation and Reduces Gap Size of a Rat Segmental Bone Defect: A Pilot Study. Front. Bioeng. Biotechnol. 6, 5, 2018.
Table 1: AO-ASIF global radiographic healing score from the mediolateral radiograph at five weeks post surgery.

| Group          | Not-united | Uncertain | United |
|----------------|------------|-----------|--------|
| 1.5mm Control  | 3/7 (43%)  | 2/7 (29%) | 2/7 (29%) |
| PBS-AMD        | 1/5 (20%)  | 0/5 (0%)  | 4/5 (80%) |
| VEGF-AMD       | 2/8 (25%)  | 2/8 (25%) | 4/8 (50%) |
| GCSF-AMD       | 3/5 (60%)  | 0/0 (0%)  | 2/5 (40%) |
| IGF1-AMD       | 2/6 (33%)  | 1/6 (17%) | 3/6 (50%) |
Table 2: MicroCT quantitative morphology data from the central 60% of the osteotomy at five weeks. Significant results (p<0.05) are shown in bold.

|           | 1.5mm | Control  | PBS-AMD | VEGF-AMD | GCSF-AMD | IGF1-AMD |
|-----------|-------|----------|---------|----------|----------|----------|
| **TV**    | (μm³) | 9.23±6.14| 15.28±3.61| 10.03±3.22| 4.33±4.72| 9.08±7.57|
| **BV**    | (μm³) | 4.31±3.08| **8.91±2.16**| 5.22±1.71| 2.50±2.60| 5.11±4.21|
| **TV/BV** | (%)   | 53.79±20.82| 58.51±6.06| 52.52±5.85| **63.07±7.29**| **59.24±5.58**|
| **TS**    | (μm²) | 62.83±45.55| 60.32±14.75| 63.56±19.88| 34.24±27.19| 39.77±30.77|
| **BS**    | (μm²) | 326.15±220.0| 450.92±121.4| 355.52±130.1| 133.83±147.2| 269.57±232.9|
| **Tb.Th** | (μm)  | 0.04±0.01| **0.06±0.00**| 0.05±0.01| **0.07±0.03**| **0.06±0.01**|
| **Tb.Sp** | (μm)  | 0.07±0.03| 0.09±0.03| 0.08±0.02| 0.06±0.02| 0.08±0.03|
| **Tb.N**  | (1/μm) | 14.09±9.32| 9.57±1.01| 10.99±1.08| 10.49±4.88| 9.71±1.30|
| **Total** | Porosity (%) | 46.21±20.82| 41.49±6.06| 47.48±5.85| **36.93±7.29**| **40.76±5.58**|
Figure 1: The mean±SEM tissue volume (TV) and bone volume (BV) within the osteotomy measured using microCT. * represents significant (p<0.05) differences compared with 1.5mm control. **,***,****,****** indicate significant differences (p<0.05) between paired groups.
Figure 2: The mean±SEM trabecular thickness (Tb.Th) and separation distance (Tb.Sp) of bone formed within the osteotomy measured using microCT. * represents significant (p<0.05) differences compared with 1.5mm control. ** and *** indicate significant differences (p<0.05) between different groups.
Figure 3: MicroCT 3D reconstructions of mid femoral regions, with a mid-sagittal reveal (top row). The middle row shows a 3D reconstruction of the central 60% of the original osteotomy region (180 slices). A representative H and E stained histology image of the central region of the fracture is also shown. Scale bar in lower left hand corner presents 500μm in all histology images.
Figure 4: Mean±SEM percentage tissue formed within the osteotomy from 2.5x magnification histomorphometry.
Figure 5: Histology of the central region of the fracture callus (H and E), showing a large area of cartilage and hypertrophic chondrocytes adjacent to osteoid in the control; reduced cartilage, and increased woven bone formation in the PBS-AMD group, indicating increased endochondral ossification, and a highly cellular, granulation tissue in the GCSF-AMD group. Scale bar in lower right hand corner represents 100μm in all images.