A systemic provascular response in bone marrow to musculoskeletal trauma in mice

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Post-natal vasculogenesis, the process by which vascular committed bone marrow stem cells or endothelial precursor cells migrate, differentiate and incorporate into the nascent endothelium and thereby contribute to physiological and pathological neovascularisation, has stimulated much interest. Its contribution to neovascularisation of tumours, wound healing and revascularisation associated with ischaemia of skeletal and cardiac muscles is well established. We evaluated the responses of endothelial precursor cells in bone marrow to musculoskeletal trauma in mice.

Bone marrow from six C57 Black 6 mice subjected to a standardised, closed fracture of the femur, was analysed for the combined expression of cell-surface markers stem cell antigen 1 (sca-1⁺) and stem cell factor receptor, CD117 (c-kit⁺) in order to identify the endothelial precursor cell population. Immunomagnetically-enriched sca-1⁺ mononuclear cell (MNC^{sca-1+}) populations were then cultured and examined for functional vascular endothelial differentiation. Bone marrow MNC^{sca-1+,c-kit+} counts increased almost twofold within 48 hours of the event, compared with baseline levels, before decreasing by 72 hours.

Sca-1⁺ mononuclear cell populations in culture from samples of bone marrow at 48 hours bound together Ulex Europus-1, and incorporated fluorescent 1,1'-dioctadecyl- 3,3,3',3' - tetramethylindocarbocyanine perchlorate-labelled acetylated low-density lipoprotein intracellularly, both characteristics of mature endothelium.

Our findings suggest that a systemic provascular response of bone marrow is initiated by musculoskeletal trauma. Its therapeutic manipulation may have implications for the potential enhancement of neovascularisation and the healing of fractures.

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©2007 British Editorial Society
of Bone and Joint Surgery
doi:10.1302/0301-620X.89B1.
18222 \$2.00

J Bone Joint Surg [Br]
2007;89-B:116-20.

Received 6 June 2006;
Accepted after revision 8
August 2006

Healing of fractures is a complex phenomenon of repair which recapitulates many aspects of skeletal development in the embryo. Endochondral and intramembranous ossification, two distinct interactive responses, which are ultimately responsible for bone healing, require an adequate blood supply. Neovascularisation of the fracture is therefore a prerequisite for normal healing. The formation of new blood vessels in adults has traditionally been understood to result exclusively from angiogenesis, the process of local proliferation, migration, and remodelling of endothelial cells (ECs) from a mature pre-existing endothelium.^{1,2} Efforts to enhance neovascularisation by supplementation with recognised pro-angiogenic cytokines have yielded promising results.³⁻⁶ However, the resident population of ECs which is responsive to available levels of angiogenic growth factors may potentially limit the extent to which supplementation enhances tissue neovascularisation. Situations which compromise endothelial function and angiogenesis are well recognised

as contributing to delayed healing of the fracture and atrophic nonunion. These include diabetes mellitus,⁷⁻¹⁰ hypercholesterolaemia,¹¹ smoking^{12,13} and advancing age.^{14,15}

The recent recognition that vasculogenesis, a process by which stem cells in bone marrow which are committed to the vascular endothelial lineage, termed endothelial precursor cells (EPCs), can migrate from the marrow to a site of developing neovasculature in adults, has stimulated much interest.¹⁶⁻¹⁸ This was previously believed to be an exclusively embryonic process. However, EPCs have now been shown to differentiate and incorporate into the nascent endothelium at sites of post-natal physiological and pathological formation of new vessels.¹⁸

Dual cell-surface antigen expression (stem cell antigen 1 (sca-1⁺) and CD117, stem cell factor receptor (c-kit⁺)) identifies a population of murine EPCs which participate in neovascularisation and give rise to functional endothelial cells in models of ischaemia.¹⁹⁻²² Our aims

Fig. 1

Diagram of stem cell antigen 1 (sca-1)(ly6A/E)+ CD117, stem cell factor receptor (c-kit) + dual-positive quadrants. Samples were subjected to a two-dimensional side-scatter fluorescence dot-plot analysis.

were to demonstrate a systemic vascular cellular response in bone marrow to the fracture of long bones by the mobilisation of sca-1⁺/c-kit⁺ mononuclear cells (MNCs^{sca-1+,c-kit+}) and to demonstrate their differentiation into phenotypic and functional endothelial cells.

Materials and Methods

Murine fracture model. A murine fracture model was used to examine systemic changes in bone marrow in response to musculoskeletal trauma. Six C57 Black 6 female mice (Jackson Laboratories, Bar Harbour, Maine) were randomised to an unfractured control group, while six mice per time point were randomised to a group with an isolated closed fracture of the shaft of the right femur produced by using a standardised three-point loading technique.²³ The recognised model for this involved intramedullary fixation of the femur before fracture.²³ However, in view of the surgical trauma involved in inserting this device, a confounding traumatic stimulus in itself, the fracture group had external splinting of the extended injured hind limb provided by the application of bulky circumferential tape (3M micropore, 3M, Aycliffe, Co. Durham). All the procedures were performed under licence from the Department of Health of Ireland and with the approval of the Ethics Committee of Cork University Hospital.

The mice were anaesthetised with intraperitoneal xylazine (10 mg/kg). Their right hip, thigh and knee were shaved. A three-point loading technique was used to create a consistent, closed, comminuted fracture of the mid-shaft of the femur.²³ Post-operatively, all the animals received oral ketoprofen (2.5 mg/kg to 5.0 mg/kg 12-hourly) for relief from pain and all were housed individually. After 24, 48 and 72 hours, all animals were killed by cervical dislocation. An identical protocol, excluding the fracture was followed for the control group.

Bone marrow harvest and precursor quantification by flow cytometric analysis. The right femur was dissected to confirm the presence of a fracture and the intact left femur and both tibiae were harvested. In order to exclude any local response of the bone marrow to the injury, the marrow was not retrieved from the fractured limb. A 18G cannula was introduced into the marrow cavity of each of the other bones distally, and the marrow was flushed of its contents with 5 ml of phosphate-buffered saline (PBS) and 2% fetal bovine serum (FBS).²¹ The suspension comprising the combined marrow contents for each individual was then filtered to remove any bony debris, centrifuged at 400 g for ten minutes and resuspended in 3 ml of medium.²¹ The mononuclear cell layer was then isolated by density-gradient centrifugation, washed twice in PBS and resuspended in PBS to a concentration of 10⁷ cells/ml.

Two cell markers were used for the identification of the EPCs. These were chosen based upon their documented roles in vasculogenesis.^{19,22} The sca-1 protein (or lymphocyte activation protein-6A (ly-6A)) was originally identified as an antigen with increased expression on the surface of activated lymphocytes which facilitates cell signalling.²⁴⁻²⁶ The C-kit receptor tyrosine kinase plays an essential role in regulating proliferation, survival and migration of haematopoietic stem and progenitor cells.^{20,27,28}

A viable cell count was performed using Trypan Blue (Stemcell Technologies Inc., Vancouver, Canada) and the cells were resuspended in the medium to 10⁵ cells/ml. The sca-1(lymphocyte antigen complex locus A/EE ly6A/E)+c-kit(cluster degeneration (CD) antigen 117 (CD117))+population was quantified at all time points. Mononuclear cells were stained with a phycoerythrin (PE)-conjugated anti-sca-1 and fluorescein-isothiocyanate (FITC)-conjugated anti-c-kit antibodies. To avoid non-specific staining, cells were stained with PE- and FITC-conjugated mouse immunoglobulin G1 (I_gG₁). The samples were subjected to a two-dimensional side scatter-fluorescence dot-plot analysis (FACS-can; Becton-Dickinson, Oxford, United Kingdom). After appropriate gating, the sca-1(ly6A/E)+c-kit(CD117)+cell population was quantified and expressed as the number of cells per 10⁶ white blood cells (Fig. 1).

Immunomagnetic-negative selection for murine MNC^{sca-1+} and culture. Bone marrow was harvested after 48 hours as described. The cells were resuspended to 5 × 10⁷ nucleated cells/ml in PBS. A centrifugation-separation technique (Stemcell Technologies) was used for murine haematopoietic progenitor cell enrichment. An antibody cocktail bound the unwanted cell populations which were pelleted on centrifugation.²¹ The enriched cells formed a band at the centrifuge density medium-PBS interface and were removed into a separate tube in which they were washed with PBS and centrifuged at 200 g. The pellet was resuspended in PBS and 2% FBS. The enriched MNCs (5 × 10³ cells/ml) were cultured on fibronectin (2.5 µg/ml)-coated chambers (Bio-coat; Becton-Dickinson) in an endothelial basal cell medium (Clonetics, Cambrex Bioscience, Baltimore Inc.,

Fig. 2

Graph showing the time course of dual-positive bone marrow, mononuclear cell (MNC) counts expressing stem cell antigen 1 and c-kit antigen (n = 6 per group).

Baltimore, Maryland) containing 2% FBS and antibiotics (streptomycin and penicillin) in 5% CO₂ at 37°C.²⁹ The culture medium was changed every 48 hours. After seven days, cultures were assessed for the functional properties of mature ECs by incubation in 10 µg/ml of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanineperchlorate-labelled low-density lipoprotein (DiI-Ac-LDL; Molecular Probes, Eugene, Oregon) for four hours at 37°C. Endothelial cells incorporating DiI-Ac-LDL degrade the lipoprotein and the DiI (fluorescent probe) accumulates in the intracellular membranes. Similarly, cells were also incubated with FITC-conjugated Ulex Europeus-1 (UEA-1; Santa Cruz Biotechnology, Santa Cruz, California) at 50 µg/ml for one hour at 37°C to determine membrane binding, a functional property of the vascular endothelium.^{19,21,30}

Statistical analysis. Data were subjected to one-way analysis of variance and pairwise multiple comparison procedures (Holm-Sidak method (Systat Software Inc., San Jose, California)) to determine statistical significance. Values were expressed as the mean and standard deviation, and a p-value < 0.05 was considered to be significant.

Results

Figure 2 gives the time course of dual-positive bone-marrow MNC^{sca-1/c-kit+} expression in the study group. MNC^{sca-1/c-kit+} levels in the bone marrow increased from 1.16% (SD 0.19) at the baseline control level to 1.72% (SD 0.41) at 24 hours (p = 0.007), peaking at 48 hours after fracture (1.99% (SD 0.36, p = 0.0002) and decreasing at 72 hours (1.54 SD 0.29 p = 0.06).

Immunomagnetic negative selection yielded MNC^{sca-1+} cell-enriched populations in excess of five times that of the baseline levels (less than 2.0%).

From cultured bone-marrow MNCs^{sca-1/c-kit+} numerous cells were found to incorporate fluorescent DiI-Ac-LDL and to bind UEA-1, functional properties specific to mature endothelial cells (Fig. 3).³

Fig. 3a

Fig. 3b

Photomicrographs showing a) spindle cells incorporating fluorescent 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanineperchlorate-labelled low density lipoprotein, and b) binding fluorescent Ulex Europeus 1.

Discussion

While angiogenesis has traditionally been accepted as the only process of adult vascular endothelial turnover, the description of the isolation of progenitor ECs by Asahara et al¹⁶ has provoked much interest in the concept of post-natal vasculogenesis and the ability of cells derived from adult bone marrow to contribute to the formation of new blood vessels. Experiments with murine bone marrow transplants have demonstrated the incorporation of bone-marrow-derived EPCs into foci of physiological and pathological neovascularisation in growing tumours, healing wounds and ischaemic skeletal and cardiac muscles.¹⁸ Matrigel wound models have demonstrated that up to 22% of new vessel endothelium is derived from bone marrow.³¹

The vasculogenic responses of bone marrow to musculoskeletal trauma have not been previously evaluated. We hypothesised that musculoskeletal trauma acts as a stimulus for the production of cells in the bone marrow capable of differentiating into phenotypic and functionally mature ECs. Using a standardised murine closed-fracture model, we sought to quantify this response and to determine the differentiation of the ECs.

Jackson et al²² found by transplantation of bone marrow the lacZ gene-positive sca-1⁺/c-kit⁺ cells could participate in myocardial revascularisation after the induction of ischaemia by occlusion and reperfusion of coronary arteries. Bailey et al¹⁹ demonstrated that adult bone-marrow-derived stem cells (c-kit⁺, sca-1⁺) gave rise to functional ECs.

We quantified dual-positive MNC expression at 72 hours after the inciting event. Bone-marrow MNC^{sca-1⁺,c-kit⁺} counts increased significantly at 24 hours compared with control baseline levels, peaking at 48 hours. This elevation persisted at 72 hours, but not to a significant level ($p = 0.06$).

Because MNC^{sca-1⁺,c-kit⁺} gave rise to EPCs, we attempted to demonstrate differentiation of ECs on cell culture. In our study, bone-marrow MNCs enriched by immunomagnetic selection for sca-1 in culture expressed EC lineage functional activity (UEA-1 binding and DiI-Ac-LDL uptake) consistent with that of mature ECs.

The molecular mechanisms responsible for vasculogenesis are not completely understood. Gene-targeting studies have shown vascular endothelial growth factor (VEGF) to be essential for embryonic vasculogenesis.³²⁻³⁴ Evidence supporting a role in modulating adult EPC kinetics has also been established. Intraperitoneal administration of recombinant VEGF gives a marked increase in MNCs in peripheral blood, both positive for EC-specific antigens and Ac-LDL intracellular incorporation. Neutralising antibody against recombinant human (rh)VEGF protein completely attenuates these effects.¹⁷ VEGF has also been demonstrated to enhance in a dose-dependent manner the proliferation and migration of EPCs in culture as well as to exert chemotactic activity on mononuclear cells in bone marrow.^{17,35}

Our study has a number of limitations. First, despite demonstrating their mobilisation within the bone marrow, we do not know whether EPCs participate in neovascularisation at the site of musculoskeletal trauma. We have previously demonstrated an increase in circulating peripheral blood EPCs after closed tibial fracture in humans,³⁶ but because of the problems in obtaining healing fracture tissue (callus) from human patients and distinguishing bone-marrow-derived ECs from native ECs, this may be difficult to determine. Studies with lac Z (endothelium specific receptor for angioprotein 1) tie-2 and gene positive allogenic bone-marrow transplantation are necessary to confirm that bone-marrow derived EPCs do indeed participate in neovascularisation. Also, in acknowledging experimental evidence of a vasculogenic response to muscle and vascular injury, it is difficult to differentiate injury to bone or to soft tissue as the primary stimulus. We can therefore only conclude that there is a response to musculoskeletal trauma.

We have explored in isolation the neovascularisation component of bone healing. Bone-marrow progenitor cell

responses to musculoskeletal injury would not, however, be expected to be confined to the vascular component of bone healing. Shirley et al³⁷ have used a rabbit ulnar osteotomy model to trace the movement of fluorescently-labelled osteogenic cells from tibial bone marrow. The data suggest that some osteoblasts involved in fracture healing were systemically mobilised and recruited to the fracture from remote sites of bone marrow.³⁷ While the molecular mechanisms responsible for osteoprogenitor mobilisation have not been explored, a role for VEGF has been determined in both endochondral and intramembranous ossification at various stages of fracture healing.⁵

We have demonstrated that bone marrow EPCs^(sca-1⁺,c-kit⁺) with the potential to differentiate into functional endothelium are mobilised after acute musculoskeletal trauma. While recent studies have demonstrated a therapeutic role for angiogenic cytokines in promoting classical angiogenesis, in the absence of a competent responsive population of ECs the effect of administering these cytokines will be limited.

By the manipulation of EPC kinetics, the systemic vasculogenic response demonstrated in our study may offer an alternate therapeutic pathway in the management of aberrant wound and fracture healing.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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