Introduction: It is well established that the MSCs reside among the stromal compartment of bone marrow and can differentiate into functional osteoblasts under specific conditions. The presence of primitive hematopoietic cells in adult peripheral blood has been recognized for three decades. However, there is a long-standing controversy as to whether MSCs can migrate through the circulation. A number of studies suggested the presence of circulating MSCs in the peripheral blood of pig, mouse, rat and human peripheral blood, but there is no report on the presence of circulating MSCs during bone repair processes such as fracture. Utilising refined isolation procedures and molecular and immunohistochemical examinations we examined the presence of osteoblastic and osteoprogenitor cells in peripheral blood of fracture patients and non-fracture controls. We investigated the expression of osteoblast differentiation related genes (alkaline phosphatase, osteocalcin, osteonectin, bone sialoprotein, Cbfα-1, BMP-2, -4, -6, BMPR1, BMPR2) and other genes related to signal transduction pathway activation (caveolin-1 and p90) in human blood mononuclear cells.

Methods: 30 ml of peripheral blood was taken from 5 healthy donors at one time point, and from seven patients with tibial or femoral fracture at 3 time points following fracture, days 1-3, 9-12 and 16-21. Patients were randomly selected, with mean age of 31, and presented with no known metabolic bone disease and were not under long-term medication. The peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Lymphoprep density gradient (1.077 g/ml, Nycomed). 1/3 PBMCs were immediately spanned onto glass slide and fixed, subject to immunocytochemistry (ICC) with various antibodies. 1/3 of PBMCs were cultured in DMEM, supplemented with 10% FCS, antibiotics, 50 mg/ml ascorbic acid, 10 mM L-glycerophosphate, 10-8 M dexamethsone, at 37°C in 5% CO2. Half of the medium was changed at 7 days and twice weekly thereafter. After 3 weeks, adherent cells were mobilized with trypsin/EDTA, and cultured in 8 well chamber slides for a week before they were fixed and used for ICC with various antibodies. RNA was extracted from the remaining 1/3 PBMCs within 4 hours of collection using the RNeasy Midi kit (Qiagen, UK). RNA integrity was verified by ethidium bromide staining of 28 S and 18 S ribosomal RNAs. Gene expression of bone cell differentiation markers alkaline phosphatase, osteocalcin, bone sialoprotein, Cbfα-1, BMP-2, -4, -6, BMPR1, BMPR2, stromal derived factor (SDF) 1 and 2, and other genes including caveolin-1, p90/80K-H, VEGF, and the housekeeping genes acidic ribosomal protein (ARP) and b-actin was performed using semi-quantitative RT-PCR. Human osteoblastic cells derived from explants were used as positive controls in all experiments.

Results: The ICC results vary for the blood samples collected prior to cell culture. The PBMCs in about two thirds of the blood samples from fracture patients showed the presence of positive staining for Cbfα-1, BMP-2, BMPR-I or II, Endoglin, Collagen type I, and Vimentin. In contrast, there was little or no staining of these markers in the PBMCs from the controls. In the fracture patients' PBMCs culture, numerous fibroblastic cells adhered to the flasks at the first week. The greatest number of adherent cells was found in the blood samples taken at 14-20 days post-fracture. After a period of 2 weeks, these cells were in spindle or round shapes, but did not form colonies in culture. ICC examinations confirmed that many of these culture-expanded cells were positive for Cbfα-1, BMP-2, BMPR-I or II, Endoglin. Type I collagen, osteocalcin and Vimentin, but were negative for alkaline phosphatase. In contrast, we failed to observe any fibroblastic cell formation in the culture of PBMCs from the controls, and the adherent cells at the end of culture period were negative for most of the markers mentioned above.

For RT-PCR results, Alkaline phosphatase mRNA expression in PBMCs was significantly upregulated in the PBMCs of fracture patients when compared with non-fracture controls at all time points following fracture. Bone sialoprotein and osteocalcin was only expressed in the osteoblast positive controls, but not detected in any of the PBMCs examined. Expression of BMP-2, -4 and -6 was observed in all cell types investigated, although there was considerable variation in the regulatory potential of the genes in the cells obtained from control volunteers and fracture patients. PBMCs demonstrated marked upregulation of BMP-2 gene in fracture patients in a time-dependent manner. BMPR1 was expressed in all PBMCs samples but BMPR2 was only expressed in the PBMCs of fracture patients. The expression of SDF-1 in all PBMCs was negative but for SDF-2 was positive. Caveolin-1 and P90/80K-H (a phosphoprotein involved in the signal transduction pathway linked to the fibroblast growth factor 3 receptor) mRNA expression was significantly increased in PBMCs from the fracture patients. PBMCs from fracture patients also expressed OSF-1 and Cbfα-1 to varying degrees. Housekeeping genes ARP and b-actin expression was unchanged between sample groups.

Discussion: This study has confirmed for the first time that patients with a recent fracture have an increase in the number of circulating MSCs in their peripheral blood. However, it is uncertain whether these circulating MSCs arise at the fracture site and leak into blood, or arise distally from the bone marrow or other sites in response to the fracture. Further study is on the way to clarify the links between trauma stimuli and the blood-borne MSCs. The gene expression of a number of osteogenic differentiation related markers, and genes implicated in signal transduction pathways, suggest a role for circulating cells in the peripheral blood contributing to the fracture healing process. Cells in the peripheral blood expressing BMP-2 may also contribute the early induction of osteoblastic cells to osseous precursors.

The identification of such precursor cells in blood may be of therapeutic significance, may contribute to the understanding of the bone repair process and suggest new treatment concepts for fracture and bone defects.

PRESENCE OF MESENCHYMAL STEM CELLS (MSCS) AND CHANGES OF GENE EXPRESSION OF OSTEOGENIC MARKERS IN HUMAN PERIPHERAL BLOOD OF PATIENTS WITH LONG BONE FRACTURE

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