An *in vitro* study demonstrating that haematomas found at the site of human fractures contain progenitor cells with multilineage capacity

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We isolated multilineage mesenchymal progenitor cells from haematomas collected from fracture sites. After the haematoma was manually removed from the fracture site it was cut into strips and cultured. Homogenous fibroblastic adherent cells were obtained. Flow cytometry revealed that the adherent cells were consistently positive for mesenchymal stem-cell-related markers CD29, CD44, CD105 and CD166, and were negative for the haemopoietic markers CD14, CD34, CD45 and CD133 similar to bone-marrow-derived mesenchymal stem cells. In the presence of lineage-specific induction factors the adherent cells could differentiate *in vitro* into osteogenic, chondrogenic and adipogenic cells.

Our results indicate that haematomas found at a fracture site contain multilineage mesenchymal progenitor cells and play an important role in bone healing. Our findings imply that to enhance healing the haematoma should not be removed from the fracture site during osteosynthesis.

The haematoma occurring at a fracture site is known to play an important role in bone healing.¹ Mizuno et al² have reported that the fracture haematoma has an inherent osteogenic potential which contributes significantly to fracture healing. Grundnes and Reikeras³ reported that removal of an organised haematoma some days after fracture impaired bone healing. It is clear that within the haematoma there is a bone-forming complex that comprises cells and growth factors including platelet-derived growth factor (PDGF), acidic and basic fibroblast growth factors (aFGF and bFGF), the transforming growth factor-β polypeptide group (TGF-β), and insulin-like growth factor (IGF).³,⁵ Previous studies have confirmed that these are central regulators of cellular proliferation, differentiation and matrix synthesis during the fracture healing process.³,⁵ However, the literature shows no reports investigating the cells present in a fracture haematoma.

When a fracture occurs, haematoma forms at the site which, in the subsequent process of fracture healing, is associated with bone formation by two different mechanisms, intramembranous and endochondral. The two types serve specific purposes. Intramembranous bone formation is limited to a ‘collar’ of bone surrounding the fracture site, whereas endochondral ossification replaces cartilage with bone within the sheath of the intra-membranous bone at the site of the haematoma, crossing or bridging the gap between fragments of bone. The progression of endochondral bone formation in the callus to the point of bridging the fracture gap is the clinical standard for fracture healing. However, the cells from which the osteoblast and chondrocyte originate are unknown. We hypothesised that one of the origins of these cells is the haematoma. Several papers have reported that fracture haematoma contains mesenchymal cells,¹⁰-¹² so it is assumed that such haematomas are at least a reservoir for mesenchymal progenitors, if not the actual source. However, there are no reports of detailed cellular analysis. In this *in vitro* study we investigated whether fracture haematoma cells (HCS) had the capacity for multilineage mesenchymal differentiation.

**Patients and Methods**

Fracture haematomas were obtained from five consecutive male patients with a mean age of 55.3 years (34 to 66) during osteosynthesis a mean of 4.2 days (2 to 8) after sustaining a fracture. The fracture sites involved were the patella in two patients, the clavicle in one, the ulna in one and the tibia in one. Patients taking anticoagulants, steroids or non-steroidal anti-inflammatory drugs in the three months prior to injury were excluded. Informed consent was obtained from all patients. The project was approved by the Ethics of Human Experiments at the Faculty of Medicine, Kobe University.
Isolation and culture of haematoma cells. Following exposure of the fracture site, haematoma containing organised fibrin clots was removed manually before any manipulation or irrigation, and placed in sterile polypropylene containers. The mean weight of haematoma obtained was 1.1 g (0.4 or irrigation, and placed in sterile polypropylene containers. Fibrin clots was removed manually before any manipulation

Sure of the fracture site, haematoma containing organised

Of 15.8 days (13 to 19) later, the adherent cells were harvested after the first passage and a total of $5 \times 10^5$ was resuspended with PBS-3% FBS and incubated with the phycoerythrin (PE)-conjugated antibodies for 30 minutes at 4°C. The fluorescence intensity of the cells was analysed using a fluorescence activated cell sorting (FACS aria) flow cytometry system (BD Biosciences). In order to compare the data with those of bone marrow-derived mesenchymal stem cells (BM-derived MSCs), BM samples were obtained with informed consent from five separate patients with a mean age of 45.6 years (15 to 75) who were undergoing hip or knee surgery, and cultured under the same conditions as the HCs (Table I). The samples were scored as ++ if more than 75% of the cells were positive, + if more than 40% but less than 75% were positive, - if less than 1% were negative for a specific antibody.

### Differentiation of haematoma cells

**Osteogenic induction.** Adherent cells were cultured for 21 days in an osteogenic medium consisting of the original medium, plus 10 nM dexamethasone (Dex)(Sigma), 10 mM β-glycerophosphate (Sigma) and 50 µg/ml ascorbic acid (Wako). After three weeks, osteogenic differentiation was evaluated by calcium deposition, which was stained with 1% alizarin red S (Hartman Leddon, Philadelphia, Pennsylvania). Expression of osteoblast-related genes, alkaline phosphatase (ALP) and osteocalcin was also measured by reverse transcription polymerase chain reaction (RT-PCR). After three weeks, ALP activities were assayed. The cell layer from each well was washed twice with PBS, sonicated with a Microson Ultrasonic Cell Diaruptor XL2000 (Misonix, Farmingdale, New York) and stored at -20°C until assayed for ALP activity. ALP activity was measured by optically monitoring the change in p-nitrophenyl phosphate solution density at 410 nm caused by the ALP-mediated release A p-nitrophenol. The optical densities obtained were compared with a p-nitrophenol standard solution (Sigma). Protein concentration in the sonicate was measured using a DC Protein Assay kit (BIO-Rad, Hercules, California) with the results expressed as p-nitrophenol produced in nmol/mg of protein.

**Chondrogenic induction.** For chondrogenic differentiation, a pellet culture was performed for three-dimensional culture. About $2.5 \times 10^5$ cells in the 15 ml polypropylene tube were centrifuged at 2000 rpm for four minutes to form a pellet. The cells were resuspended in chondrogenic medium consisting of high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, California) with $10^{-7}$ M Dex, 50 µg/ml l-ascorbic acid-2-phosphate (Sigma), 0.4 mM proline (Sigma), 1% ITS*1

### Table I. Immunophenotypical comparison of fracture haematoma cells (HCs) and bone marrow-derived mesenchymal stem cells (BM-MSCs)

<table>
<thead>
<tr>
<th>Cell-surface markers</th>
<th>HCs</th>
<th>BM-MSCs</th>
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<tbody>
<tr>
<td>CD14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD29</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD34</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD44</td>
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<td>++</td>
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<tr>
<td>CD45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD105</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CD133</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD166</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>*</sup>, less than 1% were negative for a specific antibody; +, more than 40% but less than 75% were positive; ++, more than 75% cells were positive

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**Immunophenotyping of haematoma cells by flow cytometry.** Antibodies against cluster differentiation (CD)29, CD34, CD45, CD133 and CD166 were purchased from BD Biosciences (San Jose, California). Antibody against CD105 was purchased from Ancell (Bayport, Minnesota), and antibodies against CD14 and CD44 were purchased from Exalpha Biologicals (Wartown, Massachussets). The adherent cells were harvested after the first passage and the cells were analysed using a fluorescence activated cell sorting (FACS aria) flow cytometry system (BD Biosciences). In order to compare the data with those of bone marrow-derived mesenchymal stem cells (BM-derived MSCs), BM samples were obtained with informed consent from five separate patients with a mean age of 45.6 years (15 to 75) who were undergoing hip or knee surgery, and cultured under the same conditions as the HCs (Table I). The samples were scored as ++ if more than 75% of the cells were positive, + if more than 40% but less than 75% were positive, - if less than 1% were negative for a specific antibody.
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(Sigma), 10 ng/ml recombinant human TGF-β3 (R&D Systems, Minneapolis, Minnesota), and 500 ng/ml recombinant human bone morphogenic protein-6 (BMP-6) (Sigma). After 21 days, chondrogenic differentiation was assessed by staining with toluidine blue (Muto Pure Chemicals, Tokyo, Japan). Expression of chondrocyte-specific genes, type II collagen (Col II) and sry-type high-mobility group box 9 (Sox9) was also measured by RT-PCR. Adipogenic induction. To induce adipogenic differentiation, cells were cultured for three weeks in an adipogenic medium consisting of low-growth DMEM (Sigma) with 1 µM Dex, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 10 µg/ml insulin (Sigma), 0.2 mM indometacin (Sigma), and 10% FBS. After three weeks, adipogenic differentiation was evaluated by the cellular accumulation of neutral lipid vacuoles that were stained with oil-red O (Muto Pure Chemicals). Expression of adipocyte-specific genes, lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor (PPAR-γ2) was also measured by RT-PCR.

**Table II.** Primers used for amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (sense/antisense)</th>
</tr>
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<tbody>
<tr>
<td>ALP</td>
<td>5'-CCCAAAGGCTCTCCCTGG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CGTGTAGTTGGTGCAGC-3'</td>
</tr>
<tr>
<td>OC</td>
<td>5'-TCACACTCTTCGCCCCCTGCTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGCGAAGGGGAGAAGGAAGA-3'</td>
</tr>
<tr>
<td>Col II</td>
<td>5'-TCTGCAACATCGAGACTGCG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GAAGAGCACAGGCGGCTATGT-3'</td>
</tr>
<tr>
<td>Sox9</td>
<td>5'-AACATGACATATCAAGGCG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ACGATTTCATCATCTCCG-3'</td>
</tr>
<tr>
<td>LPL</td>
<td>5'-GAGATTCCCTGATGCGACC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CTGCAATGACAGACTTTCTC-3'</td>
</tr>
<tr>
<td>PPAR-γ2</td>
<td>5'-TGGGTGAAACTGCTGGAGATTCC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CATGAGGGCTTATTTGAGACCTG-3'</td>
</tr>
<tr>
<td>GADPH</td>
<td>5'-CCACCATGGCAATTCATGCA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TCTAGACGGCAGTCAGGTCCACC-3'</td>
</tr>
</tbody>
</table>

ALP, alkaline phosphatase; OC, osteocalcin; Col II, type II collagen; Sox9, sry-type high-mobility group box 9; LPL, lipoprotein lipase; PPAR-γ2, proliferator-activated receptor; GADPH, glyceraldehyde-3-phosphate-dehydrogenase

**Fig. 1a** Osteogenic differentiation capacity of haematoma cells. Alizarin red S staining after 21 days incubation in a) osteogenic medium or b) undifferentiated medium (x 40).

**Fig. 1b**

**Results**

**Morphological characteristics and immunophenotypes of adherent haematoma cells.** In the primary culture, the adherent HCs gave rise to colonies that first became visible around the fifth day of culture as cells exhibiting a fibroblast-like spindle shape. HCs formed colonies of fibroblast-like cells observed in BM-derived MScs. The colony size increased rapidly, and after three to four weeks the cells merged and formed a subconfluent monolayer of fibroblas-
toid cells. The cells were long-lived in culture and had a significant capacity for expansion. From the calculation of populations doubling, HCs could be cultured through at least ten passages with a minimal decline in their proliferative capacity.

The cell-surface antigen profile of adherent HCs was analysed and compared with that of BM-derived MSCs (Table I). Both HCs and BM-derived MSCs were positive for MSC-related markers CD29, CD44, CD105 and CD166, but negative for haematopoietic markers CD14, CD34, CD45 and CD133.

Adherent haematoma cells exhibited in vitro osteogenic, chondrogenic and adipogenic potential. Adherent HCs were cultured under conditions favourable for osteogenic, chondrogenic or adipogenic differentiation, respectively. After a three-week incubation under osteogenic conditions, induced cells formed a mineralised matrix as evidenced by alizarin red S staining (Hartman-Leddon Company, Philadelphia, Pennsylvania) in the osteogenic medium on the 21st day (Fig. 1a) contrasting with an absence of mineralised matrix under undifferentiated conditions after the same duration (Fig. 1b).

The level of ALP activity under osteogenic conditions was significantly higher (*p = 0.017) than under control conditions on day 21 (Fig. 2). This osteogenic potential was further confirmed by RT-PCR analysis, showing the expressions of ALP and osteocalcin under osteogenic conditions after a three-week culture period (Fig. 3). The expression of ALP and osteocalcin under osteogenic conditions was higher than under undifferentiated conditions in the control group (LPL = ad+: 578.23, ad-: 100, p = 0.040; PPAR-γ2 = ad+: 170.25, ad-: 100, p = 0.043).

After a three-week incubation under chondrogenic conditions, cell pellets had a spherical and glistening transparent appearance. The development of a cartilage matrix from cell pellets was shown by staining the pro-
teoglycans with toluidine blue (Fig. 4). Expression of mRNA of Col II and Sox9 was enhanced by RT-PCR after a 21-day induction (Fig. 3).

After a three-week incubation period under adipogenic conditions, adherent HCs showed the formation of neutral lipid vacuoles, visualised by staining with oil red O (Fig. 5a). In contrast, in undifferentiated conditions no oil-red O-positive lipid vacuole was observed (Fig. 5b). The RT-PCR analysis showed the expression of LPL and PPAR-γ2 under adipogenic conditions after a three-week culture period (Fig. 3). The expression of LPL and PPAR-γ2 under adipogenic conditions was higher than that under the undifferentiated conditions in the control group.

Discussion
For the first time it has been demonstrated that cells derived from the haematoma present at a fracture site have the potential for multilineage mesenchymal differentiation in vitro. This suggests that HCs as well as growth factors play a significant and dynamic role in fracture healing.

The primary culture of adherent HCs showed the formation of colonies of fibroblast-like cells. Cell-surface markers analysed using FACS revealed that the adherent HCs expressed classical MSC marker proteins, namely CD29, CD44, CD105, and CD166, but lacked the haematopoietic markers CD14, CD34, CD45 and CD133. The capacity of HCs to differentiate into osteoblast-lineage cells that produce mineralised matrices, chondrocyte-lineage cells that produce proteoglycans, and adipocyte-lineage cells that accumulate lipid vacuoles in the presence of established lineage-specific differentiation factors in vitro was consistent with that reported for BM-derived MSCs,16,17 and was confirmed by RT-PCR analysis and histochemical evaluation. Taken together, these findings indicate that fracture haematomas contain multilineage mesenchymal progenitor cells that exhibit characteristics similar to those of BM-derived MSCs.

The process of fracture healing can be divided into five distinct stages, including an initial stage in which a haematoma is formed at the fracture site associated with an inflammatory response, a subsequent stage in which angiogenesis develops and cartilage begins to form, and then three successive stages of cartilage calcification, cartilage removal and bone formation, and ultimately a more extended stage of bone remodelling. The intramembranous bone is formed by committed osteoprogenitor cells that reside in the periosteum.3,5 The presence of growth factors, specifically the TGF-β, IGF, PDGF, aFGF, and bFGF, within the haematoma is a prerequisite for the regulation of the processes that occur during fracture healing. Some reports have described that mesenchymal stem cells differentiate into osteoblasts and/or chondrocytes under the influence of several growth factors.16,18 Based on our results, one possible mechanism for fracture healing is that these growth factors may act on the HCs at different stages of healing, and the HCs then differentiate into osteoblasts and/or chondrocytes at different stages in an autocrine and/or paracrine manner.3-9

The most likely origin of HCs is the bone marrow. Several reports have described that bone marrow stromal cells (BMSCs) are essential for bone formation after fracture.18-21 In our study, FACS analysis revealed that the phenotype of HCs was similar to that of BM-derived MSCs. Mizuno et al1 proved that fracture haematoma possesses osteogenic potential, as new bone formation was seen when the haematoma was transplanted to an intramuscular site; however, in the absence of a fracture the BM did not display this osteogenic potential. In other words, the influence of a number of factors is necessary for the differentiation of BMSCs to osteogenic cells. We postulate that exposure to
the environment of a fracture may differentiate HCs into osteogenic and chondrogenic lineage cells. Alternatively, the origin of the osteogenic and chondrogenic lines might be the peristeum and injured muscle, as periosteum-derived cells\(^ {22-24}\) and muscle-derived cells\(^ {15,26}\) have been shown to differentiate into these cell lines both \textit{in vivo} and in vitro. HCs may also be derived from circulating mesenchymal stem cells that enter the fracture site from the circulation via the damaged vasculature.\(^ {27,28}\) These hypotheses will be investigated in further studies.

In fracture healing a more satisfactory result is generally achieved if stabilisation is obtained with minimal disturbance of the fracture haematoma.\(^ {29,30}\) Usually open reduction and internal fixation result in removal of the haematoma and causes some damage to the peristium. Our findings suggest that to enhance healing of a fracture, the haematoma should not be evacuated from the fracture site during osteosynthesis. In addition, HCs could provide a useful source of cells for tissue engineering in the future treatment of nonunion and long-bone defects. Further experiments are needed to examine whether HCs have multilineage potential \textit{in vitro}.

We have shown for the first time that HCs can differentiate into osteogenic, chondrogenic and adipogenic cells \textit{in vitro}, and thus contain multilineage mesenchymal progenitor cells.

**Supplementary Material**

A further opinion by Professor Eng-Hin Lee is available with the electronic version of this article on our website at www.jbjs.org.uk

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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.

**References**


