Characterization and Purification of Osteogenic Cells From Murine Bone Marrow by Two-Color Cell Sorting Using Anti–Sca-1 Monoclonal Antibody and Wheat Germ Agglutinin

By P. Van Vlasselaer, N. Falla, H. Snoeck, and E. Mathieu

Osteogenic cells were sorted from bone marrow of 5-fluorouracil (5-FU)-treated mice based on light scatter characteristics, Sca-1 expression, and their binding to wheat germ agglutinin (WGA). Four sort gates were established using forward (FSC) and perpendicular (SSC) light scatter and were denominated as FSC<sup>high</sup> SSC<sup>low</sup>, FSC<sup>low</sup> SSC<sup>high</sup>, FSC<sup>high</sup> SSC<sup>low</sup>, and FSC<sup>low</sup> SSC<sup>high</sup> cell. Cells from the FSC<sup>high</sup> SSC<sup>high</sup> gate, but not from the other gates, synthesized alkaline phosphatase, collagen, and osteocalcin and formed a mineralized matrix in culture. The number of osteoprogenitor cells was significantly enriched after depleting the 5-FU bone marrow from cells of the lymphoid and myeloid lineage, eg, T cells, B cells, natural killer cells, granulocytes, macrophages, and erythrocytes. Approximately 95% of the FSC<sup>high</sup> SSC<sup>high</sup> cell population of this "lineage-negative" (Lin<sup>-</sup>) marrow

Bone marrow stroma forms a network of fibroblasts, adipocytes, endothelial cells, and macrophages that supports and regulates hematopoiesis<sup>12,13</sup> and harbors cells of the osteogenic lineage.<sup>3,4</sup> The latter is illustrated by the fact that marrow cells differentiate into bone when transplanted into ectopic sites<sup>5,7</sup> or when cultured in the presence of vitamin C and β-glycerophosphate.<sup>8,9</sup> In addition, a number of immortalized and transfected cell lines, generated from bone marrow stroma, elicited osteogenic characteristics in culture or when transplanted in vivo.<sup>10,11</sup>

In previous reports, we showed that mouse bone marrow contains 5-fluorouracil (5-FU)-resistant, low-density, non-adherent cells that bind wheat germ agglutinin (WGA) and that synthesize bone proteins, including alkaline phosphatase (ALP), collagen type I, and osteocalcin, and form mineralized nodules in culture.<sup>12-14</sup> Apart from these biophysical characteristics, little information is available about the immunologic phenotype of these cells. This is not surprising because osteogenic cells represent only 0.006% of flushed marrow from 5-FU-treated mice.<sup>12</sup> Moreover, no detailed enrichment procedures for these cells were available until today. Nevertheless, it is clear that the understanding of the role of cell-cell interactions, growth factors, and hormones during osteogenic differentiation largely depends on the characterization and purification of the distinct maturation stages of the osteogenic lineage. The goal of this work was to purify and to determine the immune phenotype of osteogenic cells from the murine bone marrow using fluorescence-activated cell sorting (FACS) technology. This report describes a FSC<sup>high</sup> SSC<sup>high</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> WGA<sup>high</sup> KM16<sup>-</sup> Sab-1<sup>-</sup> Sab-2<sup>-</sup> Thy1.2<sup>-</sup> c-kit<sup>-</sup> cell population that synthesizes bone proteins, including ALP, collagen, and osteocalcin, and that forms a mineralized matrix when cultured in the presence of β-glycerophosphate and vitamin C.

**MATERIALS AND METHODS**

**Mice and bone marrow cell preparation.** Eight- to ten-week-old BALB/c mice were administered 5-FU (Roche) at 150 mg/kg body weight by tail vein injection. Five days later, the marrow was flushed from the femora and dispersed into a single-cell suspension. Red blood cells were removed by density centrifugation on a 70% Percoll (Pharmacia, Uppsala, Sweden) gradient.

**Cell culture.** Bone marrow cells were cultivated in flat-bottom 96-well plates at 5 × 10<sup>5</sup> cells per well in Iscove's medium supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin, streptomycin, ascorbic acid (100 μg/ml), and β-glycerophosphate (0.6% wt/wt). The cloned osteoprogenitor cell lines MN<sub>7</sub> and MC3T3 E1<sup>15</sup> were cultured in the same medium. For the experiments in which the self-renewal of sorted cells was studied, the bone marrow cells were cultured until subconfluence, eg, approximately 15 days. These cultures were then passed every 4 days. The cells were detached from the culture vessel using trypsin.

**Measurement of ALP activity.** ALP activity was determined on day 15 of the culture as described elsewhere.<sup>16</sup> The cultures were incubated with 0.1 mol/L sodium acetate solution supplemented with 0.1% Triton X-100 and 5 mmol/L p-nitrophenol phosphate (Sigma 104; Sigma, St Louis, MO), pH 9.6, for 1 hour at 37°C. Absorbance was determined at 405 nm and compared with a p-nitrophenol standard titration curve. ALP activity was expressed as nanomoles of p-nitrophenol formed per minute.

**Collagen synthesis.** Collagen synthesis was measured on day 18 of the culture by the incorporation of [<sup>3</sup>H]-proline (Amersham, Amersham, UK) into collagenase digestible protein (CDP).<sup>17</sup> Cell cultures were incubated with [<sup>3</sup>H]-proline (1 μCi/well) for 18 hours at 37°C and then washed three times with phosphate-buffered saline (PBS). Collagenase (0.1 mg/ml) was added for 1 hour and the CDP was measured in a liquid scintillation counter. Collagenase was

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**REFERENCES**

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purchased from Worthington (UK) and was substantially free of nonproteic activity.

Osteocalcin cell enzyme-linked immunosorbent assay (ELISA). The osteocalcin cell ELISA was performed as described elsewhere. Briefly, cultures were washed with 4% cold formaldehyde for 30 minutes at 4°C and then washed with TRIS/HCl buffer (0.05 mol/L, pH 7.6). Endogenous peroxidase activity was blocked with 3% H2O2 for 5 minutes. The samples were rinsed with TRIS/HCl buffer and blocked with normal goat serum (1/5 dilution; Tago, Burlingame, CA) for 1 hour at 37°C. Rabbit-antimouse osteocalcin antisemur (kindly provided by Dr R. Bouillon, Katholic University, Leaven, Belgium) was added (1/15,000 dilution) for 2 hours at 4°C. The cultures were washed with TRIS/HCl buffer and incubated with horseradish peroxidase-conjugated goat-antirabbit Ig serum (1/12,000 dilution; Tago) for 30 minutes at 4°C. After rinsing, the cultures were incubated at 37°C with substrate solution (1 ng/mL, ABTS + 0.1 μL/mL H2O2 in 10.5 g citric acid/14.2 g Na2HPO4/500 mL H2O) and absorbance was read at 450 nm. Osteocalcin was quantitated on day 24 of culture. Nonspecific binding of the antimouse osteocalcin antisemur was determined using nonimmune rabbit serum under the same conditions. The amount of osteocalcin incorporated in the culture was determined in comparison with a standard ELISA of puriﬁed mouse osteocalcin (kindly provided by Dr R. Bouillon, Katholic University, Leaven, Belgium). The sensitivity of this assay was 0.3 ng/mL. No reactivity was observed with FCS.

Calcium determination. Calcium was determined on day 27 of culture, as described elsewhere. The cell cultures were washed with Ca2+- and Mg2+-free PBS and incubated overnight with 0.6 N HCl. The extracted calcium was complexed with o-cresol-phthalein complex (Test Combination Calcium; Boehringer Mannheim, Mannheim, Germany) and the colorometric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium provided by the vendor.

Limiting dilution analysis. Bone marrow cells were plated at different densities and stained with the von Kossa technique after 30 days of cultivation. The percentage of wells showing no von Kossa-positive nodules was calculated for each cell plating density and was plotted against the number of bone marrow cells plated per well. The number of cells required to form one bone nodule, which reflected the proportion of osteogenic cells in the entire bone marrow population, was then determined from the point at which the line crossed the 37% level. That is, Fo = e − x, where Fo is the fraction of wells without bone nodules and x is the mean number of osteogenic cells per well. Based on a Poisson distribution of progenitor cells, Fo = 37% corresponds to the dilution at which there is one progenitor cell per well.

FACS. Cells were sorted on a FACStar Plus flow cytometry system (Becton Dickinson Inc, San Jose, CA) equipped with a 488-nm Argon ion air cooled laser and a 70-μm nozzle. Sorting was performed at 40 mW laser beam energy, 10 psi sheath pressure, and 2 psi sample differential pressure. A threshold was set on forward scatter to gate out debris and dead cells. The cells were sorted in PBS and collected in FCS-coated tubes. Sorting windows were established for four separate parameters: forward (FSC) light scatter, perpendicular (SSC) light scatter, fluorescein isothiocyanate (FITC) fluorescence, and phycoerythrin (PE) fluorescence. For dual-fluorescence labeling, the cells were incubated with anti–Sta-1 antibody (E13 161-7) and biotinylated WGA (Boehringer Mannheim) for 30 minutes on ice. The cells were washed and incubated with FITC-conjugated rabbit antirabbit Ig serum (Dako, Glosstrup, Denmark) and streptavidin-PE conjugate (Becton Dickinson) for 30 minutes on ice. Nonspecific staining was determined by incubating the cells with equivalent concentrations of FITC and biotinylated isotype-matched antibodies of irrelevant specificity. Less than 1% of the cells stained with the negative controls were beyond the gates set for determining positive Sta-1 and WGA staining. Cells were maintained at room temperature during sorting. Two sorting protocols were established. In protocol 1, the cells were sorted on light-scatter characteristics according four sort gates denominated as FSClow SSClow, FSClow SSCmed, FSCmed SSCmed, and FSCmed SSChigh cell. In protocol 2, FSChigh SSChigh cells were gated and then sorted based on Sta-1 and WGA staining. To this end, three sort gates were created: Sta-1 WGA−, Sta-1 WGA+, and Sta-1 WGA+. For some experiments, bone marrow was depleted from T cells, B cells, and natural killer (NK) cells, macrophages, granulocytes, and erythrocytes by panning as described elsewhere. Bacteriologic petridishes were coated with rabbit antirat Ig serum or rabbit antinmune Ig serum (Dako). Marrow cells were incubated for 30 minutes on ice with the following monoclonal antibodies (MoAbs): RA3-6B2.1 (B220, mature, and progenitor B cells), RB6-8C5 (Gr-1 and granulocytes), kindly provided by Dr R. Coffman, DNAX, Palo Alto, CA), M1/70.15.11.5HL (anti-Mac-1 and macrophages; American Type Culture Collection [ATCC], Rockville, MD), M3/38.1.2.8.HL.2 (anti-Mac-2 and macrophages; ATCC), M3/84.6.34 (anti-Mac-3 and macrophages; ATCC), GKI.5 (anti-LT14 and helper T cells; ATCC), 116-13.1 (anti-Lyt2 and cytotoxic and suppressor T cells; ATCC), PK136 (anti-NK cells; ATCC), M1/75.16.4.HLK (anti-heat stable antigen [HSA] and mouse red blood cells), and J11d.2 (antimature and antiprogenitor erythroid cells; ATCC).

The marrow cells were then incubated on the coated petridishes for 2 hour at 37°C. Finally, the respective phenotypes were depleted for more than 98% as determined by single fluorescence labeling in FACS analysis.

Phenotypic analysis. Bone marrow from untreated and 5-FU-treated mice, sorted stroma cells collected after the second passage of cultivation, and cloned MN7 cells were stained using the panel of MoAbs described above extended with J1j.10 (anti-Thyl.2; ATCC), ACK-2 (anti–c-kit; kindly provided by Dr S. Nishikawa, Institute for Medical Immunology, Kuramoto, Japan), SA-1 and SA-2 (endothelial cells; kindly provided by Dr B.A. Imhof, Basel Institute for Immunology, Basel, Switzerland), and KM16 (stromal cells; kindly provided by Dr D.G. Osmond, McGill University, Montreal, Canada). Statistical analysis was performed using the Lysys II program (Becton Dickinson).

Histologic procedures. Cultures were ﬁxed in neutral buffered formalin and selected areas of the cell layer were removed, decalciﬁed with EDTA, embedded in glycol methacrylate, and cut in 3-μm sections. The sections were stained with hematoxylin and eosin. For scanning electron microscopy, the cultures were rinsed with PBS and fixed for 1 hour with sodium-cacodylate buffer in 0.1 mol/L phosphate buffer (pH 7.2). After rinsing, the cultures were postﬁxed for 1 hour with 1% osmium tetroxide in the same buffer. The cultures were subsequently rinsed and progressively dehydrated with alcohol. They were processed for critical point drying (Balzers Union, Liechtenstein) in CO2 and coated with gold (50 nm; Balzers Union). The cultures were observed in a JEOL JSM-F15 microscope.

RESULTS

Osteogenic cells exhibit FSChigh and SSCmed characteristics. An initial set of experiments was performed to determine the light-scatter characteristics of the osteogenic cell population from the bone marrow of 5-FU–treated mice. To this end, the marrow was sorted, ungated, or, according to four sort gates, denominated as FSClow SSClow, FSCmed SSCmed, FSCmed SSClow, and FSCmed SSChigh (Fig 1). The respective gates represent approximately 4%, 5%, 61%, and 23% of the 5-FU marrow. Because in vivo 5-FU treatment resulted in a 98% depletion of mononuclear cells of the
Fig 1. FACS contour plot showing FSC and SSC characteristics of bone marrow from 5-FU-treated mice. Different gates used for sorting are identified as FSClowSSClow, FSClowSSCmid, FSCmedSSCmed, and FSCmedSSCmed cells.

marrow (data not shown), these gates represent, respectively, 0.08%, 0.1%, 1.2%, and 0.46% of the marrow cells in untreated animals. From the gated sorted cell populations, only those displaying FSCmedSSCmed light-scatter characteristics synthesized bone proteins and mineralized (Table 1). The other cells, sorted according to FSClow SSCmed, FSCmed SSCmed, and FSCmed SSCmed light-scatter characteristics, showed no osteogenic activity, not even when their cell number per well was increased fourfold (data not shown). It is clear from the results that, even though equal numbers of cells were plated, the sorted cells show less osteogenic activity than do the unsorted cells. Consequently, the effect of the sorting procedure on the osteogenic potential of marrow cells was verified. Whereas unsorted marrow, plated at 5 x 10⁶ cells per well, synthesized detectable amounts of ALP, collagen, and osteocalcin and formed a mineralized matrix, equal numbers of ungated sorted marrow cells did not. Microscopic observation of 1-day-old cultures showed that, in contrast to the unsorted cells, a significant number of the sorted cells failed to adhere. In addition, trypan blue staining showed that, whereas immediately after sorting 95% of the cells were viable, only 30% of them remained alive after 24 hours of culture (data not shown). Only when the number of ungated sorted cells was increased fourfold (2 x 10⁶ cells per well) was significant bone protein synthesis and mineralization observed (Table 1).

Enrichment of osteogenic cells by positive depletion of lymphoid and myeloid cells. It is clear from the data above that osteoprogenitor cells represent only a minor population of the 5-FU bone marrow. The goal was to enrich the osteoprogenitor cell population by panning, using the immunophenotypic characteristics of the 5-FU-resistant cells. To

Table 1. Osteogenic Activity of Cells Sorted From Bone Marrow Based on Their Light-Scatter Characteristics

<table>
<thead>
<tr>
<th>Population*</th>
<th>ALP p-Nitrophenol (nmol/min)</th>
<th>[³¹P]-Proline (cpm)</th>
<th>Osteocalcin (ng/well)</th>
<th>Calcium (µg/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td>5.2 ± 1.1</td>
<td>6,200 ± 828</td>
<td>11 ± 0.2</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>Ungated</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ungated†</td>
<td>1.1 ± 0.2</td>
<td>863 ± 115</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>FSClowSSClow</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSClowSSCmed</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSCmedSSClow</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSCmedSSCmed</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSCmedSSCmed</td>
<td>2.3 ± 0.4</td>
<td>1,750 ± 150</td>
<td>2.1 ± 0.01</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD of triplicate cultures from one representative experiment. ALP, collagen, osteocalcin, and calcium were determined on days 15, 18, 24, and 27 of culture, respectively. Three experiments were performed. All cells were plated at 5 x 10⁶ cells per well unless stated otherwise.

Abbreviation: ND, not detectable.

* Gates are defined in Fig 1.
† A concentration of 2 x 10⁶ cells/well was plated.
Determination of the frequency of osteogenic cells. Cells from the bone marrow that was depleted from cells of the lymphoid and myeloid lineage (Lin-) were plated at different cell densities in a limiting dilution fashion. Based on this end, FACS analysis was performed on the bone marrow of untreated and 5-FU–treated mice using lymphoid- and myeloid-specific MoAbs. Figure 2 shows that, whereas a large proportion of the myeloid and lymphoid cells are sensitive to the 5-FU treatment, a substantial percentage of them appear not to be affected by this drug. Consequently, attempts were made to enrich the osteogenic cell population by depleting the marrow from 5-FU–resistant T cells (L3T4, Lyt2), B cells (B220), NK cells (PK136), granulocytes (Gr-1), macrophages (Mac-1, Mac-2, and Mac-3), and erythroid cells (HSA and J11d.2) by panning. Panning completely removed the myeloid and lymphoid cells, as verified by FACS analysis, and depleted the 5-FU marrow from lymphoid and myeloid cells. Consequently, the marrow was first gated on FSC<sup>high</sup> SSC<sup>high</sup> characteristics and then sorted according to three sort gates based on their double staining with anti–Sca-1-FTC and WGA-PE. More precisely, these sort gates were established based on the binding intensity of the 5-FU marrow cells to WGA and were denominated as Sca-1<sup>+</sup> WGA<sup>high</sup>, Sca-1<sup>+</sup> WGA<sup>medium</sup>, and Sca-1<sup>+</sup> WGA<sup>low</sup> (Fig 5). These gates represent 10%, 13%, and 34% of the ungated and 6%, 27%, and 62% of the FSC<sup>high</sup> SSC<sup>high</sup> gated 5-FU marrow, respectively. The sorted cell populations were cultivated and ALP, collagen, and osteocalcin synthesis and mineralization were scored on days 15, 18, 24, and 27 of culture, respectively. Table 2 shows that, in contrast to the untreated marrow, a substantial percentage of them were plated in 60, 60, 90, 120, 180, 240, 300, 360, and 420 wells at 5 x 10<sup>4</sup>, 4 x 10<sup>5</sup>, 2 x 10<sup>5</sup>, 10<sup>5</sup>, 8 x 10<sup>4</sup>, 6 x 10<sup>4</sup>, 4 x 10<sup>4</sup>, 2 x 10<sup>4</sup>, and 10<sup>3</sup> cells per well (○) and 4 x 10<sup>5</sup>, 2 x 10<sup>5</sup>, 10<sup>5</sup>, 8 x 10<sup>4</sup>, 6 x 10<sup>4</sup>, 4 x 10<sup>4</sup>, 2 x 10<sup>4</sup>, and 10<sup>3</sup> cells per well (●), respectively. Cultures were maintained for 25 days and then stained with the von Kossa technique. The percentage of wells without mineralized nodules ±97% confidence limits was plotted against the number of cells plated per well.
the unsorted cells, the ungated sorted cells were unable to produce detectable amounts of bone proteins or to mineralize when plated at $5 \times 10^5$ cells per well. As mentioned above, microscopic observation showed that this correlated again with reduced adherence and decreasing viability of the sorted cells during subsequent culture. From the gated cell populations, only the Sca-1$^+$ WGA$^{bright}$ cells synthesized appreciable amounts of ALP, collagen, and osteocalcin and mineralized in time. It is of interest to note that the sorted cells showed less osteogenic activity compared with the unsorted cells, even though they were plated at the same density. Hence, the osteogenic activity in the Sca-1$^+$ WGA$^{bright}$ gated cell population was not enriched in comparison with the ungated and the unsorted cells.

**Sorted osteogenic cells express stromal and endothelial but not hematopoietic cell surface antigens.** Thus far, the sorting results suggest that osteogenic cells belong to a marrow cell population with FSC$^{high}$ SSC$^{high}$ Lin$^-$. Sca-1$^+$ WGA$^{bright}$ characteristics. To further define the immunophenotype of osteogenic cells, SCA$^{high}$ SSC$^{high}$ Lin$^-$. Sca-1$^+$ WGA$^{bright}$ cells were sorted and cultured for 1 week and then screened for the expression of (1) the stromal surface antigen KM16, (2) the endothelial surface antigens Sab-1 and Sab-2, and (3) the hematopoietic surface antigens Thy1.2 and c-kit. During the 1-week culture period, the sorted cells shed the fluoresceinated Sca-1 antibody and WGA and showed no background fluorescence that interfered with the staining for the KM16, Sab-1, and Sab-2 antigens. Furthermore, during this culture period, the cells increased in number without losing their osteogenic potential (see below). The FACS analysis shown in Fig 6 illustrates that, in addition to the characteristics described above, 88%, 95%, and 55% of the sorted osteogenic cells expressed the KM16, Sab-1, and Sab-2 surface antigens, respectively. In contrast, these cells did not express the Thy1.2 or c-kit surface antigens (Fig 6).

**Self-renewal of FSC$^{high}$ SSC$^{high}$ Lin$^-$. Sca-1$^+$ WGA$^{bright}$ cells in culture.** Osteoprogenitor cells from the rat bone marrow were reported to lose their osteogenic activity on progressive subculturing. Based on this observation, we determined the self-renewal potential of osteoprogenitor cells sorted from 5-FU marrow. To this end, FSC$^{high}$ SSC$^{high}$ Lin$^-$. Sca-1$^+$ WGA$^{bright}$ cells were sorted and cultured until subcon-
Table 2. Osteogenic Activity of Cells Gated on FSC<sup>high</sup>-SSC<sup>high</sup> Characteristics and Then Sorted Based on Their Dual-Fluorescence Staining With Sca-1-FITC and WGA-PE

<table>
<thead>
<tr>
<th>Population*</th>
<th>ALP p-Nitrophenol (nmol/min)</th>
<th>[%]-Proline (cpm)</th>
<th>Osteocalcin (ng/well)</th>
<th>Calcium (µg/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td>4.2 ± 1.5</td>
<td>3,273 ± 421</td>
<td>6.8 ± 1.1</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>Ungated</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sca-1&lt;sup&gt;+&lt;/sup&gt;WGA&lt;sup&gt;dim&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sca-1&lt;sup&gt;+&lt;/sup&gt;WGA&lt;sup&gt;med&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sca-1&lt;sup&gt;+&lt;/sup&gt;WGA&lt;sup&gt;high&lt;/sup&gt;</td>
<td>3.1 ± 0.8</td>
<td>2,512 ± 221</td>
<td>3.3 ± 0.06</td>
<td>2.1 ± 0.4</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD of triplicate cultures from one representative experiment. ALP, collagen, osteocalcin, and calcium were determined on days 15, 18, 24, and 27 of the culture, respectively. Three experiments were performed. Unsorted, ungated sorted cells and sorted cells were plated at 5 × 10⁴ cells per well.

Abbreviation: ND, not detectable.

* Gates are defined in Fig 5.

fluence, eg, approximately day 15 of culture. From that point on, the cells were subcultured every 4 days. The potential of the sorted cells to synthesize bone proteins and to mineralize was screened after the third and the fifth passage. More precisely, cells of the respective passages were cultured and ALP, collagen, and osteocalcin synthesis and mineralization was scored at 3 days interval over a period of 15 days. We like to emphasize that, at the time these cultures were started, the cells harvested at passages 3 and 5 had been in culture for 27 and 35 days, respectively. Figure 7 shows that sorted cells sustained osteogenic activity at least up to three subcultures. At the fifth passage, the cells had lost their ability to synthesize osteocalcin and to mineralize, although they were still capable of synthesizing significant amounts of ALP and collagen. Hence, the osteogenic potential of sorted cells appears to be transient. It is important to note that, in contrast to the work of McCulloch et al, our cultures were performed in the absence of exogenous glucocorticoids.

Morphologic characteristics of FSC<sup>high</sup> SSC<sup>high</sup> Lin<sup>−</sup> Sca-1<sup>+</sup> WGA<sup>high</sup> sorted cells in culture. Microscopic observation showed that sorted FSC<sup>high</sup> SSC<sup>high</sup> Lin<sup>−</sup> Sca-1<sup>+</sup> WGA<sup>high</sup> cells have a polygonal fibroblastic appearance (Fig 8A). These cells form distinct three-dimensional nodules that stain positive with the von Kossa technique (Fig 8B and C). Histologic examination of hematoxylin and eosin-stained cross-sections show that these nodules are covered by elongate to cuboidal osteoblast-like cells and contain osteocyte-like cells that are embedded in a connective tissue matrix (Fig 8D and E). A better idea of the matrix component in these cultures is given by the scanning electron microscopy micrograph in Fig 8F. This picture shows cellular protrusions that are embedded in a dense collagenous matrix. Moreover, mineral deposits can be observed in conjunction with the collagen fibers.

DISCUSSION

This report describes the phenotypic characterization and purification of a cell population of the bone marrow of 5-FU-treated mice that synthesizes bone proteins including ALP, collagen, and osteocalcin and that mineralizes when cultured in the presence of β-glycerophosphate and vitamin C. In an initial set of FACS sorting experiments, osteogenic
cells displayed FSC<sup>high</sup> SSC<sup>high</sup> characteristics. This complies with the observation that fibroblast colony-forming unit (CFU-Fs) of the human marrow showed FSC<sup>high</sup> SSC<sup>high</sup> characteristics in the FACS sort. Characteristically, cells displaying a high light scatter have a large size, a low density, and a complex cytoplasm. This is in agreement with the biophysical and morphologic characteristics of osteogenic cells as described by Budenz and Bernard and Falla et al.

From the data in this report it is obvious that sorted cells show less osteogenic activity than unsorted cells. This is either caused by a direct effect on the osteogenic potency of the individual osteoprogenitor cells themselves or by a sort-induced cell loss. Although the first possibility cannot be ruled out at this point, it is at least clear from our results that a significant number of the sorted cells fail to adhere to the culture flasks and die within a culture period of 24 hours. It is conceivable that the sorting procedure inhibits the synthesis of extracellular matrix proteins and adhesion molecules that are essential for the survival of osteoprogenitor cells in culture. In other words, we believe that the sorting procedure affects the number and the biochemistry of osteoprogenitor cells rather than their potential to synthesize bone proteins and to form a mineralized matrix. Not only osteoprogenitor cells suffer during sorting. Indeed, significantly less numbers of CFU-F were observed in sorted as compared with unsorted bone marrow (data not shown). Most likely, this "sorting-effect" is caused by the shearing forces induced by the sorting procedure. In support of this, we experienced that acceptable cell yields were obtained only when sorting was performed under conditions in which the shearing forces were reduced, eg, low sheath fluid and sample pressure and clean tubing. In addition, in our hands, overall cell viability was greatly improved when reduced laser beam energy was applied and when the cells were sorted in PBS and collected in undiluted FCS. Taken together, these findings underline once more the fragile nature of osteogenic cells as mentioned initially by Turksen and Aubin. Further, this may explain the troubles people encounter in trying to purify these cells by flow cytometry means.

To sort osteogenic cells by FACS on the basis of their immunologic phenotype, a number of presort enrichment procedures were performed. In addition to in vivo treatment with 5-FU, which resulted in a 95% reduction of the mononuclear cells of the marrow, we initially tried to deplete the marrow cell number using magnetic bead separation and complement lysis (data not shown). These techniques proved not to be satisfactory because osteogenic cells bind nonspecifically to the beads and because only minor depletions were obtained using complement. Alternatively, marrow cell depletion was tried by panning. Using MoAbs directed to lymphoid and myeloid cell surface antigens, this technique reduced 5-FU marrow for 98%. Because the "lineage"-depleted marrow showed increased osteogenic activity in limiting dilution, panning can be considered as a useful procedure for the enrichment of osteogenic cells. Furthermore, this observation suggests that osteogenic cells from the mouse bone marrow do not express lymphoid or myeloid cell surface markers. This is in agreement with the data of Piersma et al showing that murine CFU-F do not express B220, Mac-1, and Thy1 surface antigens. We like to emphasize that our data refer to osteoprogenitor cells of the bone marrow and that by no means can it be excluded that cells with a more mature osteoblastic phenotype may express a different immunologic phenotype, including the expression of cell surface markers, especially those of the lymphoid or myeloid lineage.

The low frequency of osteoprogenitor cells in the bone marrow made it technically difficult to determine the immunophenotypical parameters by which these cells could be sorted by FACS. Therefore, we first defined the immunologic phenotype of established osteoprogenitor cell lines and used that information to make an educated guess on which parameters would be useful to sort the osteoprogenitor cells from the marrow. In a number of preliminary experiments, FACS

![Diagram of ALP, Collagen, Osteocalcin, and Calcium levels over time](https://www.bloodjournal.org)

Fig 7. Effect of subcultivation on the osteogenic potency of FSC<sup>high</sup> SSC<sup>high</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> WGA<sup>low</sup> sorted cells. FSC<sup>high</sup> SSC<sup>high</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> WGA<sup>low</sup> cells were sorted and cultured. At subconfluence (day 16), the cells were passed every 4 days. Cells of passage numbers 3 (○) and 5 (●) were taken and cultivated in 96-well multwell plates. ALP activity and collagen and osteocalcin synthesis and mineralization was determined at 3-day intervals for a period of 15 days.
Fig. 8. Morphologic characteristics of sorted FSC$^{\text{high}}$ SSC$^{\text{high}}$ Lin$^{-}$ Sca-1$^{+}$ WGA$^{\text{high}}$ cells in culture. (A and B) Phase-contrast micrographs of 15-day-old (original magnification x 360) and 27-day-old (original magnification x 50) cultures, respectively. Note the polygonal cells in (A) and the three-dimensional nodule in (B). (C) A 25-day-old culture showing a nodule stained with the von Kossa technique (original magnification x 200). (D and E) Hematoxylin and eosin-stained cross-sections through a 25-day-old, demineralized nodule. Osteoblast-like cells (arrow) cover the top of the nodule and osteocyte-like cells (arrowheads) can be observed in the nodules. (F) Scanning electron microscopy picture of a 26-day-old culture. Note the collagenous matrix (white arrow) and the calcium phosphate mineral deposits (black arrow) in between the cell protrusions (white arrowheads).

Analysis showed that osteoprogenitor cells such as MN7 and MC3T3.E1 expressed the Sca-1 antigen and bound intensely to WGA. The latter is in agreement with the observation of Falla et al.\textsuperscript{2} that osteogenic cells were removed from the marrow after WGA agglutination and sedimentation. According to these findings, osteogenic cells were sorted from Lin$^{-}$ 5-FU marrow on the basis of their Sca-1 expression and WGA binding. As described by Ploemacher and Bruns,\textsuperscript{39} the mouse marrow can be subdivided in four populations based on its binding intensity to WGA: WGA$^{\text{negative}}$, WGA$^{\text{high}}$, WGA$^{\text{medium}}$, and WGA$^{\text{high}}$. In analogy with these investigators, we sorted 5-FU bone marrow cells according to these
sort gates. We were repeatedly able to sort osteogenic cells from the Sca-1+ WGA-bright population. This implies that the osteogenic cells of the marrow share at least a number of cell membrane characteristics with immortalized osteoprogenitor cell lines.

Recently, Huang and Terstappen²⁰ described a CD34⁺ HLA-DR⁺ CD38⁻ pluripotent stem cell in the human fetal bone marrow that can differentiate into hematopoietic precursors and stromal cells that are capable of supporting these precursors. Moreover, in culture, these cells showed ALP activity and extensive chondroitin sulphate immunoreactivity consistent with osteoblast and chondroblast differentiation. Thus, there is mounting evidence that hematopoietic and stromal cells originate from the same undifferentiated precursor. This idea was further supported by the work of Ardavin et al.⁴¹ showing that thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. Furthermore, the fact that the cell surface antigen CD34 is expressed on cells of both the hematopoietic and stromal lineages⁴² further illustrates this idea. In this context, Sca-1 expression on osteogenic cells may be of particular interest because Spangrude et al.¹² showed that Sca-1 in combination with Thy-1 expression could be used to sort hematopoietic stem cells from the marrow. In addition, hematopoietic stem cells were shown to bind to WGA.²⁹ Thus, Sca-1 expression and WGA binding by osteogenic cells may refer to the existence of a common pluripotent stem cell for hematopoietic and stromal cells. However, some observations indicate that sorted FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright cells from adult 5-FU-treated bone marrow are irreversibly differentiated and committed to the stromal lineage. First of all, FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright cells showed no hematopoietic activity in culture as determined by CFU-granulocyte-macrophage assay (data not shown). Second, they are WGAbright, whereas cells of the hematopoietic lineage display a WGA⁺ phenotype.²⁹ Furthermore, FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright cells do not express the hematopoietic cell surface antigens Thy-1 and c-kit. However, on the other hand, they express the cell surface antigens KM16, Sab-1, and Sab-2, which are restricted to cells of the stromal lineage.³³,³⁴

It was previously shown by McCulloch et al. that osteoprogenitor cells of the rat bone marrow exhibit self-renewal in culture. At least we were able to sustain osteogenic activity during subcultivation for up to four passages. In addition, Bellows et al.⁶³ showed that nodule formation and maintenance of rat calvaria cells in culture is significantly increased in the presence of dexamethasone. Indeed, mass population studies²¹,²⁴ and limiting dilution analysis³¹ suggest that a subpopulation of calvaria cells is dependent on dexamethasone or natural glucocorticoids for expression of bone nodule formation. Similarly, our data show that the self-renewal of FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright cells is limited and diminishes during subculturing. In addition, the time course of osteocalcin synthesis and calcium uptake by the sorted cells in the self-renewal study are different from the unsorted cells shown previously by Falla et al.¹² This report shows a time course of bone protein synthesis and mineralization in primary cultures of bone marrow cells from 5-FU-treated mice. In these cultures, osteocalcin synthesis and mineralization was insignificant during the first 12 days of the culture and rapidly increased from then on. In contrast, the fractionated cells in the self-renewal study did not show such a latency period and significant levels of osteocalcin synthesis and mineralization were observed from the onset of the cultures. In situ hybridization data in the report by Falla et al.¹² showed that cells flushed from the bone marrow do not express detectable levels of osteocalcin. Therefore, the latency period in osteocalcin synthesis and mineralization in these cultures reflects most likely the time necessary for "naive" cells to progress through the different steps of osteoblastic differentiation. To study the self-renewal potency of fractionated cells, FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright cells were cultured until subconfluence before they were passaged at 4-day intervals. In other words, Fig 7 reflects the time course of bone protein synthesis and mineralization of cells that, at the time of plating, had been cultured for 27 (passage 3) and 35 (passage 5) days, respectively. Thus, at the moment of plating, the cells used for the self-renewal experiments had already reached a distinct level of osteoblastic differentiation, e.g., they already expressed osteocalcin. Hence, in our opinion, this explains the difference in the time courses of osteocalcin synthesis and mineralization between unfractionated and fractionated cells. On the other hand, no real differences were observed in the time courses of ALP activity and collagen synthesis. Both proteins are continuously expressed by unfractionated fresh bone marrow cells, which explains why they are synthesized from the onset of cultures of both unfractionated and fractionated cells. No studies were performed with dexamethasone because a previous study indicated that the osteogenic activity of mouse bone marrow was significantly reduced in the presence of 10⁻⁷ to 10⁻¹⁰ mol dexamethasone.¹² In addition, cultured FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright cells readily detached from the culture dish and died in the presence of 10⁻⁷ mol hydrocortisone (data not shown). Whereas it is perfectly possible that the osteogenic activity of these cells depends on the presence of endogenous glucocorticoids in the medium, they definitely do not require exogenous supplementation of glucocorticoids. Further studies using steroid-depleted medium are necessary to determine the requirement of glucocorticoids for osteoblastic differentiation in this model.

In a previous report, we showed that bone marrow from 5-FU-treated mice form three-dimensional structures that stain with the Von Kossa technique and that contain osteoblast and osteocyte-like cells. Scanning electron microscopy illustrated the presence of a mineralized matrix filling up the extracellular spaces.¹² Morphologic studies showed that FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright sorted cells form nodules that stain with the Von Kossa technique. Furthermore, these nodules contain osteoblast and osteocyte-like cells that resemble those formed by unsorted bone marrow and calvaria cells from the mouse¹² and from other species such as the rat and human.⁴⁵,⁴⁶ Under no circumstances were we able to obtain mineralized cultures using cells sorted according to characteristics different from FSChigh SSChigh Lin⁻ Sca-1⁺ WGAbright (data not shown).

In conclusion, FACS sort technology appears to be useful
for the characterization of osteoprogenitor cells of the marrow. However, because this technology results in a significant loss of viable cells, more efforts have to be taken to fine tune this technology. In this context, large nozzle sorting may be a plausible option.

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Characterization and purification of osteogenic cells from murine bone marrow by two-color cell sorting using anti-Sca-1 monoclonal antibody and wheat germ agglutinin

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