Positive and Negative Immunoselection for Enrichment of Two Classes of Osteoprogenitor Cells

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Abstract. The number of identifiable stages and expression of differentiation markers in cells of the osteoblast lineage are not well understood. In the present study, a mAb, designated rat bone marrow (RBM) 211.13, was prepared that stained selectively the osteogenic and preosteoblastic cells along the surfaces of bone in calvariae, femurs, and metatarsals. The staining was cell surface associated and coincided with that for alkaline phosphatase (APase) detected histochemically. Only cells positive for APase activity by biochemical assay and not those without APase activity (e.g., fetal rat skin) stained with RBM 211.13. By immunoblotting, RBM 211.13 recognized a band coinciding with APase activity on nonreducing/nondenaturing gels, and RBM 211.13 precipitated a protein which on reduced gels migrated with an apparent molecular mass of ~80 kD. RBM 211.13 labeling was abolished by phosphatidylinositol-specific phospholipase C, known to release APase from the cell surface. All of these data support the concept that RBM 211.13 recognizes the bone isoenzyme of APase. RBM 211.13 was used to sort by flow cytometry the APase-positive and APase-negative cells from mixed fetal rat calvaria (RC) cell populations. The osteoprogenitors we identified earlier that form bone nodules in vitro (Belows, C. G., J. E. Aubin, J. N. M. Heersche, and M. E. Antosz. 1986. Calcif. Tissue Int. 36:143–154; Bellows, C. J., J. N. M. Heersche, and J. E. Aubin. 1990. Dev. Biol. 140:132–138) were found within the APase-positive pool. By immunopanning, RC cells were separated into APase-enriched (APase-positive, adherent) and APase-depleted (APase-negative, nonadherent) populations. The APase-positive fraction was enriched two-to-threefold for bone-forming osteoprogenitors compared to unfractionated cells, while the APase-negative population formed very few nodules under the same conditions. Both populations responded to the glucocorticoid dexamethasone (DEX) with an increase in bone nodule formation. However, the fold stimulation in bone formation in the APase-negative population was ~30-fold, while the fold stimulation in the APase-positive population was only ~5-fold. These data suggest that APase expression can be used for immunoselection to fractionate osteoblastic populations into an APase-positive population and a population initially APase-negative, that virtually all osteoprogenitors forming bone in vitro in the absence of added glucocorticoids reside in the APase-positive pool, and that the only osteoprogenitors present in the APase-negative pool are those requiring DEX to differentiate.

Bone is a highly organized tissue comprising a calcified connective tissue matrix and specific bone cells (osteoblasts, osteocytes, osteoclasts, and their precursors) (Rodan and Rodan, 1984; Nijweide et al., 1986; Caplan, 1988; Marks and Popoff, 1988; Owen, 1988; Aubin et al., 1990; Heersche and Aubin, 1990). While the importance of the regulation of proliferation and differentiation of the progenitor cells for osteoblasts is recognized, it is poorly understood. The current concept is that the osteoprogenitors are undifferentiated mesenchymal cells residing in the stromal tissues surrounding bone marrow and in other connective tissue compartments, e.g., the periosteal layer of bone itself (Owen, 1985). However, the number of steps leading from uncommitted to committed osteoprogenitor through to terminally differentiated osteocyte is not known, partly due to the absence of well-defined markers for each stage of the progression through the lineage. The need for unambiguous delineation of steps in the osteogenic pathway becomes increasingly crucial as attempts are made to define commitment and restriction points in multipotential cells (e.g., Grigoriadis et al., 1988) and to define target populations for regulation by oncogenes (e.g., c-fos; Ruther et al., 1987) or growth factors (e.g., TGFβ; Joyce et al., 1990).

Recently, we have developed and used a colony assay to study the regulation of osteoprogenitor cells present within fetal rat calvaria (RC) cell populations in vitro (Belows

1. Abbreviations used in this paper: DEX, dexamethasone, RBM, rat bone marrow; RC, rat calvaria.
et al., 1986). However, studies to assess the direct versus the indirect effects of various growth factors on the osteoprogenitors have been hampered by the fact that these cells are present at a very low frequency (<1%) in the primary RC populations (Bellows and Aubin, 1989), and at even lower frequency in first subculture populations (Bellows et al., 1987, 1990). While cell cloning procedures have been useful to isolate normal osteoblastic cells (Aubin et al., 1982; Guenther et al., 1989), the stages of differentiation represented by such clonal populations are uncertain and none has yet appeared to represent the bone-forming osteoprogenitors, consistent with their low frequency in the mass populations. Therefore, other approaches are required for isolation of these cells.

Specific morphological and histochemical changes help to describe stages during osteoblast differentiation in vivo (Caplan, 1988). Acquisition of biochemical properties, e.g., alkaline phosphatase activity, and expression of certain bone-matrix-associated proteins, such as osteocalcin and osteopontin, are useful (Butler, 1989), but cannot yet be associated unambiguously with cells at precise stages of differentiation or to a subspecialization of already matured or differentiated osteoblasts. The need exists for identification of other molecules or novel use of molecules already known to aid in discrimination of different stages of differentiation from early progenitors to mature osteoblasts and osteocytes.

Here we report the production and characterization of a mAb recognizing osteoblasts and osteoprogenitors in rat bone tissue. A variety of assays suggests that this antibody recognizes an epitope on the bone isoenzyme of alkaline phosphatase (APase). We have used the antibody to identify cells in vivo and in vitro and to isolate by cell sorting and panning the APase-positive and -negative cells from freshly isolated mixed populations of RC cells. We found that the bone-forming osteoprogenitor cells can be enriched for in the APase-positive fraction, but that a second class of osteoprogenitor cell, those requiring glucocorticoids to make bone in vitro, are present in the APase-negative fraction of cells.

Materials and Methods

Cells and Cell Culture

Populations of fetal RC cells were prepared by enzymatic digestion of calvariae from 21-d-old fetuses of timed-pregnant Wistar rats as described (Rao et al., 1976; Bellows et al., 1986; Turksen et al., 1990). Briefly, calvariae were dissected aseptically, the loosely adherent soft connective tissues were removed, and the calvariae were minced and treated with a collagenase mixture. Five populations (RCI through RCV) were obtained after sequential digestion times of ≈10, 20, 30, 50, and 70 min, respectively, by collecting released cells through a sterile filter (200 mesh, pore size 74 μm). Population I (RCI) was discarded; populations II through V (RCII-RCV) were maintained separately or pooled and plated into 7-T5 tissue culture flasks in αMEM containing 15% heat-inactivated PBS and antibiotics (as above) (standard growth medium) supplemented with 10 μg/ml ascorbic acid (Fisher Scientific Comp., Fair Lawn, NJ) (AA) and 10 mM sodium-β-glucopyrophosphate (β-GP) BDH Chemicals, Poole, England, (supplemented growth medium) (day 0). The next day (day 1), the medium was changed to supplemented medium with or without 10-4 M dexamethasone (DEX).

Bone Nodule Formation

The assay for bone nodule formation was as described (Bellows et al., 1986; Turksen et al., 1990). Panned (adherent or nonadherent) cells, sorted, or unsorted fetal RC cells were counted and control and treated in each experiment were adjusted to the same cell density, a density chosen to yield adequate bone nodule formation for counting and statistics. The recovered cells were plated into 35-mm tissue culture dishes (Falcon 3001; Becton Dickinson & Co., Mountain View, CA) at a density of 3 x 105 cells per dish in αMEM containing 15% heat-inactivated PBS and antibiotics (as above) (standard growth medium) supplemented with 50 μg/ml ascorbic acid (Fisher Scientific Comp., Fair Lawn, NJ) (AA) and 10 mM sodium-β-glucopyrophosphate (β-GP) BDH Chemicals, Poole, England (supplemented growth medium) (day 0). The next day (day 1), the medium was changed to supplemented medium with or without 10-4 M dexamethasone (DEX).

The Production of Antibodies

Bone marrow stromal cells were prepared from adult male Wistar rats as described (Maniaptopoulos et al., 1988). First subculture cells were plated at 103 cells/cm2 in 150-cm2 round tissue culture dishes and grown as above until bone nodules were formed and had started to mineralize. The cells were washed 3x with PBS, recovered by gentle scraping, and pipetted repeatedly to form a largely single cell suspension. Young female Balb/c mice were immunized with three consecutive intraperitoneal injections of the stromal cells (107 cells/0.2 ml/mouse) at 3-wk intervals. 3 d after the final injection, spleen cells from immune mice were fused with the myeloma cell line SP2/0 (Shulman et al., 1978) (kindly provided by Dr. M. Shulman, Department of Immunology, University of Toronto), with 50% polyethylene glycol (PEG) (PEG 1000, Sigma Chemical Co.) as fusion agent. After fusion, cells were cultured in microtiter plates (Nunc, Roskilde, Denmark) and hybridomas were selected by growth in HAT medium as described (Kohler and Milstein, 1975; Galfre et al., 1977; Connor et al., 1983). Supernatants from wells with growing cells were assayed at 15-20 d by indirect immunofluorescence on RC frozen sections (see below). Selected hybridomas were cloned by limiting dilution. One such hybridoma was rat bone marrow (RBM) 211 and the subclone RBM 211.13 was used in all experiments reported here.

The isotype of RBM 211.13 was determined by immunoprecipitation using immunodiffusion plates (The Binding Site Ltd, University of Birmingham Research Institute, Birmingham, UK).

Preparation of Ascites Fluid

To obtain ascites fluid, 106 cloned hybridoma cells were injected intraperitoneally into Balb/c mice that had been primed with 0.5 ml Pristane (2,6,10,14-tetramethylpentadecane) (Aldrich Chemical Co., Milwaukee, WI) 10-15 d earlier. Ascites fluids were collected, centrifuged, and the IgG fraction from the ascites fluid was purified by chromatography on columns of protein A-Sepharose (Pharmacia Fine Chemicals Co., Montreal, PQ).

Screening Assay for Selection of RBM 211.13

Supernatants from wells with good hybridoma growth were screened on frozen sections prepared from calvariae from 21 d fetal Wistar rats. The calvariae were removed from the fetuses in such a fashion as to leave periosteum, soft connective tissue, and skin intact. Calvariae were immersed in liquid nitrogen immediately and stored at −80°C until needed. To prepare frozen sections, the tissues were mounted on aluminum chucks in Tissue-Tek OCT (Tissue-Tek Division, Miles Laboratories Inc. Naperville, IL) and 6-8 μm sections were cut on a cryostat at −20°C. The sections were collected on 8- or 12-well slides (Carlson Scientific Inc., Peotone, IL) and used either immediately or stored at −80°C until needed. Antibodies were selected for bone-specific or bone-associated staining according to the immunofluorescence procedure outlined below.

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Immunofluorescence

On Frozen Sections. Cryostat sections were air-dried for 30 min at room temperature, fixed in 1-3.7% (vol/vol) formaldehyde (Fisher Scientific Comp.) in PBS for 5 min at room temperature and postfixed in -20°C methanol for 5 min. The sections were rinsed in PBS 3× and then incubated with 3% (wt/vol) BSA (essentially fatty acid free; Sigma Chemical Co.) prepped by heating at 80°C for 3 min in PBS. The sections were incubated with the BSA solution for 1 h at 37°C to block nonspecific sites. Sections were then overlayed with antibodies; culture supernatants were used undiluted, unpurified ascites fluids were diluted 1:800 in PBS, affinity-purified antisera were diluted 1:5 × 105 in 0.05% Tween 20 and incubated with affinity-purified, fluorescein isothiocyanate (FITC)-conjugated, affinity-purified P(ab')2 fragments of sheep anti-mouse IgG (NEI, Boston, MA) (1:75 dilution in PBS) for 30 min at room temperature. Sections were rinsed with PBS, mounted with Moviol (Hoechst Ltd, Montreal, PQ), and observed with a Zeiss Photomicroscope III (Zeiss, Oberkochen, West Germany) equipped with epifluorescence filters. Photographs were taken using Kodak (Eastman Kodak Co., Rochester, NY) T-Max 400 ASA or P3200 ASA films and developed with Kodak T-Max developer.

On Cultured Cells. Immunofluorescence on cultured cells was done essentially as described (Turksen et al., 1988). Washing, incubations with antibodies, mounting, and photography were as above.

Control samples were stained with an unrelated hybridoma supernatant or supernatant of the SP2/0 myeloma cells or FITC-(Fab')2-SAM alone.

Electrophoresis

PAGE was performed using the buffer system described by Laemmli (1970). Gels were run on a mini-gel apparatus at 150 V constant voltage. The commercially available molecular weight markers (BRL, Gaithersburg, MD) were used.

Immunoprecipitation

The immunoprecipitation procedure has been described in detail (Kubota et al., 1989). UMR 106.06 or RC II-V cells were used for these experiments. The specific immunoprecipitate was dissolved, boiled in Laemmli sample buffer, and the proteins were analyzed under reducing conditions by SDS-PAGE on a 10% gel and prepared for fluorography (Bonner and Laskey, 1974). The dried gel was exposed to Kodak SD-5 X-ray film at -70°C. Molecular weights were estimated using 14C-labeled protein standards (Gibco BRL, Burlington, Ontario).

Immunoblotting

The method of Towbin et al. (1979) was used to transfer proteins from SDS-PAGE gels to nitrocellulose sheets. At the completion of transfer, the sheets were blocked by 3% (wt/vol) BSA in PBS at 37°C for 2 h and incubated with asctes fluid of RMB 211.13 (1:1,000 dilution) overnight at room temperature on a shaker. The sheets were then washed 3× for 10 min each with PBS containing 0.05% Tween 20 and incubated with affinity-purified, peroxidase-conjugated rabbit anti-mouse IgG (Bio Rad Canada, Mississauga, Ontario) diluted 1:1,000. The reaction was developed with 4-chloro-1-naphthol. Control samples were treated with an unrelated hybridoma supernatant or supernatant of the SP2/0 myeloma cells or labeled second antibody alone.

Alkaline Phosphatase Activity

To localize APase histochemically, cells were fixed and incubated for 15 min at room temperature with 15 mg naphthol AS-TR phosphate (Sigma Chemical Co.) dissolved in 0.25 ml N,N-dimethylformamide (Fisher Scientific Comp.) and 50 ml 0.1 M Tris buffer, pH 8.9, with 30 mg Fast Red Violet LB salt (Sigma Chemical Co.). To detect APase activity in SDS-PAGE, the method of Trepanier et al. (1976) was used. APase activity was measured quantitatively in samples of cell homogenates as described by Turksen et al. (1990).

Treatment with PI-PLC

UMR 106.06 cells grown on coverslips were rinsed 3× with serum-free αMEM, incubated with 20 ng/ml PI-PLC (ICN) for 60 min at 37°C and then prepared for immunofluorescence as described above. Intensity of labeling with RMB 211.13 antibodies was measured in PI-PLC treated and untreated cells.

Treatment with PNGase F

UMR 106.06 cell homogenates were electrophoretically transferred as above. After the transfer, the nitrocellular strips were incubated with PNGase F (0.6 U/ml) (Boehringer Mannheim Biochimica, Montreal, PQ) for 24 h at 37°C as described by Tarentino et al. (1985). After enzyme treatment, the strips were processed for immunoblotting as above.

Flow Cytometry

RC cells were prepared as above and plated. After 1 d, individual populations were trypsinized (0.01% trypsin in citrate saline) and counted. For the quantitation of RMB 211.13 positive cells, we plated 2.5 × 105 cells per T75 flask from each population. Where indicated, RC II-V mixed populations were prepared by mixing equal volumes of cells from each separate population. These cultures were maintained for 3-5 d in culture in supplemented medium as above and labeled with RMB 211.13 antibody as follows. Cells were trypsinized and collected by gently pipeting with a Pasteur pipet and centrifuged. The cells were resuspended in fresh growth medium at 2-3 × 106 cells/ml and incubated at 37°C for 1 h to allow the cells to recover from the trypsinization. For sorting, viable cells were used. For quantitation of relative percentage positive cells were fixed in formaldehyde (see section on immunofluorescence). After that, cells were centrifuged as above and resuspended in sterile RMB 211.13 hybridoma supernatant (106 cells/ml) and incubated at 37°C for 30 min. After the incubation, cells were washed with sterile PBS 3× by resuspension and centrifugation, incubated with FITC-conjugated sheep antimouse antibodies as above for 30 min at 37°C and then washed 3× with PBS. Immediately before analysis, cells were filtered through a 30-mm mesh nylon filter. Samples were run on a Coulter Epics V cell sorter equipped with an Argon laser operated at 488 nm and 400 mV for fluorescence excitation. Fluorescence was detected with an S-11 photomultiplier tube at 550 V with a 520-nm barrier filter. For sorting, we set gates to recover the 10% of cells with highest staining and the 10% of cells with lowest staining. Control samples were stained with an unrelated hybridoma supernatant or supernatant of the SP2/0 myeloma cells or FITC-(Fab')2-SAM alone.

Panning

Preparation of Antibody-coated Plates. 100-mm Fisher-brand sterile petri dishes (Fisher) were incubated for 1 h at 37°C with 5 ml of serum-free culture medium of the RMB 211.13 hybridoma cells. After incubation, the antibody solution was recovered, plates were washed 3× with Ca2+-free PBS to remove residual unbound antibodies, and then incubated with heat-inactivated (80°C for 3 min) 3% BSA in PBS for 1 h at 37°C. The plates were then rinsed 3× with 5 ml of Ca2+-free PBS and used immediately for panning. For controls, we used dishes coated with antibodies in medium conditioned by other hybridomas or the 3% BSA solution alone.

Panning. Fig. 8 shows a schema of the panning procedures. Primary cultures of RC cells, grown in supplemented medium (as above) for 3-4 d, were collected by brief trypsinization, centrifuged, washed, resuspended at 1-2 × 105 cells/ml in Ca2+-free PBS, and then filtered to remove cell clumps and assure a single cell suspension. 2-4 × 106 cells/dish were incubated at 37°C on 100-mm Petri dishes previously coated with the RMB 211.13 antibody as above. After 40 min, nonadherent cells were gently removed by washing with Ca2+-free PBS. Adherent cells were incubated in regular growth medium for 3-4 h and then recovered by trypsinization. Cells adherent on dishes coated with RMB 211.13 antibodies were compared with the cells nonadherent to these dishes and the cells nonadherent to BSA-coated dishes. Nonadherent cells were treated similarly to adherent cells, in that they were recovered and incubated in tissue culture dishes with growth medium for the same period of time as antibody-adherent cells, then trypsinized. For further analyses, cells recovered after trypsinization were counted and replated at appropriate densities for measurement of APase activity (1-5 × 105 cells/35 mm) or bone nodule formation (106 cell/35-mm dish).

Statistical Analysis

Levels of significance for comparisons between samples were determined using the two-tailed t-test distribution. Results are expressed as the mean ± SD of three to five dishes or samples per point.
Results

In the fusion in which the RBM 211.13 antibody was isolated, the supernatant from one well showed staining restricted to osteoblasts and the cells close to bone as described below. This well was cloned by limiting dilution and one clone, designated RBM 211.13, was used to prepare all supernatants and ascites fluid used in these experiments. The RBM 211.13 antibodies were determined to be of the IgG subclass.

Cell and Tissue Staining with RBM 211.13

Staining of sections of the top of fetal rat heads, including the calvaria bone with periosteum, soft connective tissue,

![Figure 1](image-url)

Figure 1. Immunofluorescence with RMB 211.13. (A) Hematoxylin-eosin stain of 6 µm paraffin section of the top of the head of a 21-d fetal rat. e, epithelium of the skin; c, soft connective tissue; m, muscle; b, calvaria bone. The white bracket denotes approximately the same area as the stained area in B and an equivalent area at higher power in C; the bracket encompasses the mineralized bone, osteocytes, osteoblasts, and preosteoblasts. (B) The antigen recognized by RBM 211.13 was restricted to the osteoblasts along the bone surfaces, the preosteoblasts in the periosteum for three to four cell layers behind the osteoblasts, and the preosteoblasts on the endocranial surface of the bone. Most osteocytes and the bone matrix did not label nor did the fibrous connective tissue. e, epithelium of the skin; b, bone. (C) Higher magnification of the bone and adjacent osteoblasts and preosteoblasts. B–C are 6 µm frozen sections. (A, 225×; B, 225×; C, 450×).

and skin intact showed that RBM 211.13 labeled only osteoblasts and preosteoblasts in the region three to four cell layers out from the bone surfaces and most osteocytes were not labeled (Fig. 1). Brief treatments of the sections with collagenase, hyaluronidase, trypsin, or pronase did not expose any cryptic antigenic sites nor obliterate the bone cell-associated staining (not shown). In fetal rat long bones, including the metatarsal (Fig. 2, A and B) and femur (not shown), the osteoblasts, the periosteum, chondrocytes of the hypertrophic zone of cartilage, and the associated periosteum-perichondrium were all intensely stained. Resting zone and proliferating zone chondrocytes were unlabeled. Similar to sections from the head, soft connective tissue and muscle were negative (not shown).

Because the staining pattern and cells expressing the antigen were similar to those that are known to express high APase activity as detected histochemically, we compared APase labeling by a histochemical assay (Fig. 2 C) with RBM 211.13 labeling on the metatarsal and the labeling patterns were nearly identical (see Fig. 2, B and C). RBM 211.13 did not label fetal kidney but labeled the collecting tubules of adult kidney and labeled a very few cells in sections of fetal and adult liver, all similar to the histochemical APase staining (not shown). There was no labeling in fetal intestine, brain, and skin (dermis or epidermis) sections with RBM 211.13 antibody (not shown).

A variety of osteoblastic cells and cell lines were also tested in vitro. Among the cells of two rat osteoblastic osteosarcoma cell lines, virtually all cells were labeled in the UMR 106.06 population (Fig. 3 A), while a large variation in intensity of individual cells was obvious in ROS 17/2.8 (Fig. 3 B). The pattern was typical of a membrane-associated molecule with the epitope exposed on the external surface of live, fixed, or fixed and permeabilized cells. A gradation of labeling intensity was also obvious in isolated RC populations in which some cells were not labeled at all by RBM 211.13, others were labeled slightly, and others were intensely stained (Fig. 3 C) (see quantitation below). In stromal bone marrow populations, which were used for immunization in this case, a proportion of cells labeled with a pattern similar to that seen in fetal RC cells (not shown). The proportion of cells labeled in all populations was similar to the proportion labeling for APase activity by the histochemical assay. Fetal rat skin fibroblasts were not labeled with RBM 211.13. The RBM 211.13 antibody did not cross-react with human, mouse, rabbit, and chick osteoblastic cells.

Analysis of the Molecule Recognized by RBM 211.13

Previously, Noda et al. (1988) showed that the APase of osteoblastic cells was attached to the external cell surface via a glycosyl-phosphatidylinositol (GPI) linkage that is sensitive to phosphatidylinositol-specific phospholipase C (PI-specific PLC). When we treated UMR 106.06 cells for 1 h at 37°C with 20 µg/ml PI-PLC, the labeling with RBM 211.13 was diminished to almost background (not shown). Next, we did an immunoprecipitation from 35S-methionine-labeled macromolecules of UMR 106.06 using the RBM 211.13 antibody. One protein was precipitated with an M, ~80 kD under reduced conditions (Fig. 4 A). On immunoblots, on the other hand, RBM 211.13 recognized a band only when proteins were run under nonreducing conditions and without boiling samples. Under these conditions, RBM
Figure 2. Labeling of frozen sections of metatarsal bones of 21-d fetal rats. (A) Metatarsal bones of the rat stained with hematoxylin-eosin. Note the development of the bony core, the cartilage, and the surrounding fibrous tissue. (B) Immunofluorescence with RBM 211.13. The osteoblasts of the bony core were labeled, but not the bone matrix. Hypertrophic chondrocytes labeled, but no other chondrocytes or the cartilage matrix. The periosteum labeled intensely, but not the fibrous connective tissue. (C) Localization of alkaline phosphatase (APase) activity by histochemical stain showed virtually coincident staining of the APase and the labeling with RBM 211.13. (A, 175×; B, 250×; C, 150×).

RBM 211.13 recognized a very broad band with $M_r \sim 120-150$ kD. This band corresponded to the region containing APase activity as detected histochemically (Fig. 4 B). This band was PNGase F sensitive (Fig. 4 C), suggesting that the RBM 211.13 antibody recognizes an epitope carbohydrate in nature.

**RBM 211.13-positive Cells in Different Fetal RC Populations**

Since individual RC cells in culture were stained with different intensities from very bright to undetectable with the RBM 211.13 antibody, we determined by flow cytometry the
Figure 3. Immunofluorescence with RBM 211.13 on cultured osteoblastic cells. RBM 211.13 labeled a surface-associated antigen present (A) in all UMR 106.06 cells and (B) to varying degrees in individual ROS 17/2.8 cells. (C) The gradation of labeling intensities from cells with intense staining to cells with undetectable label was also evident in primary cultures of fetal RC cells. (A and B, 480×; C, 325×).

Table I. Quantitation of Sequentially Released RC Populations with RBM 211.13

| Population  | % Positive |
|-------------|------------|
| RC I        | 3          |
| RC II       | 13         |
| RC III      | 53         |
| RC IV       | 74         |
| RC V        | 61         |
| mixed RC II-V | 59       |

Cells isolated from 21-d fetal RC with collagenase digestion were labeled with RBM 211.13 and analyzed by flow cytometry. Populations sequentially released with time of collagenase treatment are populations RC I through RC V, released after 10, 20, 30, 50, and 70 min, respectively. RC II–V is a population comprising RC II to RC V mixed together as used in subsequent experiments. See Materials and Methods for further details.
the positive cells in the higher population were "diluted" by negative cells in the lower populations (Table I).

**Cell Sorting with RBM 211.13 and Isolation of the Nodule-forming Osteoprogenitor Cell**

Previously we have shown that when RC cells are grown in medium supplemented with ascorbic acid and β-glycerophosphate, three-dimensional nodular structures form which have the histological, immunohistological (Bellows et al., 1986), and ultrastructural (Bhargava et al., 1988) appearance of woven bone (Fig. 5). In a typical population of RC cells grown under standard conditions to form bone nodules, the number of osteoprogenitors is <1%, based on limiting dilution analysis (Bellows and Aubin, 1989). We therefore sought to determine whether the bone nodule-forming osteoprogenitor cell could be enriched in a population by sorting cells on the basis of RBM 211.13 binding. Primary RC cells were plated for 4–5 d after enzymatic isolation to allow recovery, then labeled with RBM 211.13, and the fluorescence intensity profile was determined (Fig. 6). Windows were set to collect the 10% of cells with brightest staining and the 10% with little or no staining. The sorted cells were then assessed for their ability to form bone nodules under standard conditions. No nodule-forming osteoprogenitors were recovered in the sorted cells negative for RBM 211.13 labeling. Bone nodule-forming cells were present in the cells sorted for intense RBM 211.13 staining (Fig. 7A). However, a series of control experiments with labeled and unlabeled cells indicated that not all the nodule-forming cells expected on a per plated cell basis were being recovered, suggesting that mechanical trauma caused by the shear forces in the flow stream caused at least some of the nodule-forming osteoprogenitor cells to be lost (Fig. 7).

![Image](image_url)

**Figure 5.** Morphological features of bone nodules formed in RC cell populations grown in medium supplemented with ascorbic acid and β-glycerophosphate. (A) By day 10, the RC cells had grown to multilayered cell sheets. (B) In discrete areas of the culture dish, cells with a marked polygonal morphology, tight packing and surrounded by refractile matrix were evident by days 12–13. (C) These areas became noticeably three-dimensional and became opaque as they mineralized. (D) A hematoxylin-eosin stain of a 6-μm paraffin section of a nodular structure as in C, embedded and cut in cross-section perpendicular to the floor of the culture dish. The nodule has all the morphological features of woven bone with a cuboidal layer of osteoblastic cells on the upper (medium) surface and osteocyte-like cells embedded in a dense collagenous matrix. For further details, see Bellows et al., 1986 and Bhargava et al., 1988.

![Image](image_url)

**Figure 6.** Flow cytometry with primary RC cells. Mixed populations RC II–V were cultured for 5–6 d before labeling with RBM 211.13. (X axis) Log fluorescence intensity, arbitrary scale channels 1–256. (Y axis) Relative cell frequency. The gate for collection of the 10% of cells with lowest intensity labeling is indicated in the top panel and that for the 10% with most intense labeling is shown in the bottom panel.
Figure 7. Bone nodule formation by cells sorted for low or high staining with RBM 211.13. RC cells sorted as described in Fig. 6 were collected, plated at 10^5 cells/35-min tissue culture dish, and grown for bone nodule formation as described in Materials and Methods. (A) In the absence of DEX; (B) in the presence of DEX. Cells labeled with antibody and passed through the flow stream without sorting (PASS) had fewer nodules than the same cells without passing through the flow stream (CO) in the absence (A) or in the presence (B) of DEX. Significantly different from the corresponding control, * P < 0.05, ***, P < 0.001. Cells sorted on the basis of intense staining with RBM 211.13 (HIGH) formed many bone nodules, while those sorted for low staining (LOW) made very few nodules. Significantly different from PASS or HIGH, ***, P < 0.001. DEX stimulated nodule formation in all populations; the fold stimulation in DEX treated versus untreated populations in CO was 10 ± 0.3, in PASS was 9 ± 0.2, in the HIGH population 7 ± 3, and in the LOW population 11 ± 1. Bars represent the means ± SD of five dishes.

Panning with RBM 211.13

Because of the apparent sensitivity of nodule-forming osteoprogenitor cells to passage through the flow stream of the cell sorter, we used immunopanning on RBM 211.13-coated petri dishes for further experiments (Fig. 8). Over a 30–45-min period, 40–60% of the RC population became attached to petri dishes coated with RBM 211.13. Over the same period, virtually no RC cells attached to dishes coated with BSA. As expected, APase activity was enriched in cells panned by adherence to RBM 211.13 and depleted in the population not adherent to RBM 211.13 (Fig. 9).

We next sought to determine whether the bone nodule-forming osteoprogenitors could be enriched in the adherent or the nonadherent fraction, i.e., if they were present in the APase-positive or APase-negative pools. A time course of adherence to RBM 211.13 indicated that among the pool attaching in the first 15 min were few bone nodule-forming cells; concomitantly they were found in the nonadherent population. By 30–45 min, however, the majority of the bone nodule-forming cells was found in the adherent pool (Fig. 10). Therefore, an adherence time of 45 min was used in all subsequent experiments. Taking the BSA-nonadherent fraction as representative of the mixed RC population, valid

Figure 8. Schema of the immunopanning procedure. Petri dishes were prepared by pretreatment with BSA or the antibody RBM 211.13. Primary populations RC II–V were collected and divided into equal aliquots. One aliquot was incubated on the BSA-coated plates; virtually no cells attached yielding the BSA nonadherent control (BSA NA) population. One aliquot was added to the RBM 211.13–coated dishes, yielding an RBM 211.13 adherent population and an RBM 211.13 nonadherent population. All populations were recovered and replated at equivalent cell numbers for determination of alkaline phosphatase (APase) activity or for bone nodule formation.

Figure 9. Determination of alkaline phosphatase (APase) activity in the control (unfractionated, BSA NA) population compared to the RBM 211.13 adherent (211 A) and the RBM 211.13 nonadherent (211 NA) population. Cells panned as in Fig. 8 were collected and plated at equivalent cell numbers in 35-mm dishes. 48 h later, APase was determined as in Materials and Methods. APase activity was enriched in the 211 adherent population and depleted in the nonadherent population compared to control population. Bars represent the means ± SD of triplicate dishes. Significantly different from BSA NA, **, P < 0.03, ***, P < 0.001.
Conditions

Figure 10. Time course of adhesion of bone nodule-forming osteoprogenitor cells to RBM 211.13-coated petri dishes. Cells were panned as in Fig. 8 and the 211 adherent (211 A) and the 211 nonadherent (211 NA) populations were collected at various times and replated under the conditions of bone nodule formation. At 15 min, most nodule-forming cells were still in the nonadherent population. By 30-45 min, most nodule-forming cells were in the adherent fraction. Bars represent the means ± SD of five dishes. Significantly different from 211A15', *** P < 0.001.

given that no cells attached to the BSA, we found an approximately two- to threefold enrichment for bone-forming osteoprogenitor cells in the RBM 211.13-adherent cells and a concomitant depletion in the RBM 211.13 nonadherent pool (Fig. 11).

Figure 11. Enrichment of the bone nodule-forming cells after immunopanning. Primary RC cells were panned for 45 min as in Fig. 8, collected and plated at equivalent cell numbers for bone nodule formation in the absence (A) or the presence (B) of DEX. 211 adherent (211 A) and 211 nonadherent (211 NA) populations were compared with the unfractionated control, BSA nonadherent (BSA NA) population. The number of nodules formed on a per plated cell basis was greater in the 211 A and less in the 211 NA population compared to control (BSA NA). DEX stimulated nodule formation in all populations, however the fold stimulation in DEX treated versus untreated cells in BSA NA was 8 ± 0.3, in 211 A was 5 ± 0.4 and in 211 NA was 33 ± 0.8. Bars represent the means ± SD of five dishes. Significantly different from BSA NA, *** P < 0.001.

Earlier, we reported that glucocorticoids such as DEX stimulate bone nodule formation in RC populations by inducing expression of bone formation by cells that do not form bone in the absence of DEX (Bellows et al., 1987, 1990). We therefore determined whether the cells requiring DEX for bone formation were restricted to either of the extreme ends of the populations sorted by flow cytometry and/or the RBM 211.13-adherent or the nonadherent fraction of cells or were present in both. DEX caused a large increase in expression of nodule formation in the APase-negative sorted cells and a somewhat smaller response in the APase-positive sorted cells (Fig. 7 B). Similarly, in a typical panning experiment, bone nodule-forming cells were enriched in the 211-adherent fraction and depleted from the 211-nonadherent fraction (Fig. 12 A). DEX elicited an approximately eightfold increase in the number of nodules formed in the control (unpanned; BSA-nonadherent) population. In the RBM 211.13-adherent population, DEX elicited a 5-fold increase, and in the RBM 211.13-nonadherent population, a 33-fold increase in the number of bone nodules formed compared to the same populations in the absence of DEX (Fig. 12 B).

Discussion

In this paper, we have described the isolation of a mAb reacting selectively with osteoblastic cells in vivo and in vitro. Characterization of the antibody showed that it recognizes the bone-liver-kidney isoenzyme of rat APase. This evidence
includes the localization of the staining coincident with the histochemical staining for APase, the release of the antigen by PI-PLC, immunoprecipitation, and immunoblotting. Our data further indicate that RBM 211.13 recognizes an epitope present in the nonreduced form of the native molecule and possibly carbohydrate in nature. Since the antibody recognizes an epitope present on the external face of the plasma membrane of living cells, we have used it to separate APase-positive and APase-negative cells, and have determined that the nodule-forming osteoprogenitor cell present at low frequency (<1%) in typical RC mixed populations can be recovered from the heterogeneous population in the fraction of cells expressing APase. On the other hand, the osteoprogenitor cells requiring glucocorticoids to form bone were enriched in the APase-negative fraction. We have, therefore, described a combined approach in which expression of surface molecules detected immunologically can be used to study the osteoblast lineage by isolation or enrichment for cells that functionally can make bone in culture.

Data from several labs have confirmed that the populations of cells isolated from fetal rat calvariae are heterogeneous for the expression of osteoblastic properties (Wong, 1980; Aubin et al., 1982; Heersche and Aubin, 1990). Although several procedures have been used to enrich for or purify subpopulations, no technique has yet been reported in which a subpopulation has been isolated specifically on the basis of expression of a particular osteoblast marker. Using the mAb RBM 211.13, we have enriched for the cells expressing high APase activity in RC populations and also obtained a pool in which APase activity is low. In vivo, APase-positive cells are heterogeneous and include the postmitotic functional osteoblast on the bone surface and the proliferative cells comprising the three to four cell layers residing behind the osteoblasts, which are thought to be preosteoblasts and osteoprogenitor cells at different stages of differentiation. The level of APase has been used routinely in in vitro experiments as a marker of osteoblast differentiation. It is generally accepted that as the specific activity of APase in a population changes there is a corresponding change in the state of differentiation of cells, i.e., increases in APase activity reflect a maturation from an earlier to a more mature stage of osteoblast differentiation (Rodan and Rodan, 1984). Clearly, some cells in our APase-positive pool did proliferate and we could visualize mitotic, APase-positive cells, probably representative of the preosteoblast pool rather than mature osteoblasts. Over a time of 2–3 d after panning, the cells with "high" or "low" APase continued to express relatively high or low activities, respectively. In preliminary experiments, we have found that with longer time (>5 d), however, APase activity decreased in the initially high APase population, perhaps reflecting terminal differentiation to APase-negative cells that would be consistent with maturation of APase-positive cells to osteocyte-like cells and increased in DEX in the initially low APase population, perhaps reflecting their maturation to more differentiated cells (data not shown).

The time course for adherence of RC cells to RBM 211.13–coated dishes showed that maximum attachment of cells occurred after 30–45 min at 37°C. It was of particular note that the cells capable of forming bone nodules in the absence of DEX were specifically enriched in the APase-positive (211-adherent) population and depleted from the APase-negative (211-nonadherent) population with time. This is consistent with the cells sorted by flow cytometry in which, without DEX, nodule-forming cells were found in the fraction of cells sorted for high intensity staining with the APase antibody and were not present in the fraction with low intensity staining. Thus, it appeared that the bone nodule–forming osteoprogenitor could be recovered in the APase-positive fraction, suggesting that the cells forming bone in the absence of glucocorticoids are relatively late in the differentiation pathway from committed cell to osteoblasts. Consistent with this, earlier we found that the bone nodule–forming cell also has a relatively small proliferative capacity, i.e., is present in populations only up to a population doubling level of \( \sim 10 \) (Bellows and Aubin, 1989; Bellows et al., 1990). One must keep in mind, however, that under standard conditions, \( \sim 0.3\% \) of the cells in a mixed RC population are bone nodule–forming osteoprogenitors while 40–60% are APase-positive. Thus, the osteoprogenitors sorted on the basis of APase activity are a small subpopulation of the total APase-positive pool. We have argued elsewhere that the osteoprogenitor must go through approximately five to six doublings to form a visible nodule (Bellows and Aubin, 1990), suggesting that these cells are a subpopulation of APase-positive cells still with some capacity, albeit limited, to divide.

Earlier, we found that DEX stimulated the proliferation of bone nodule–forming osteoprogenitor cells, resulting in the average size of a nodule being bigger in cultures grown in DEX than in cultures without DEX (Bellows et al., 1990). In addition, a second class of bone nodule–forming osteoprogenitor was present in RC populations, which made bone in culture only if DEX was present in the culture medium (Bellows et al., 1987, 1990). The additional nodules formed in the presence of DEX could have resulted from DEX increasing the proliferative capacity of more mature osteoprogenitor cells so that they achieved a sufficient number of cell divisions to form a visible nodule. However, another possibility was that DEX was acting upon a population of osteoprogenitors that required glucocorticoids to proliferate and/or differentiate along the osteogenic pathway. The second major conclusion to be drawn from our current experiments is that the DEX–requiring osteoprogenitor may be a less mature cell, not yet expressing APase on its surface (and therefore present in the APase-negative or nonadherent population), and that this cell type either will not proliferate and/or differentiate into bone nodule–forming cells in the absence of DEX. Consistent with their being relatively immature was the finding that in the presence of DEX nodules continued to form "later" in the culture period than in its absence (Bellows et al., 1987). The smaller fold increase in nodule number in the APase-positive population may result from DEX acting to increase the proliferative capacity of the more mature, APase-positive cells so that a detectable nodule may form as we had suggested earlier. However, an equally likely possibility is "contamination" of the adherent cells by the DEX–requiring cells in the nonadherent, APase-negative fraction, because it is difficult technically to remove all nonadherent cells from the adherent population.

Even though it is clear that panning is well adapted to large scale preparations, it does suffer from the limitation that a lack of purity is achieved in many instances, especially when the desired population is present in low frequency. Nevertheless, it is clear that we did achieve enrichment for populations of cells present at <1% of the whole cell population.
Figure 13. A proposed lineage diagram with differentiation steps for cells of the osteoblast lineage. Immunoselection with antibodies against APase allowed discrimination of an APase-positive osteoprogenitor (OP) making bone in vitro without DEX (late OP) from an earlier APase-negative OP (early OP), which in vitro proliferates and/or differentiates only in the presence of DEX. We have superimposed on this scheme where we think these types of OP cells reside in comparison to cells expressing other osteoblast markers. For conciseness and ease of comparison, this table is based primarily on immunocytochemical evidence for the expression of osteoblast-associated markers. However, other biochemical approaches support the data referenced (for reviews see Rodan and Rodan, 1984; Nijweide et al., 1986; Martin et al., 1987; Butler, 1989). J. Doty and Schofled, 1976. 2, Mark et al., 1987a. 3, Turksen and Aubin, current paper. 4, Bronckers et al., 1985. 5, Mark et al., 1987b. 6, Chen et al., 1991. 7, Rouleau et al., 1988, 1990. 8, Rodan and Rodan, 1984; Martin et al., 1987. 9, Tenenbaum and Heersche, 1985. 10, Bellows et al., 1987; Bellows and Aubin, 1989.

As importantly, we have shown that expression of APase activity can be used to separate osteoprogenitors that do not require glucocorticoids (positive selection) from those that require glucocorticoids (negative selection) to make bone. Our data are consistent with the former being a more mature, less proliferative population and the latter a less mature, more proliferative population. On the basis of the immunoselection and functional assay for bone formation described here, we propose a lineage scheme in which we now identify a less mature, APase-negative, glucocorticoid-requiring osteoprogenitor separate from a later APase-positive osteoprogenitor, and we have tentatively placed these cells along a pathway encompassing cells expressing other osteoblastic markers (Fig. 13).

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