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Molecular Cloning of the Cell Surface Antigen Identified by the Osteoprogenitor-Specific Monoclonal Antibody, HOP-26

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Abstract Bone is a highly organized structure comprising a calcified connective tissue matrix formed by mature osteoblasts, which develop from the proliferation and differentiation of osteoprogenitor cells. The osteogenic cell lineage is thought to arise from a population of uncommitted multipotential stromal precursor cells (SPC) which reside close to all bone surfaces, in the bone marrow spaces and the surrounding connective tissue. These SPC also give rise to related cell lineages which form cartilage, smooth muscle, fat, and fibrous tissue. Due to the lack of well defined cell surface markers, little is known of the precise developmentally regulated changes in phenotype which occur during the differentiation and maturation of human osteoprogenitor cells into functional osteoblasts and ultimately, terminally differentiated osteocytes. In order to identify antibody reagents with greater specificity for osteoprogenitors we generated a series of antibodies following immunization with freshly isolated human bone marrow stromal fibroblasts. One such antibody, HOP-26, reacts with a cell surface antigen expressed by SPC and developing bone cells. We now demonstrate that this mAb identifies a member of the tetraspan family of cell surface glycoproteins, namely CD63. Western blot analysis of human bone marrow stromal cells (HBMSC) has revealed that like a well defined CD63 mAb 12F12, HOP-26 interacts with a heavily glycosylated cell surface protein with an apparent molecular weight of 50–60 kD. J. Cell. Biochem. 89: 56–66, 2003.

Key words: HOP-26; osteoprogenitor; stromal precursor cell; CD63; fibroblast colony forming unit; CFU-F; bone; marrow fibroblast

The development of the active bone-forming cell, the osteoblast, from their precursor cells (osteoprogenitors) occurs through a series of cellular transitional stages which can be characterized by morphological, biochemical, and molecular criteria [Owen, 1985; Aubin et al., 1993; Triffitt, 1996]. To date, the most highly characterized cells of the osteogenic lineage are the osteoblasts, which are responsible for the production of the bone matrix constituents [Aubin and Triffitt, 2002]. Active osteoblasts are identified morphologically by their cuboidal appearance and by their association with newly synthesized bone matrix at sites of active bone formation. These committed osteogenic cells are also characterized histologically by their expression of high levels of alkaline phosphatase (AP) activity and biochemically by the synthesis of certain bone-associated matrix proteins such as collagen type I, osteocalcin, bone sialoprotein, osteonectin, and osteopontin [Robey et al., 1992; Robey and Boskey, 1995; Robey, 1996; Aubin and Triffitt, 2002].

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A major obstacle in the study of human bone tissue has been the inability to phenotypically define and specifically isolate early progenitor cells of the osteogenic and related stromal lineages. Putative stromal precursor cells (SPC) have been identified in the bone marrow of a number of species, including humans, by their ability to form clones of cells morphologically resembling fibroblast (fibroblast colony-forming cells or CFU-F) [Castro-Malaspina et al., 1980], when single cell suspensions of bone marrow are explanted at appropriate densities in adherent cultures [Friedenstein, 1976; Friedenstein et al., 1978; Castro-Malaspina et al., 1980]. In recent years, the direct isolation of these cells has been facilitated to some extent by the generation of a limited number of monoclonal antibodies with apparent specificity for these cells. A number of groups, including our own, have reported the generation of monoclonal antibodies which demonstrate particular reactivities to SPC and their progeny, including SH-2, SH-3, SH-4, SB-10 [Bruder and Caplan, 1989, 1990a,b; Bruder et al., 1997, 1998], and STRO-1 [Simmons and Torok-Storb, 1991].

Previous studies from our laboratory have shown that monoclonal antibody STRO-1 reacts with an as yet unidentified cell surface molecule whose expression is restricted to a minor subpopulation of cells in fresh aspirates of human bone marrow [Simmons and Torok-Storb, 1991], including all assayable CFU-F. Moreover, within this population are cells with the potential to give rise to a range of stromal cell lineages including fibroblasts, smooth muscle cells, adipocytes, chondrocytes, and osteoblasts [Simmons and Torok-Storb, 1991; Gronthos et al., 1994]. In addition to STRO-1, we have recently described a mAb HOP-26, which exhibits reactivity to stromal fibroblast-like cells both in vivo and in vitro, and with candidate osteoprogenitor cells in sections of adult trabecular bone tissue [Joyner et al., 1997]. In this report we demonstrate that HOP-26 identifies a member of the tetraspan family of cell surface glycoproteins, namely CD63, a lysosomal membrane glycoprotein which is translocated to the plasma membrane following activation with a variety of agonists including thrombin and PMA [Metzelaar et al., 1989, 1991; Vischer and Wagner, 1993]. This is the first report documenting the expression of CD63 in human bone tissue and given its specific distribution on putative osteoprogenitors and cells within the marrow fibroblast lineage, suggests an important role for this molecule in bone development.

MATERIALS AND METHODS

Human Bone Marrow Stromal Cell (HBMSC) Cultures

Stromal cultures were established, essentially as described by Simmons et al. [1989]. Bone marrow mononuclear cells (BMMNC) were prepared by buoyant density gradient centrifugation as previously described [Zannettino et al., 1995]. The use of normal BM cells for these studies was approved by the Human Ethics Committee of the Royal Adelaide Hospital. After washing thrice in "HHF" (Hanks Buffered Salt Solution (HBSS) supplemented with 20 mM HEPES and 5% fetal calf serum (FCS)), the $1–5 \times 10^7$ BMMNCs were resuspended in 10 ml of alpha-modification of Eagles' medium (α-MEM: Flow Laboratories, Irvine, Scotland) supplemented with Folic acid (0.01 mg/ml), myo-inositol (0.4 mg/ml) (Sigma Chemical Co., St. Louis, MO), 50 mM/l 2-mercaptoethanol, 1 mM/l hydrocortisone sodium succinate (Sigma), 12.5% FCS, and 12.5% horse serum (CSL, Melbourne, Australia) and cultured in 25 cm^2 flasks (Becton Dickinson Labware, Franklin Lakes, NJ). Upon development of a confluent stromal layer, the cells were detached using 0.05% (w/v) trypsin-EDTA in PBS (Gibco Invitrogen Austrailia, Victoria, Austrailia) and replated in the same medium at approximately $1–2 \times 10^4$ cells per cm^2 in 2×75 cm^2 tissue culture flasks (Becton Dickinson Labware).

Normal Human Bone Cell (NHBC) Cultures

Trabecular bone specimens were obtained from normal patients (58–80 years old) during routine knee and hip replacements from the Department of Orthopaedic Surgery and Trauma at the Royal Adelaide Hospital. The use of trabecular bone specimens for these studies was approved by the Human Ethics Committee of the Royal Adelaide Hospital. Bone explants were cultured as previously described [Gronthos et al., 1997]. Briefly, bone chips were grown in T-75 tissue-culture flasks in α-MEM supplemented with 10% FCS, 2 mM L-glutamine and 100 mM L-ascorbate-2-phosphate (Novachem, Melbourne, Australia). Cultures were fed three times per week for 5–6 weeks and were incubated at 37°C in 5% CO_2 until confluent.

In Situ Immunofluorescence Staining of Cultured Human Bone Marrow Stromal and NHBCs

Cultures of human bone marrow stromal and bone cells, were trypsinized as described above and seeded at 2×10^4 cells per well of an 8-chamber slide (Nunc, Inc., Naperville, IL) 16 h prior to immunostaining. Cultures were washed three times with ice-cold HHF and then fixed in acetone/methanol 1:1 at $-20^{\circ}C$ for 15 min. After washing three times in PBS the cells were blocked in 5% (v/v) normal goat serum (NGS) for 1 h at room temperature. The blocking buffer was removed and saturating levels of the mAbs 12F12 [Zannettino et al., 1996], HOP-26 [Joyner et al., 1997], or isotype-matched non-binding controls were added for 60 min at room temperature. The slides were washed three times in PBS + 0.05% (v/v) TritonX-100 (Sigma). To reveal primary antibody reactivity, cells were incubated with a 1/50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse $F(ab)_2$ antisera (Silenus, Hawthorn, Victoria, Australia) for 60 min at room temperature. The cells were then washed as above and mounted in aqueous mountant (Uvinert, BDH). The labeled specimens were examined using an Olympus BH2-RFCA fluorescence microscope.

Western Blotting

Cultured HBMSC were detached by trypsin digestion as described above, washed twice with PBS, and resuspended at 2×10^7 cells per ml in PBS. Cells were lysed by the addition of an equal volume of 1% (v/v) NP40/PBS as previously described [Cole et al., 1987]. Lysates comprising 1×10^6 cell equivalents were resuspended in an equal volume of 2 × non-reducing sample buffer (62.5 mM Tris, 3% (w/v) SDS, 10% (v/v)), boiled for 3 min, and analyzed by 10% (w/v) SDS–polyacrylamide gel electrophoresis [Laemmli, 1970]. After electrophoresis, proteins transferred to Polyvinyl-difluoroacetate (PVDF; MSI Membranes, Geneworks, Adelaide, Australia) at 30 mA overnight in a wet blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA). After blocking for 2 h with 5% (w/v) skim milk powder, 0.05% (v/v) Tween-20 in PBS, filter strips were incubated with either mAb HOP-26, 12F12 antibodies, or isotype-matched controls (all culture supernatants) for 1 h at room temperature. Filter strips were washed with 0.05% (v/v) Tween-20 in PBS and subsequently incubated with goat anti-mouse conjugated to AP (Amersham, Poole, England). Immunoreactive proteins were visualized on a FluorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Molecular Dynamics) as recommended by the manufacturer.

Expression Cloning of the cDNA Encoding HOP-26

The cDNA encoding the cell surface antigen identified by the mAb HOP-26 was isolated from a HBMSC cDNA library in the retroviral vector, pRUF*neo* as recently described [Zannettino et al., 1996]. Briefly, cDNA synthesized from mRNA from HBMSC cultures was cloned into the retroviral vector pRUF*neo*. Plasmid DNA from the library was used to transfect a viral packaging line (PA317). Virus containing supernatant from these cells was used to infect the packing cell line ψ_2, which in turn was used to infect the murine factor-dependent cell line FDC-P1. Infected cells were selected for G418 resistance, labelled with HOP-26 antibody and cells specifically binding the antibody were isolated by immunomagnetic bead selection (Dynabead, Dynal, Oslo, Sweden). After expansion of the initially selected cells in culture, immunomagnetic bead selection was repeated a further two times. FDC-P1 cells which demonstrated specific binding of HOP-26 antibody (approximately 60%) were purified by fluorescence-activated cell sorting (FACS) and clones isolated following culture in semi-solid media as previously described [Zannettino et al., 1996]. To recover proviral cDNA inserts corresponding to the HOP-26 antigen, the polymerase chain reaction (PCR) using retroviral specific primers was performed on genomic DNA prepared from six HOP-26^+ FDC-P1 clones, as previously described [Zannettino et al., 1996].

Partial-Sequencing of PCR-Rescued cDNA Clones and Computer Analysis

As described previously [Zannettino et al., 1996], cDNA clones generated by PCR were gel purified and subcloned into the pGEM-T vector (Promega, Madison, WI) as recommended by the manufacturer. Double-stranded DNA was prepared by standard alkaline lysis "mini-prep" method [Sambrook et al., 1989] and 1–2 μg was used per sequencing reaction. Reactions were prepared using the PRISM™ Ready Reaction Cycle sequencing kit (Applied Biosystem, Foster City, CA), as recommended by the

manufacturer. Reactions analyzing both cDNA strands were run on a Applied Biosystems 373 automated sequence analyzer and 500–600 bp of 5′ and 3′ sequence data was routinely obtained per clone. Sequence data were then analyzed by accessing the Genbank and European Molecular Biology laboratory (EMBL) databases at the National Centre for Biotechnological Information (NCBI).

Recloning of the HOP-26 cDNA Clone Into pRUF*neo* and Validation of Surface Antigen Expression

Following PCR recovery of proviral cDNA inserts from genomic DNA, unique *Bam* HI and *Xho* I restriction sites present in the 5′ and 3′ flanking regions, respectively, were utilized to "reclone" the cDNA into the MCS of the retroviral vector pRUF*neo*. *E. coli* DH10B cells were transformed and plasmid DNA isolated using Qiagen-tip 100 columns (Qiagen, Victoria, Australia) as recommended by the manufacturer. Stable, G418 resistant ψ_2 virus-producing cell lines were produced by calcium phosphate transfection and used to infect FDC-P1 cells by co-cultivation, as described previously [Zannettino et al., 1996]. G418 resistant FDC-P1 cells were then analyzed for antigen expression by indirect immunofluorescence and flow cytometry.

Enzymatic Digestion of NHBC Cultures for Immunofluorescence and Flow Cytometry

Single cell suspensions were obtained from confluent primary NHBC cultures by enzymatic digestion [Gronthos et al., 1997]. The cultures were washed twice in phosphate buffered saline pH 7.4 and then digested in a solution of collagenase (3 mg/ml) (Collagenase Type I; Worthington Biochemical Co., Freehold, NJ) and dispase (4 mg/ml) (Neutral Protease Grade II; Boehringer Mannheim, GMBH, W. Germany) for 90 min at 37°C. Cell suspensions were then washed with growth medium before being passed through a Falcon cell strainer (Becton Dickinson Labware) to obtain a single cell suspension.

Indirect Immunofluorescence and Flow Cytometric Analysis

Prior to immunolabeling, cells (normal human bone or bone marrow mononuclear cells) were incubated in blocking buffer (HBSS + 20 mM HEPES, 1% normal human AB serum, 1% bovine serum albumin (BSA: Cohn fraction V, Sigma Aldrich Pty Ltd, NSW, Australia), and 5% FCS for 20 min on ice. Aliquots of 5×10^5 cells were resuspended in 100 μl of saturating concentrations of HOP-26 supernatant (mouse IgM mAb) or 12F12 (mouse IgG1 anti-CD63 mAb) for 45 min on ice. The isotype-matched, non-binding control antibodies, antibodies, IgG1 (3D3), and IgM (1A6.12) (kindly provided by Dr. L.K. Ashman, Hanson Centre for Cancer Research, Adelaide, South Australia) were used as culture supernatants under identical conditions. The cells were then washed in HBSS with 5% FCS and incubated with a goat anti-mouse IgG (γ-chain specific) phycoerythrin (PE) (1/50) and a goat anti-mouse IgM (μ-chain specific) FITC (1/30) (Southern Biotechnology Associates, Birmingham, AL) for 45 min on ice. Prior to analysis, cells were washed twice in HBSS with 5% FCS and resuspended in PBS/1% paraformaldehyde. Flow cytometric analysis was performed using a Coulter Excel flow cytometer (Coulter Corp., Hialeah, FL). Positivity for each antibody was defined as the level of fluorescence greater than 99% of what was observed when isotype matched, non-binding control antibodies were used. 20,000 events were collected per sample as list mode data and analyzed using Coulter ELITE software.

RESULTS

Molecular Cloning of the HOP-26 Antigen

The cDNA encoding the cell surface antigen identified by the mAb HOP-26 was isolated from a HBMSC cDNA library in the retroviral vector, pRUF*neo* as previously described [Zannettino et al., 1996]. Partial sequence of the human cDNA insert recovered was subjected to FASTA alignment analysis (National Centre for Biotechnology Information) and revealed 100% homology with the human CD63 coding sequence (Fig. 1). Furthermore, complete sequence analysis of this clone confirmed complete identity to open reading frame of the CD63 cDNA sequence (Genbank accession no. M58485; data not shown). In order to confirm that the CD63 cDNA was responsible for the HOP-26 binding phenotype, the recovered cDNA was subcloned into the retroviral vector pRUF*neo* and introduced into FDC-P1 cells as described in the Materials and Methods. As shown in Figure 2, a clone of FDC-P1 cells transduced with the HOP-26 cDNA, as expected, demonstrated specific

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>gb|X07982|HSME491 Human mRNA for melanoma-associated antigen ME491; Plus Strand HSPs:
Score = 815 (225.2 bits), Expect = 2.8e-58, P = 2.8e-58
Identities = 275/279 (99%), Positives = 275/279 (99%), Strand = Plus

Query:   162 GCCTNGGNAAGCCCAGGCCCACCAGCCATGGCGGTGGAAGGAGGAATGAAATGTGTGAAG 221
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Sbjct:     1 GCCTTGGGAAGCCCAGGCCCGGCAGCCATGGCGGTGGAAGGAGGAATGAAATGTGTGAAG 60

Query:   222 TTCTTGCTCTACGTCCTCCTGCTGGCCTTTTGCGCCTGTGCAGTGGGACTGATTGCCGTG 281
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    61 TTCTTGCTCTACGTCCTCCTGCTGGCCTTTTGCGCCTGTGCAGTGGGACTGATTGCCGTG 120

Query:   282 GGTGTCGGGGGCACAGCTTGTCCTGAGTCAGACCATAATCCAGGGGCTACCCCTGGCTCTC 341
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   121 GGTGTCGGGGGCACAGCTTGTCCTGAGTCAGACCATAATCCAGGGGCTACCCCTGGCTCTC 180

Query:   342 TGTTGCCAGTGGTCATCATCGCAGTGGGTGTCTTCCTCTTCCTGGTGGCTTTTATGGGCT 401
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   181 TGTTGCCAGTGGTCATCATCGCAGTGGGTGTCTTCCTCTTCCTGGTGGCTTTTGTGGGCT 240

Query:   402 GCTGCGGGGGCCTGGAAAGAGAACTATTGTCTTATGATCA 440
             |||||||||||||||||||||||||||||||||||||||
Sbjct:   241 GCTGCGGGGGCCTGCAAGGAGAACTATTGTCTTATGATCA 279
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Fig. 1. FASTA alignment analysis of HOP-26-derived PCR products. Following partial sequence analysis, the resultant nucleotide sequence was compared with sequences submitted to the combined Genbank/EMBL database via standard "FASTA alignment analysis," and revealed 100% homology with the CD63 complementary DNA sequence (Genbank accession no. M58485).

binding of HOP-26 antibody. In addition, this clone also bound 12F12, a monoclonal antibody shown in a previous report to identify CD63 [Zannettino et al., 1996]. Collectively, therefore, these data demonstrate that HOP-26 identifies the product of the *CD63* gene.

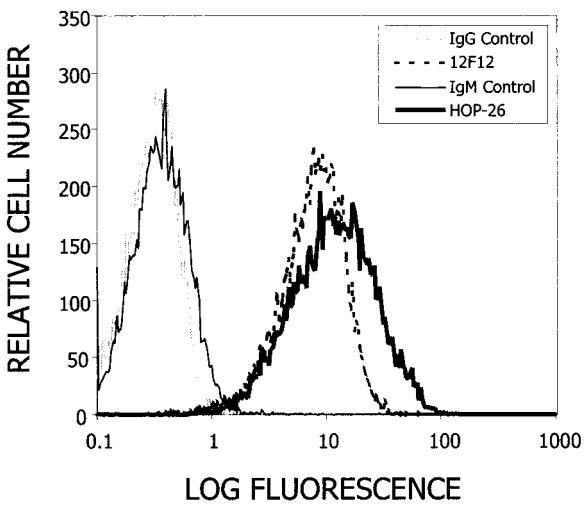

Fig. 2. HOP-26 and 12F12 mAbs recognise the same glycoprotein antigen on transfectants expressing the CD63 cDNA. A 1.7 kb *Bam*HI-*Xho*I restriction fragment of the CD63 cDNA (harboring both the entire coding sequence and the 5′ and 3′ non-coding regions) was subcloned into the pRUF*neo* vector and subsequently introduced into FDC-P1 cells by retroviral transduction (refer to Materials and Methods). The resultant G418-resistant cell population, was stained by indirect immunofluorescence and analyzed by flow cytometry. Data are displayed as single-parameter fluorescence (FITC) histograms of 1×10^4 light-scatter gated events, collected as list mode data. IgG control (thin grey line); IgM control (thin black line); mAb HOP-26 (thick black line); mAb 12F12 (dashed line).

HOP-26 and a Defined CD63 mAb Display Comparable Reactivity With Cultured HBMSCs

As CD63 is a lysosomal membrane glycoprotein, which is translocated to the plasma membrane, we examined the subcellular localization of the epitope of CD63 identified by HOP-26 compared with the mAb 12F12 on cultures of human bone and bone marrow stromal cells. As can be seen in Figure 3, both antibodies demonstrated a punctate perinuclear pattern of staining consistent with the previously reported pattern of staining of these cells with HOP-26 [Joyner et al., 1997]. In addition, to confirm that the apparent molecular mass of the protein identified by HOP-26 was consistent with CD63, detergent lysates of cultured HBMSCs were resolved by SDS–PAGE. Total cellular proteins were transferred to a PVDF membrane and immunoblotted with HOP-26 and mAb 12F12. A broad band ranging in molecular weight of 35–100 kD, with a predominant species of 50–60 kD was observed under non-reducing conditions with both HOP-26 and 12F12 (Fig. 4).

HOP-26 and a Defined CD63 mAb Display Identical Reactivity With Cultured Human Bone Cells and Bone Marrow Mononuclear Cells

Dual-color immunofluorescence and flow cytometry was utilized to compare the pattern of expression of CD63 by bone marrow-derived mononuclear and human bone cells as detected by monoclonal antibodies HOP-26 and 12F12. As demonstrated in Figure 5B,C, monoclonal antibodies HOP-26 and 12F12 identified

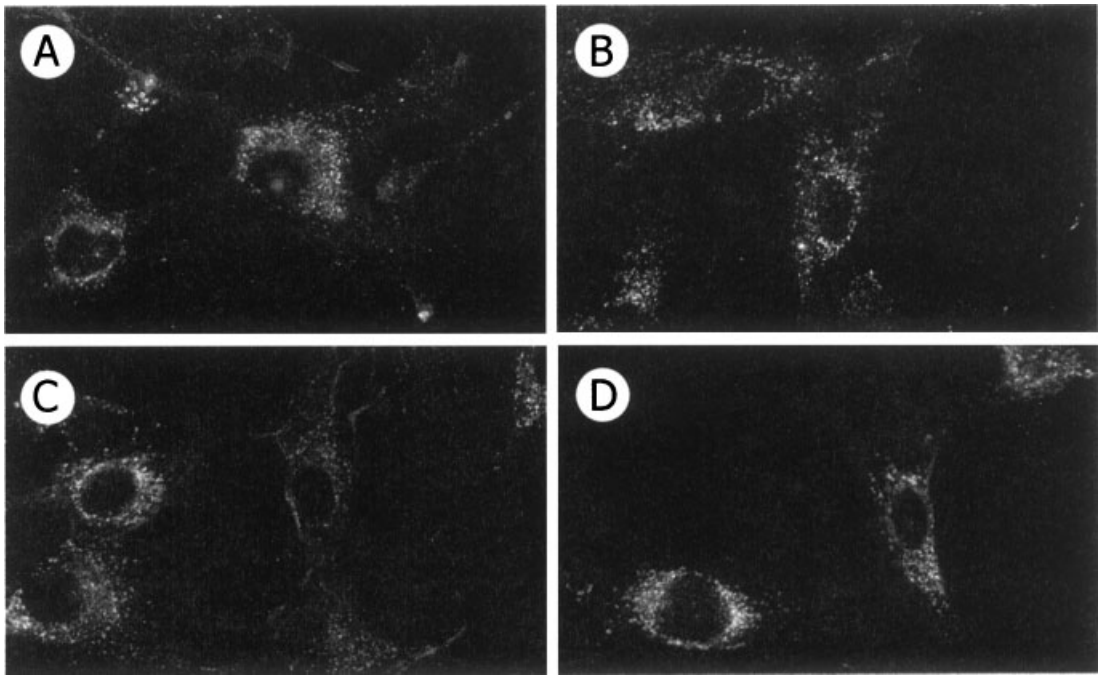

Fig. 3. Monoclonal antibodies HOP-26 and 12F12 identify an antigen expressed by cultured NHBCs and HBMSCs. The expression of the antigen identified by mAbs HOP-26 and 12F12 was assessed by indirect in situ immunofluorescence (as described in Materials and Methods). Bone marrow stromal cells (**A** and **B**) and NHBCs (**C** and **D**) demonstrate cytoplasmic and membrane staining for both 12F12 (A and C) and HOP-26 (B and D), (original magnification X200). No staining was observed when isotype-matched, non-binding monoclonal antibodies were used under identical staining conditions (data not shown).

essentially all of the human bone cell population, whilst 10.5% (Fig. 6C) and 11.5% (Fig. 6B) of the mononuclear cell population were stained, respectively. When used together and detected by isotype-specific secondary fluorescent conjugates, co-linear staining of the human bone cell population was observed (Fig. 5D). Interestingly, when BM mononuclear cells were similarly analyzed, co-linear staining of the positive fraction was also observed, however approximately 3% of the $12F12^{DIM/-}$ population were found to be HOP-26^{+}. This suggests that although HOP-26 and 12F12 appear to recognize distinct antigenic determinants (i.e., limited or no cross-blocking), HOP-26 appears to identify a cryptic epitope of CD63 on BM mononuclear cells, that is not detected by the mAb 12F12 (Fig. 6D).

DISCUSSION

Due to the diversity of the BM stromal cell population, attempts to characterize the biological properties of each cellular component have been complicated in part by the lack of lineage-specific markers which might facilitate the precise identification and isolation of each cell type. In a previous report [Joyner et al., 1997], we demonstrated that the monoclonal antibody HOP-26, raised against human marrow stromal fibroblasts, demonstrated specific reactivity to cells close to new bone formation in periosteum and between bone trabeculae in sections of human fetal limb. Similarly, in adult trabecular bone tissue, HOP-26 was found to react with a minor population of cells within the bone marrow spaces but not with osteoblasts, adipocytes, fibrous tissue, skin, muscle, appendix, brain, and tonsil. Moreover, immunopanning methods enabled isolation of cells with fibroblast colony forming (CFU-Fs) potential by selecting cells with the highest level of cell surface expression. These findings demonstrated the value of this antibody in histopathology and in providing enriched populations of progenitor cells capable of osteoblastic differentiation. In this current communication, we conclusively demonstrate that HOP-26 identifies the lysosomal and cell surface glycoprotein, CD63.

CD63 is a member of an emerging superfamily of tetraspan glycoproteins (TM4SF),

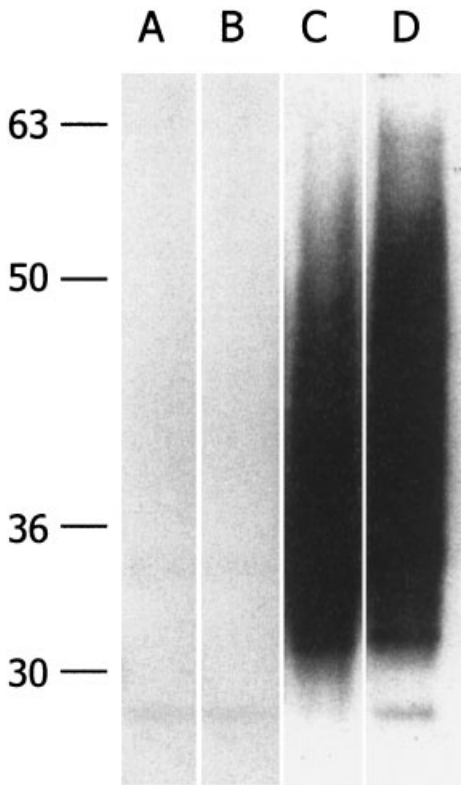

Fig. 4. Western blot analysis cultured human BM stromal cells (HBMSC) with mAb HOP-26 and 12F12. Membrane preparations from cultured HBMSC were separated by 10% SDS–polyacrylamide gel electrophoresis under non-reducing conditions and transferred to PVDF. The filters were successively incubated with either the HOP-26, 12F12 supernatant or isotype matched, non-binding controls and anti-mouse-AP. The immunoreactive proteins were detected as described in the Materials and Methods. **Lane A**, IgG negative control; **Lane B**, IgM negative control; **Lane C**, HOP-26; **Lane D**, 12F12.

which include CD9 [Boucheix et al., 1991], CD37 [Classon et al., 1989], CD53 [Angelisova et al., 1990], and the recently cloned CD151 (PETA-3) [Fitter et al., 1995]. Moreover, CD63 has been shown to be identical to the melanoma-associated antigen, ME491, originally described as an antigen associated with early stages of melanoma tumor progression [Metzelaar et al., 1991]. This heterogenously glycosylated TM4SF member is expressed on activated platelets [Metzelaar et al., 1989] and endothelium [Vischer and Wagner, 1993] and is a lysosomal membrane glycoprotein which is translocated to the plasma membrane following activation. CD63 is also expressed on monocytes, macrophages, and is weakly expressed on granulocytes, B and T lymphocytes.

Although the precise function of this molecule in bone tissue is yet to be determined, studies in other systems have shown that mAbs to CD63 inhibit adhesion of neutrophils to activated endothelium [Kitani et al., 1991] and monocyte adherence to serum-coated plates [Toothill et al., 1990], suggesting a role in adhesion. Moreover, functional inferences can be made from studies examining other family members, which function in a number of key cellular processes including signal transduction [Olweus et al., 1993], cell activation [Tai et al., 1996], proliferation [Hadjiargyrou and Patterson, 1995; Wice and Gordon, 1995; Geisert et al., 1996], motility [Ikeyama et al., 1993], and adhesion [Hadjiargyrou and Patterson, 1995]. In addition, TM4SF molecules have been shown to be components of multi-protein complexes involved in these cellular processes. Recently, CD63 and CD81 were shown to complex with phosphatidylinositol 4-kinase in fibrosarcoma cells [Berditchevski et al., 1997]. Consistent with the notion that TM4SF members form components of multiprotein complexes involved in cellular adhesion and migration processes, several members of the TM4SF have been shown to associate with a subset of β_1 integrins. In particular, CD63 has been shown to specifically associate with integrins $\alpha_3\beta_1$ [Rubinstein et al., 1994; Berditchevski et al., 1995, 1996; Nakamura et al., 1995; Hadjiargyrou et al., 1996; Mannion et al., 1996] and $\alpha_6\beta_1$ [Berditchevski et al., 1995, 1996; Hadjiargyrou et al., 1996] in a variety of cell types. As osteoprogenitor cells express many of these integrin heterodimers [Gronthos et al., 1997], CD63 may function to mediate regulation of integrin avidity ("inside-out" signaling) [Hynes, 1992], or may be a component of the signaling pathway initiated by integrin ligation ("outside-in" signaling) [Ginsberg et al., 1992].

Our previous report describing the distribution of the HOP-26 reactive epitope [Joyner et al., 1997] relied almost exclusively on histochemical methods on fixed cells and/or paraffin embedded tissues. In contrast, studies presented here and elsewhere [Metzelaar et al., 1989, 1991], have examined the expression of CD63 in freshly isolated and or viable, cultured cells. Like other anti-CD63 monoclonal antibodies, HOP-26 identifies a range of cell types in preparations of fresh BM, including monocytes, lymphocytes, and neutrophils. It is now clear that fixation renders the epitope identified by the HOP-26 antibody partially denatured and unrecognizable on most cell types which express

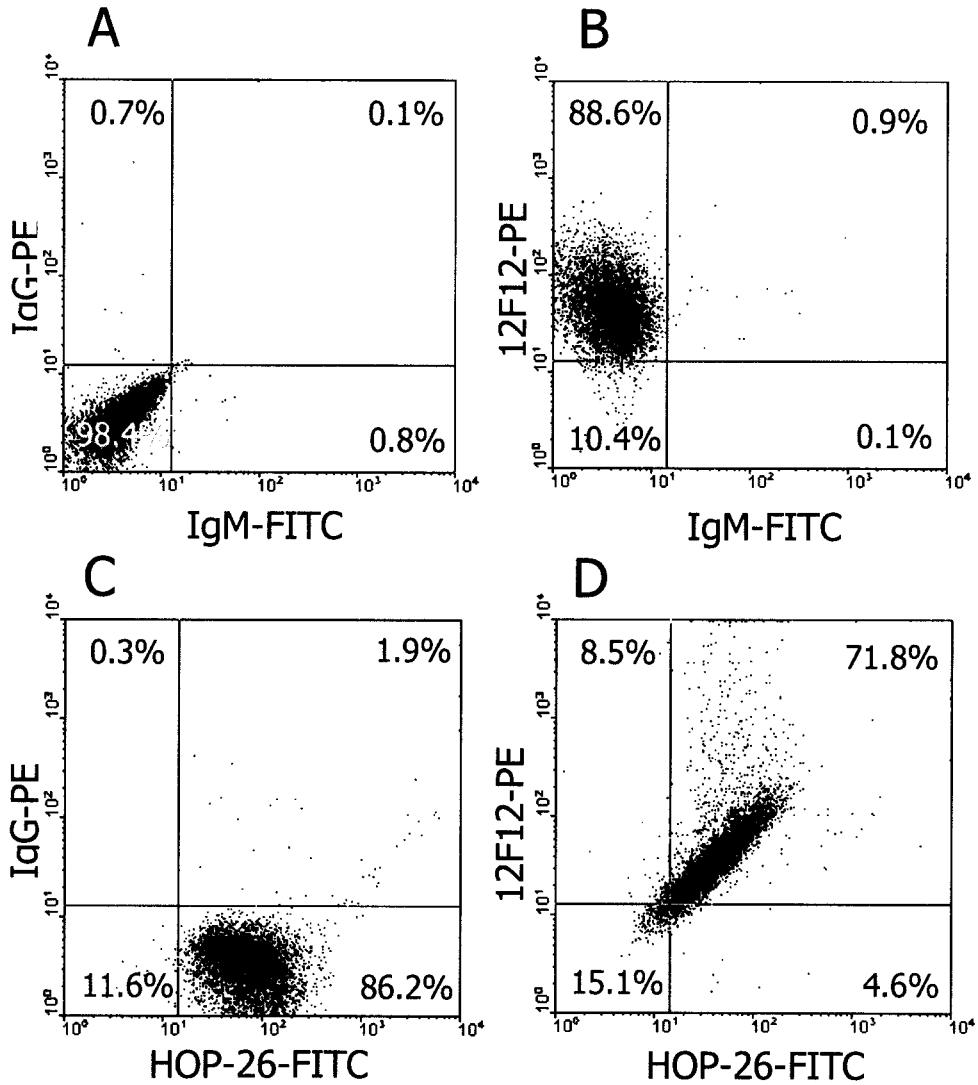

Fig. 5. Monoclonal antibodies HOP-26 and 12F12 display comparable reactivity with NHBCs. NHBCs were stained by dual-color indirect immunofluorescence with HOP-26 or 12F12 in combination with non-binding isotype-matched controls (**B** and **C**), or together (**D**) or with both non-binding isotype-matched controls (**A**). Primary antibodies were detected by incubation with isotype-specific FITC or PE-conjugated secondary antibodies. Data are displayed as two-parameter dot plots of 1×10^4 light scatter gated events collected as list-mode data and analyzed with Coulter Epics software.

CD63 at low levels with the exception of osteoprogenitor cells. In addition to this, it is apparent from Figure 6 that HOP-26 and 12F12 react with overlapping, yet marginally different subsets of marrow mononuclear cells. Although beyond the scope of this present communication, it would appear that monoclonal antibodies to CD63, including 12F12 and HOP-26, identify subtle differences in cell-specific differential glycosylation of the CD63 glycoprotein.

As clearly demonstrated in this present study, and by a number of other previous reports [Terstappen et al., 1993; Hill et al., 1996], monoclonal antibodies provide sensitive probes of structural detail beyond the determination of the presence of a given molecule, and have proved exceedingly useful in the identification and isolation of particular cell populations. Although we have previously reported the expression of CD63 by cultured bone marrow stroma [Zannettino et al., 1996], to the authors' knowledge, this represents the first report documenting the expression of CD63 in human bone tissue. Unlike other antibodies to CD63, including 12F12, HOP-26 identifies a unique epitope on the CD63 glycoprotein, which is preserved on osteoprogenitors following fixation, making this antibody a valuable reagent in

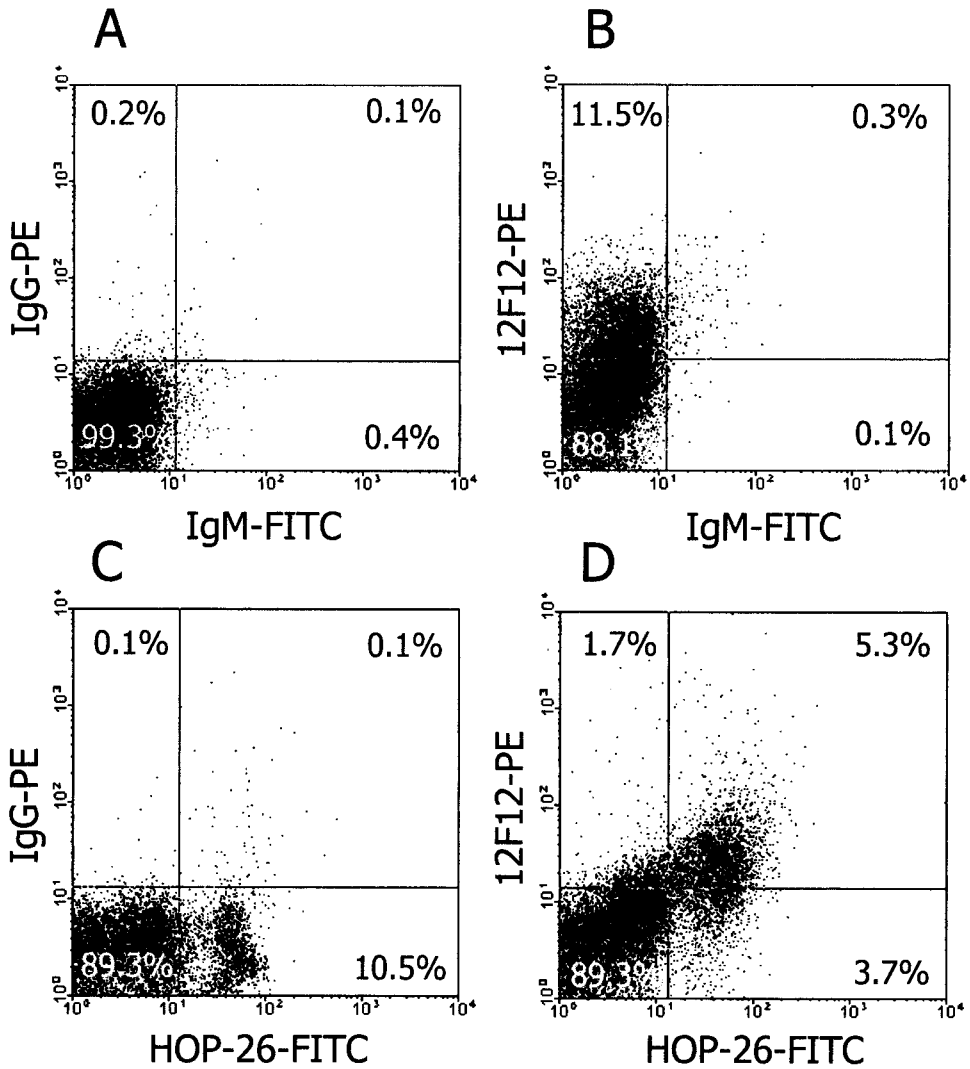

Fig. 6. Monoclonal antibodies HOP-26 and 12F12 display comparable reactivity with bone marrow mononuclear cells. Bone marrow mononuclear cells (BMMNC) were stained by dual-color indirect immunofluorescence with HOP-26 or 12F12 in combination with non-binding isotype-matched controls (**B** and **C**), or together (**D**) or with both non-binding isotype-matched controls (**A**). Detection and analysis was carried out as described in Figure 5, with the exception that 1×10^5 light scatter gated events were collected as list mode data.

determining the role played by CD63 in bone formation and bone homeostasis.

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