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Expression of stem cell factor by osteoblasts in normal and hyperparathyroid bone: relation to ectopic mast cell differentiation

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Abstract Mast cells accumulate in hyperparathyroid bone, but the reason is not clear. We compared the distribution of mast cells and related growth factors in normal and hyperparathyroid bone. Mast cell formation was strongly affected by proximity to bone-forming surfaces of hyperparathyroid bone. Hyperparathyroidism greatly increased the production by active, bone-synthesizing osteoblasts of stem cell factor (SCF) but not of IL-3. Osteoblast SCF was distributed to the basolateral cell membranes, and its cDNA sequence (GenBank AF119835) is homologous to the murine membrane-bound SCF. Quiescent osteoblasts did not produce detectable SCF. Synthetic osteoblasts in normal bone were SCF positive, but comprised a much smaller population of cells, in keeping with the slow turnover of normal bone. Major SCF isoforms on immunoblot analysis of osteoblast-fraction proteins from high-turnover bone had M_rs of about 48 and 40 kDa. Similar SCF isoforms were produced by MG63 osteoblast-derived cells and were identified by several anti-SCF antibodies. SCF is expressed in several mesenchymal cell types in a complementary fashion with cells bearing its receptor. SCF potently facilitates differentiation of mast cells, so the increase in paratrabecular mast cells in hyperparathyroid bone is probably driven by osteoblastic SCF. However, since mast cells are not normal components of bone, osteoblastic SCF probably regulates other cells, with mast cell differentiation occurring as a side effect greatly increased osteoblastic activity.

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Introduction

Bone is periodically degraded by osteoclasts and replaced by osteoblasts, although the rate of bone turnover varies widely. In acute osteoporosis with rapid bone loss, bone marrow mast cells are increased [2], and, with the extremely high rates of bone formation and degradation that occur in hyperparathyroidism secondary to chronic renal insufficiency, there is striking accumulation of mast cells. Serious histamine-release-associated problems including intractable pruritus [7, 30], bone pain and fractures are also common in these patients [32], but whether these are associated with the mast cells is not known. When hyperparathyroidism is reversed by kidney transplantation, the bone-associated mast cells and pruritus typically disappear [8].

Bone turnover is complex, and many cytokines function in bone metabolism [1]. Mast cell development is linked to stem cell factor (SCF) [13, 24] and IL-3 (multi-CSF) [17, 24], although other cytokines also affect mast cell formation in specific circumstances and IL-3 alone is insufficient for human mast cell maturation [33]. The association with SCF is striking, and in malignant mastocytosis autocrine SCF causes mast cell nodules to grow [20]. SCF is typically expressed in mesenchymal or stromal cells in a complementary fashion with cells bearing its receptor, the proto-oncogene c-*kit*. SCF is an enabling or synergistic factor in several cell maturation processes that have multiple differentiation steps [26]. IL-3 is an early differentiation factor, produced mainly by T-lymphocytes, that is synergistic with SCF in mast cell development [24]. IL-3 also has significant effects on monocyte differentiation. This is relevant to bone turnover, because osteoclasts develop from granulocyte-monocyte colony-forming unit (GM-CFU) precursors. IL-3 is one of several complementary cytokines that affect osteoclast

formation [29]; GM-CFUs are expanded by IL-3 synergistically with SCF [4]. Both SCF [5, 15] and IL-3 [6] function in normal bone cell differentiation, although IL-3 affects several cell lines.

This study was conducted to determine whether a biochemical basis for mast cell accumulation in high-turnover bone could be identified. We studied mast cell formation in bone sections from normal or hyperparathyroid subjects, and examined the occurrence of SCF and IL-3 by the unlabelled antibody technique. Results were followed up with immunoblot analysis of bone cell and marrow fractions from high-turnover human bone and of osteoblast-derived MG63 osteosarcoma cells.

Materials and methods

All procedures were approved by the Institutional Review Board for research involving human subjects.

Bone sections were prepared from plastic-embedded tissue. Materials studied were transiliac bone biopsy specimens 8–10 mm in diameter or sections of thoracic vertebrae obtained at autopsy. Plastic-embedded bone sections were fixed in neutral 5% formalin with isotonic sucrose overnight prior to dehydration in graded alcohols, methacrylate embedding, and sectioning [9]. Sections were stained with toluidine blue to demonstrate mast cells by the metachromatic (red) reaction of histamine-containing granules. Biopsy tissue had been tetracycline labelled for identification of bone-forming regions. Measurements in tissue sections were performed with an ocular micrometer and cells per unit area were measured with an ocular grid.

Monoclonal anti-human SCF 7H6 was a kind gift from K. Langley, Amgen, Thousand Oaks, Calif.). Rabbit polyclonal antibody to the human SCF aa 151–164 (DAFKDFVVASETSD, in exon 5 [22]), was from Medical and Biological Laboratories, Nagoya, Japan. Antibody to C-terminal human SCF aa 263–272 (EDNEISMLQ in exon 8 [22]) was from Research Genetics (Huntsville, Ala.). Anti-human c-*kit* was generously supplied by L. Ashman, Leukaemia Research Unit, Division of Haematology, Hanson Cent for Cancer Research, South Australia. Anti human IL-3 was from Sigma Chemical Co., Saint Louis, Mo. Unlabelled antibody reactions in tissue were performed on 7-µm undecalcified sections after de-plasticizing with xylene overnight at 42°C. Reactions were conducted as described elsewhere [3] in phosphate-buffered saline with 0.05% polyethoxyethylene sorbitan monolaureate; nonspecific reaction was reduced by preincubating sections with 3% goat serum. Primary antibody reactions were 2 h, and subsequent antibody reactions 1 h, with three washes between steps. Unless otherwise indicated, primary antibodies were used at 2 µg/ml for monoclonal antibodies or 1:500 v/v for antisera. Detection reactions used goat-anti mouse or anti-rabbit F_c linkers (Sigma) at 1:200 v/v and appropriate peroxidase-anti-peroxidase complexes (Sigma) at 1:1000. Visualization used 100 µg/ml diaminobenzidine activated by 0.1% H_2O_2, and hematoxylin counterstain to show cellular detail. Nonimmune sera or irrelevant hybridoma supernatants were used as controls.

For immunoblot analysis, trabecular bone was separated from marrow by vortexing 0.5-cm fragments three times in PBS. Bone trabeculae were collected after each wash using 100-µm nylon filters. The trabecular bone was collected by centrifugation after three washes. The first marrow cell filtrate was collected by centrifugation; adipocytes were removed by decanting; and erythrocytes were lysed in 0.1% NaCl. The remaining cells were used as the marrow cell fraction. From bone and marrow fractions, cellular protein was extracted using 10% SDS with 2 mM dithiothreitol, 5 min, 95°C. For immunoblot analysis, 100-µg aliquots (50-µg aliquots for chemiluminescence detection) of cell protein were separated on SDS-13% (Fig. 5) or –14% (Fig. 6) polyacrylamide gels. Following transfer to polyvinylidine difluoride membranes, proteins were localized by immune reactions as described [35]. These were similar to the reactions for tissue sections, but nonspecific reactions were blocked in 2% condensed milk in phosphate-buffered saline with 0.05% polyoxyethylene sorbitan monolaureate; antibody reaction steps were 30 min, with three washes after each step before the next. Primary antibodies were used at 1:1000 or 1 µg/ml, with detection of alkaline-phosphatase-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad, Richmond, Calif.) using *p*-nitrophenylphosphate substrate and fast blue to visualize alkaline phosphatase labels, or horseradish peroxidase-coupled goat anti-rabbit (Amersham, Arlington Heights, Ill.) and luminol (Amersham) for chemiluminescence detection (Fig. 6B).

A 633-bp SCF cDNA segment was made by PCR using the primers GCCTTTCCTTATGAAGAAGAC and TGCTGTCATTCCTAAGGGA (–10 to +11 b and complement of 604–623 b, GenBank M59964) and then placed into the pcDNA3 eukaryotic expression vector (Invitrogen, San Diego, Calif.) in antisense orientation at Eco RI and Xba I, and confirmed by sequencing. This vector includes cytomegalovirus immediate-early enhancer-promoter, polyadenylation, and transcription termination signals, *Neo* resistance, and SV40 *ori*. For transfection studies, human MG63 osteosarcoma cells (ATCC, Baltimore, Md.) were used. These cells produced immunoreactive SCF proteins indistinguishable from primary osteoblasts; nontransformed osteoblasts were not suitable for transfection. Empty vector and vector with antisense SCF were transfected into 60% confluent MG63 cells by the cationic lipid method (Tfx 20, Promega, Madison Wis.) using 0.5 µg vector and 3:1 charge excess of cationic lipid, 1 h at 37°C, followed by selection in 50 µg/ml G418.

Results

Toluidine blue-stained mast cells in hyperparathyroid bone are shown in (Fig. 1). Analysis of several sections showed that these cells were most abundant within approximately 20 µm of bone-forming osteoblasts; a repre-

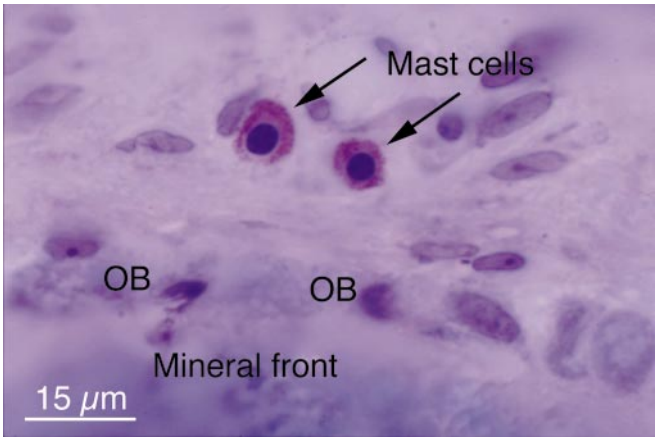

Fig. 1 Mast cells in paratrabecular tissue of hyperparathyroid bone. Toluidine blue was used to demonstrate mast cells by the metachromatic staining of their histamine-containing granules. In this photograph, two mast cells with abundant red granules (*arrows*) contrast with the blue colour of the paratrabecular tissue, in which only the nuclei are stained. The mast cells are ~10 µm in diameter. A line of active osteoblasts (OB) and the mineralizing front are identified for orientation

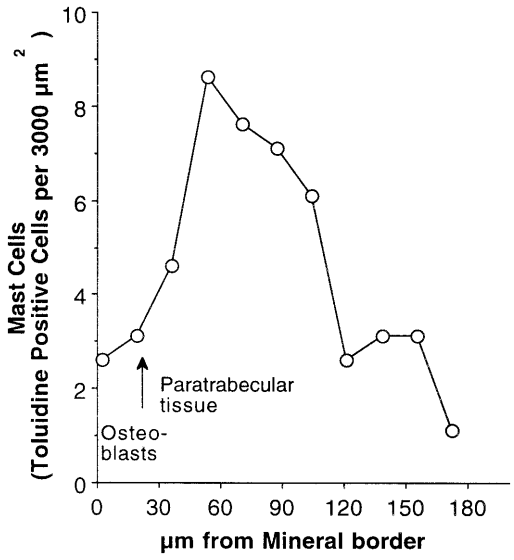

Fig. 2 Distribution of mast cells in hyperparathyroid human bone as a function of distance from bone formation sites. Results from a typical section are shown; peak distribution of mast cells was consistently near the osteoblast layer, although absolute numbers varied widely between hyperparathyroid patients. Normal bone sections had too few paratrabecular mast cells for meaningful comparison. Cells were counted in 32-µm grids ($10^3 \mu m^2$), as a function of distance from the mineralizing site; the approximate position of the basolateral border of the active osteoblasts, about 15 µm from the mineralizing front (Fig. 1), is indicated by an *arrow*. Mast cells were present in marrow (>100 µm from the mineral), but the largest concentration was near the site of bone formation

sentative distribution plot is shown in Fig 2. While a similar distribution was noted in each of seven hyperparathyroid patients, the absolute numbers varied. Paratrabecular mast cells were too infrequent in normal PTH controls for meaningful comparisons; see Peart and Ellis (1975) [28]. Occurrence of mast cells in hyperparathyroid bone so close to the mineralizing osteoblast layer was particularly remarkable. This tissue is dense paratrabecular mesenchyme that accumulates in hyperparathyroid bone, traditionally described as "osteitis fibrosa" although it is not inflammatory. Mineral-secreting regions were identified with tetracycline labels (not illustrated) in addition to their characteristic cuboidal-columnar morphology (OB, Fig. 1).

In situ unlabelled antibody reactions for SCF, SCF receptor, and IL-3 were performed. Assays were done on seven hyperparathyroid and four normal specimens; typical results are illustrated in Fig. 3. The major finding was that synthetic paratrabecular osteoblasts produced large quantities of SCF on their basolateral surfaces (Fig. 3A), while inactive osteoblasts did not produce detectable SCF (Fig. 3B). The membrane-associated appearance of SCF at oil-immersion magnification (Fig. 1A) is typical of high-molecular-weight forms of SCF (see Discussion). Curiously, nonhyperparathyroid bone showed a similar pattern, although positive osteoblasts were much less frequent (Fig. 3C, D), in keeping with the much lower bone-forming activity is normal bone. The density of staining for SCF in active osteoblasts varied somewhat between sections; replicates using adjacent sections (not illustrated) suggested that these differences were caused by minor inconsistencies in processing of sections. Only active osteoblasts (Fig. 3B, D) demonstrated membrane-associated SCF. The association of osteoblastic activity state, SCF expression, and mast cell formation suggests that PTH cause osteoblast synthesis of SCF that, at high levels, causes mast cells to differentiate in the paratrabecular region (see Discussion).

Antibody to IL-3 identified scattered cells in marrow (Fig. 3E), but not in the paratrabecular tissue (Fig 3F). No difference was seen between control and hyperparathyroid bone was seen in IL-3-positive cells. The IL-3-positive cells were 6–7 µm in diameter, a result consistent with the expression of IL-3 by lymphocytes. The lack of association with the region of mast cell formation or rate of bone turnover suggests that IL-3 is not related to increased mast cell formation in hyperparathyroid bone. Antibody to c-*kit* labelled membranes of some paratrabecular and marrow cells, but was too weak to be interpreted reliably (not illustrated). Preimmune or nonimmune controls were run for each antibody, and produced no significant labelling (nonimmune controls for SCF are shown in Fig. 5).

Unlabelled antibody localization in tissue is subject to artifacts, and the expression of SCF was therefore confirmed by immunoblot analysis. To obtain sufficient fresh bone cells, vertebral medullary bone was taken at autopsy from a 52-year-old woman who had increased bone formation and degradation due to resulting from recent menopause and high-dose corticosteroid therapy. Approximately 50% of the trabecular bone surface showed active osteoblasts (Fig. 4). The osteoblast-enriched bone cell fraction, but not the marrow fraction, contained easily detectable quantities of SCF (Fig. 5A). The SCF was predominantly a large form with M_r about 48 kDa, but smaller amounts with M_r ~40 kDa were also seen. Several immunoblot reactions gave similar results, and control hybridoma supernatant was negative. There was no detectable SCF in 100 µg of marrow cell protein run in parallel with 100 µg of bone cell protein. Coomassie blue staining confirmed that essentially equal amounts of bone and marrow cell proteins were assayed (Fig. 5B). The osteoblast-enriched fraction had a larger number of identifiable proteins, reflecting the derivation mainly from one cell type, while the marrow fraction was a mixture of many cell types. Other cells in marrow are known to produce SCF under some circumstances, and it is likely that the very distinct difference between SCF in the osteoblast and marrow fractions is due to the large amount of SCF produced by high-turnover bone (see Discussion).

The SCF receptor *kit* was present at levels too low for accurate assessment by tissue staining. In keeping, with this, reaction of anti-*kit* with osteoblast and marrow cell fractions was weak (not illustrated). More selective approaches, such as cell- sorting, have detected c-*kit*-posi-

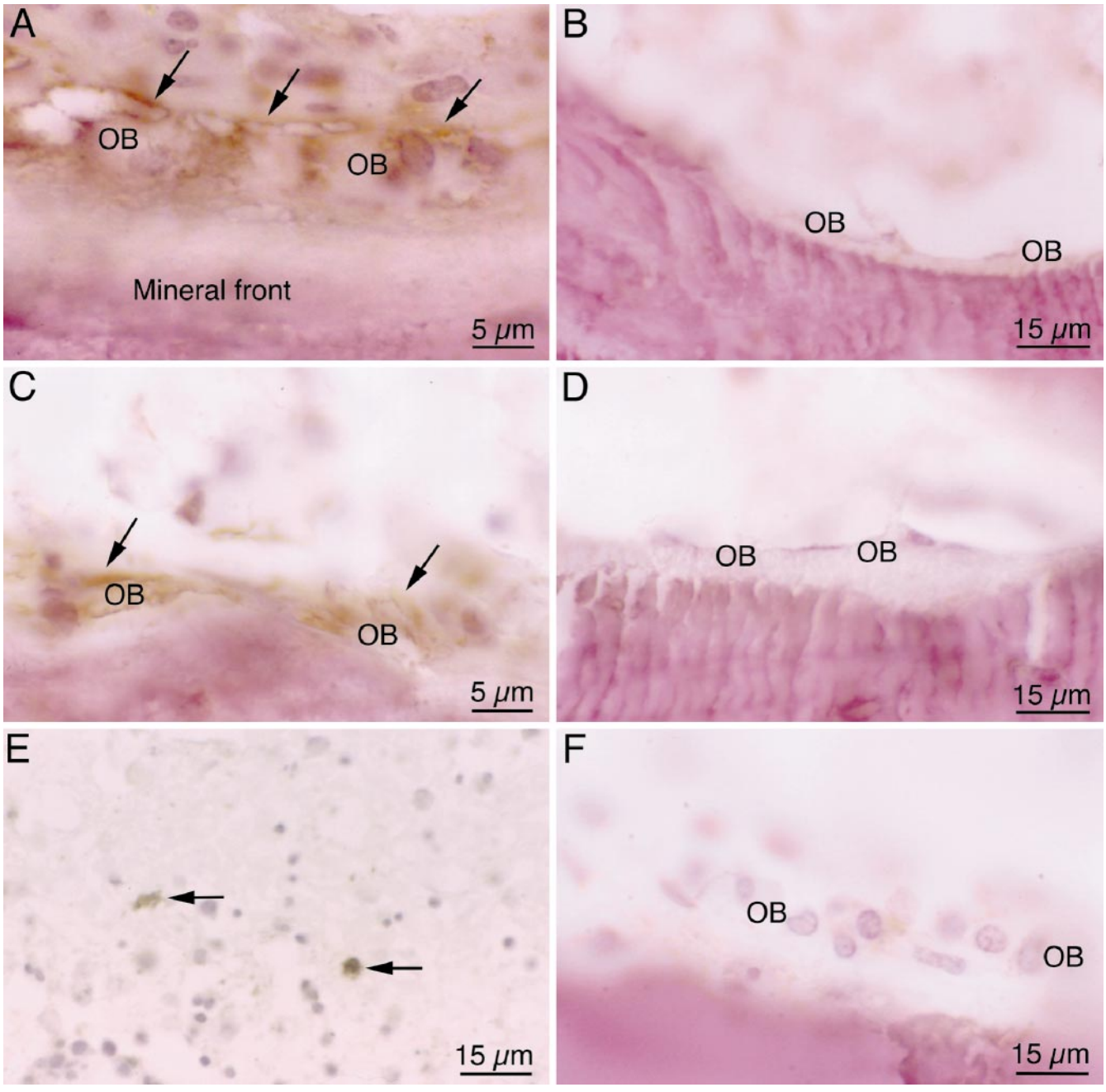

Fig 3A–F Stem cell factor (SCF) and IL-3 immune reaction in hyperparathyroid and normal human bone. **A–D** SCF production by osteoblasts. **A, B** Two areas from a single section of hyperparathyroid bone, with synthetic cuboidal and inactive flattened osteoblasts (*OB*), respectively. **C, D** Similar areas from a single section of normal bone with synthetic and inactive osteoblasts. Immunoreactive SCF (brown colour) was present at the basolateral membranes of synthetic osteoblasts (arrows, **A, C**). In both hyperparathyroid and normal bone, however, quiescent, attenuated osteoblasts (**B, D**) were unreactive. **E, F** IL-3 expression in bone. Scattered IL-3 expressing cells were present in marrow (**E**), but were not seen in the paratrabecular tissue (**F**). The unlabelled antibody method was applied to deplasticized 7-μm sections of formalin–fixed, methacrylate-embedded bone as described in Methods. Examples shown are representative of seven hyperparathyroid and four normal bone sections. Preimmune and nonimmune controls showed no consistent reaction and are not illustrated

tive cells in bone (see Discussion). Anti-IL-3 reaction on immunoblot was undetectable or extremely weak (not illustrated).This was consistent with the tissue staining results, which showed small numbers of positive cells in the marrow without associated of IL-3-producing cells with paratrabecular bone (Fig. 3E, F).

The SCF identified in osteoblasts using the 7H6 antibody (Fig. 5) was unusually large. It was therefore possible that either a new form of SCF or cross-reactive proteins were present. Therefore, results using the 7H6 monoclonal antibody were confirmed with polyclonal antibodies specific for two regions of SCF. Osteoblast-derived MG63 cells, in which expression of SCF could be controlled by transfection (Methods), were studied to

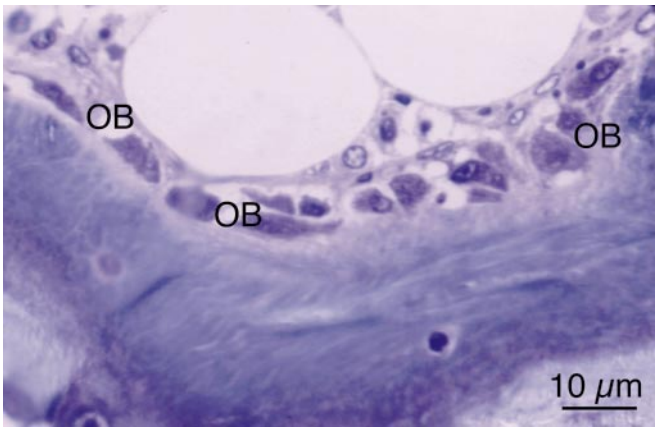

Fig 4 Characteristics of medullary bone used for immunoblot analysis. Toluidine blue stain demonstrating the cuboidal morphology of active osteoblasts present on about 50% of the medullary bone surface. Bone cells were separated from marrow by vigorous washing, which simply and efficiently separates marrow and bone cell fractions for comparison by immune analysis (Fig. 5)

demonstrate the relation between SCF mRNA expression and the ~40–48 kDa glycoprotein products (Fig. 6A, B). Immunoblot studies illustrated in Fig. 6, showed that antibody to amino acids translated from exon 5 recognized isoforms, of M_r ca. 40 kDa, while antibody to amino acids translated from exon 8 recognized only the approx. 48 kDa isoform, indicating that the smaller isoform is truncated (see Discussion). In either case, when expression of SCF was eliminated by stable transfection of plasmids producing antisense SCF mRNA, the immunoreactive material was also eliminated, indicating that the products with M_r around 40–48 are SCF isoforms.

Discussion

In hyperparathyroid bone, mast cells occur in increased numbers near sites of bone formation (Figs. 1, 2). Since mast cell development is stimulated by IL-3 and SCF, we studied the effect of increased bone turnover on the expression these cytokines. IL-3 was not detected in hyperparathyroid bone cells by unlabelled antibody localization. IL-3-producing cells were present in the marrow fraction (Fig. 3), but did not increase measurably in hyperparathyroid bone. IL-3 alone does not support mast cell differentiation independently in humans [33], which is in keeping with our results.

However, SCF was strongly expressed on the nonmatrix surfaces of synthetically active osteoblasts, but not on quiescent osteoblasts (Fig. 3). Synthetic osteoblasts act in contiguous groups connected by gap junctions, and SCF-positive cells were typically groups of associated columnar cells demonstrated clearly in hyperparathyroid bone (Fig. 3A). Bone synthesis is increased over 10-fold in hyperparathyroidism, and SCF expression was also amplified dramatically. Expression of SCF in the osteoblast fraction of high-turnover bone was confirmed by immunoblot analysis (Fig. 5). It was somewhat puzzling that the marrow fraction was entirely negative, since osteoblasts are derived from marrow stroma [11]. However, the preosteoblastic component represents less than 1% of marrow cells [31], most of which consist of erythrocyte and leucocyte precursors. Thus, while marrow mesenchymal cells probably produce small amounts of SCF that support the infrequent formation of marrow mast cells, it is not surprising that SCF was not detected in marrow cell lysates.

SCF is a glycoprotein that occurs in multiple forms because of alternative mRNA splicing and post-transla-

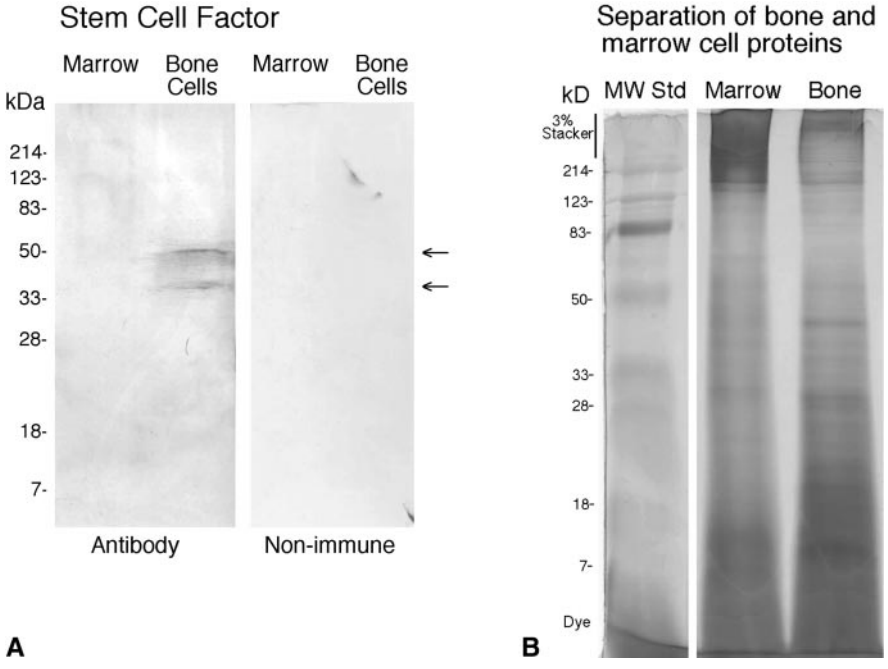

Fig 5A, B Immunoblot analysis of SCF and IL-3 in trabecular bone and marrow cells. **A** The bone cell fraction contained two SCF isoforms, about 40 and 48 kDa, in easily detectable quantities. The marrow cell fraction showed no SCF, and nonimmune controls were negative. This result is representative of several similar determinations using alkaline phosphatase detection of bound antibody (Methods). **B** A duplicate SDS-PAGE gel of that used for the immunoblot in **A**, stained with Coomassie blue to demonstrate that the marrow and bone cell fractions contained similar protein quantities. The bone cell fraction is largely (approx. 50%) osteoblasts, and more individual proteins are distinguished than in the marrow fraction, which is a complex mixture

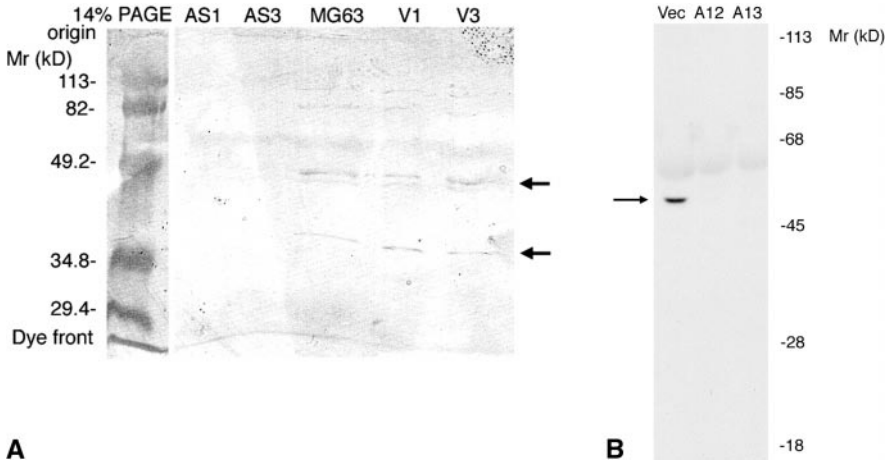

Fig 6A, B Identification of isoforms of SCF in osteoblast-derived cells by antibodies to synthetic peptides from different regions of SCF, and the effect of eliminating SCF by stable transfection of SCF antisense RNA-producing plasmids. **A** Polyclonal antibody specific to a sequence in exon 5 recognized SCF isoforms of M_r ~40 and 48 kDa (*arrows*), indistinguishable from those reacting with the 7H6 monoclonal antibody (Fig. 5). Osteoblasts in which expression of SCF was abolished by antisense transfection (AS1, AS3) had no reaction. Unmodified MG63 cells or cells containing the vector only (MG63, V1, V3) gave similar results; alkaline phosphatase detection of bound antibody is shown, as in Fig. 5A. **B** Antibody to a sequence in exon 8 recognized only the isoform with M_r about 48 kDa isoform (*arrow*), indicating that the smaller isoform is truncated (see Discussion). Enhanced chemiluminescence detection of bound antibody was used to increase the sensitivity of this assay

tional processing. A membrane-associated form was cloned from murine cells [14]; it lacks exon 6, which contains a proteolytic cleavage site that produces smaller soluble forms [21]. Other soluble forms of M_r 20–25 kDa are produced by splice variants missing additional exons [22]. The murine membrane-bound form has an isoform with M_r approx. 50 kDa, although smaller variants also occur [14]. In osteoblasts, we detected two major SCF isoforms with M_rs near 40 and 48 kDa. Because these are high M_rs for SCF, sequence characterization was done. As reported in GenBank AF119835, the human osteoblast is, not surprisingly, a homologue of the murine membrane-bound KL-2 form, missing exon 6. Additional confirmation that the approx. 40- and 48-kDa products detected with the 7H6 monoclonal antibody are indeed SCF isoforms was obtained using antibodies to regions of SCF translated from exons 5 and 8, and using stable transfectants of MG63 osteoblast-derived cells in which antisense mRNA SCF expression abolishes the expression of immunoreactive material (Fig. 6). Curiously, the antibody specific for exon 8 does not recognize the 40-kDa SCF isoform (Fig. 6B), suggesting that this is truncated, but whether this represents proteolytic cleavage or an mRNA variant is unknown.

Interaction of SCF and its receptor c-*kit* is important in mast cell differentiation [13, 20]. SCF is expressed by stromal cells, such as fibroblasts, and its receptor is found on mast cell precursors [18]. Experimental hyperparathyroidism in rats caused by either PTH administration or a hypocalcaemic diet produced mast cells [30]. It is possible that parathyroid hormone increases SCF expression in other organs, such as skin [25]. The occurrence of mast cells in hyperparathyroid bone may thus be related to the increased SCF synthesis driven by abnormally high PTH.

That PTH-stimulated osteoblasts did not make soluble 20- to 25-kDa SCF isoforms suggests, however, that this cytokine serves a different, cell-surface-specific purpose in bone. There is no apparent function for mast cells in bone trabeculae, and paratrabecular mast cells do not occur at measurable frequency in normal patients. Osteoblasts in nonhyperparathyroid bone make membrane-associated SCF (Fig. 3C), and occasional mast cells occur in normal marrow. In addition, the frequency of mast cells in hyperparathyroid patients is highly variable [28]. This may reflect that a variable fraction (usually small) of the membrane-bound osteoblast SCF escapes, as a result of proteolysis or mRNA-processing variation. This soluble SCF would induce nearby mast cell differentiation.

Other findings suggest that SCF has functions in bone cell differentiation that are unrelated to mast cells. Preosteoclasts express c-*kit* [10], a finding confirmed by flow cytometry [27], and osteoblast SCF production in tissue culture is PTH responsive [10]. Van T Hof et al. [34] showed that SCF promotes osteoclast differentiation and that osteoblast-like cells, which support osteoclastic differentiation in vitro, produce SCF. However, SCF is unlikely to be a dominant osteoclast differentiation signal, except possibly in hyperparathyroidism. Contact of supporting stromal cells and preosteoclasts dramatically increases osteoclast formation [16]. However, the recently identified osteoblast-produced PTH-responsive tumour necrosis factor homologue RANKL (osteoprotegerin ligand) [19] binds to intermediate committed osteoclast precursors and stimulates osteoclastogenesis without stromal cells, 1,25 dihydroxyvitamin D_3, or glucocorticoids. Further, a cytokine cocktail of IL-1, IL-3, and CSF-1 [23] or an antibody for cell-fusion-related protein [12] are sufficient for osteoclast dif-

ferentiation in some circumstances. Thus, a likely role for SCF would be to coordinate or synergize bone cell formation or activity, but SCF is not required for osteoclast differentiation.

In conclusion, our in situ data in bone from hyperparathyroid patients show a direct relationship between osteoblastic activity and SCF expression. These data suggest that osteoblastic expression of cell-surface SCF functions in bone metabolism in vivo. High levels of SCF promote formation of mast cells from precursors in the absence of normal synergistic agents such as IL-3 [20, 24]. Thus, it is likely that mast cells proliferate in hyperparathyroidism because large amounts of SCF are produced because of the amplified osteoblastic activity. When bone formation rates are normal, synthetic osteoblasts produce membrane–bound SCF, but apparently the concentration that reaches mast cell precursors is insufficient to cause mast cell formation. While many factors are involved, the selective expression of membrane-associated SCF in active osteoblasts suggests that SCF may facilitate maintenance of bone structure by promoting bone degradation near the site of bone formation, and the association with hyperparathyroidism suggests a PTH-related function. Determining whether this attractive hypothetical mechanism is valid will require substantial molecular characterization.

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