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Kinetics of Osteoprogenitor Proliferation and Osteoblast Differentiation In Vitro

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Abstract Fetal rat calvaria cells plated at very low density generate discrete colonies, some of which are bone colonies (nodules) from individual osteoprogenitors that divide and differentiate. We have analyzed the relationship between cell proliferation and acquisition of tissue-specific differentiation markers in bone colonies followed individually from the original single cell to the fully mineralized state. The size distribution of fully formed nodules is unimodal, suggesting that the coupling between proliferation and differentiation of osteoprogenitor cells is governed by a stochastic element, but distributed around an optimum, corresponding to the peak colony size/division potential. Kinetic analysis of colony growth showed that osteoprogenitors undergo 9–10 population doublings before the appearance of the first morphologically differentiated osteoblasts in the developing colony. Double immunolabeling showed that these proliferating cells express a gradient of bone markers, from proliferative alkaline phosphatase-negative cells at the periphery of colonies, to postmitotic, osteocalcin-producing osteoblasts at the centers. An inverse relationship exists between cell division and expression of osteocalcin, the latter being restricted to late-stage, BrdU-negative osteoblasts, while the expression of all other markers is acquired before the cessation of proliferation, but not concomitantly. Bone sialoprotein expression is biphasic, detectable in some of the early, alkaline phosphatase-negative cells, and again later in both late preosteoblast (BrdU-positive) and osteoblast (BrdU-negative, osteocalcin-positive) cells. In late-stage, heavily mineralized nodules, staining for osteocalcin and bone sialoprotein is not detectable in the oldest/most mature cells. Our observations support the view that the bone nodule "tissue-like" structure, originating from a single osteoprogenitor and finally encompassing mineralized matrix production, recapitulates successive stages of the osteoblast differentiation pathway, in a proliferation/maturation sequence. Understanding the complexity of the proliferation/differentiation kinetics that occurs within bone nodules will aid in the qualitative and/or quantitative interpretation of tissue-specific marker expression during osteoblastic differentiation. J. Cell. Biochem. 74:616–627, 1999.

Key words: osteoprogenitors; proliferation; differentiation; colony assay; osteoblast phenotype; bone cell differentiation

Osteoblastic cells comprise a great diversity of morphologies and activities, ranging from preosteoblasts, defined by their polygonal shape and proximity/juxtaposition, to cuboidal osteoblasts, through cuboidal, matrix- synthesizing osteoblasts, to matrix and mineral-embedded osteocytes and very flattened or thin bone-lining cells. These various morphotypes are generally considered different maturational stages of the same cell lineage, originating from the proliferation and differentiation of osteoprogenitor cells of pleiomorphic or fibroblastic (spindle-shaped) morphology [for review, see Aubin and Liu, 1996]. When cells enzymatically isolated from fetal rat calvaria (RC) are grown in medium supplemented with ascorbic acid and β-glycerophosphate, discrete three-dimensional mineralizing bone nodules form in the culture. Bone nodules have the histological, ultrastructural, and immunohistochemical appearance of woven bone [Bellows et al., 1986; Bhargava et al., 1988; Nefussi et al., 1985]. Limiting dilution analysis has indicated that less than 1% of the cells in the isolated RC population are osteoprogenitor cells capable of dividing and differen-

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tiating to form bone nodules in vitro and that one osteoprogenitor cell gives rise to one bone nodule under standard culture conditions [Bellows and Aubin, 1989]. Thus, by plating fetal RC cells at very low densities, it is possible to obtain single isolated colonies that have arisen from individual cells that have attached and a few of these colonies represent the osteoprogenitor cells, which through clonal expansion undergo a series of amplifying divisions ultimately to give rise to a colony of differentiated osteoblasts depositing a mineralizing matrix [Aubin et al., 1990; Bellows and Aubin, 1989]. Other colonies represent other lineages including, e.g., fibroblastic, adipocytic, or other osteoblastic stages not capable of forming bone nodules in vitro. Therefore, primary cultures of single colonies arising from fetal RC cells provide an in vitro model system in which to address experimentally the relationship between progenitor cell proliferation and osteoblast differentiation, for which there is believed to be an approximately inverse relationship [Aronow et al., 1990; Aubin et al., 1992, 1993, 1995; Aubin and Liu, 1996; Bellows and Aubin, 1989; Bellows et al., 1986, 1990; Bhargava et al., 1988; Liu et al., 1994, 1997; Malaval et al., 1994; Pockwinse et al., 1992, 1995; Stein and Lian, 1993; Stein et al., 1990]. For example, although it is clear that alkaline phosphatase is already expressed in some proliferative osteoprogenitors and preosteoblasts [Turksen and Aubin, 1991], its expression increases further as osteoblasts mature [Rodan and Noda, 1991], and it has been reported that osteocalcin is upregulated only at the postproliferative osteoblast stage [Aronow et al., 1990; Pockwinse et al., 1992, 1995; Stein and Lian, 1993; Stein et al., 1990]. However, it unknown whether there is a requisite number of divisions/population doublings that a progenitor cell must transit before attaining the mature osteoblast phenotype, nor has the precise relationship between proliferative lifetime and acquisition of other markers associated with the differentiated osteoblast been determined. This study addressed both questions.

MATERIALS AND METHODS

Cell Culture

Cells were enzymatically isolated from the calvaria of 21-day Wistar rat fetuses by sequential digestion with collagenase as described previously [Bellows et al., 1986]. Cells obtained from the last four of the five digestion steps (populations II–V) were pooled and plated in T-75 flasks in α-MEM containing 15% heat-inactivated fetal bovine serum (FBS) (Flow Laboratories, McLean, VA) and antibiotics comprising 100 mg/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 50 mg/ml gentamycin (Sigma), and 0.3 mg/ml fungizone (Flow Laboratories). After 24-h incubation, attached cells were washed with phosphate-buffered saline (PBS) to remove nonviable cells and other debris and were then collected by trypsinization using 0.01% trypsin in citrate saline. Aliquots were counted with a Coulter counter, and the remaining cells were resuspended in the standard medium described above supplemented with 50 mg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and 10 nM dexamethasone (Merck, Sharp, and Dohme, Canada, Ltd., Kirkland, PQ). The resuspended cells were plated into 100 mm tissue culture dishes at 10–35 cells/cm^2. The medium was changed every 2–3 days. All dishes were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 incubator.

Progenitor Cell Population Doubling Analysis

At day 1 after plating, dishes were placed on a grid and individual single cells well separated from neighboring cells were identified and given an identification number and location on the dish. Each day thereafter up to the termination of the experiment (i.e., when bone colonies had mineralized, days 21–25, depending on the experiment), the same cells and their incipient colonies were located and photomicrographs were taken. As cells doubled and colonies grew, colonies were discarded from further analysis if they merged or overlapped with adjacent colonies. For estimation of the number of population doublings that each individual progenitor cell had undergone on each day of analysis, photomicrographs were printed and the cell number counted from micrographs. The number (n) of population doublings was calculated from cell number/colony$=2^n$.

Colonies comprising bone nodules were identified by morphology (cells with distinct cuboidal shape and mineralizing matrix, with or without staining for calcium deposits by the von Kossa technique) [see Liu et al., 1994].

Antibodies

The antiserum directed against rat osteocalcin was kindly provided by Dominique Mod-

rowski (INSERM U349, Hôpital Lariboisière, Paris, France). This antibody was raised in rabbits against osteocalcin purified from rat bone by gel filtration chromatography in denaturing conditions followed by ion exchange in high-perforamce liquid chromatography (HPLC) [Modrowski et al., 1992]. The antiserum directed against bone sialoprotein (BSP was obtained in rabbits immunized with a mouse BSP synthetic peptide (CYDNENGEPRGDTYRAYED) coupled to keyhole limpet's hemocyanin [Raynal et al., 1996]. The monoclonal antibody RBM211.13 (recognizing rat bone/liver/kidney type alkaline phosphatase [ALP]) has been described previously [Turksen and Aubin, 1991; Turksen et al., 1992]. The monoclonal antibodies MPIIIB10_1 (recognizing rat osteopontin) and WVID1 (9C5) (recognizing rat BSP) were obtained from the Developmental Studies Hybridoma Bank maintained at the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD) and from the Department of Biology, University of Iowa, Iowa City, IA (under contract N01-HD-6–2915 from the NICHD). Anti-BrdU monoclonal antibody was purchased from Amersham (Oakville, ON, Canada). Controls were either nonimmune rabbit serum or nonspecific mouse immunoglobulin (mouse IgG3 (KAPPA), Cappel, West Chester, PA).

Immunolabeling

Immunolabeling of cultures was done essentially as described previously [Turksen and Aubin, 1991; Turksen et al., 1992], with adaptation to single nodule labeling [Liu et al., 1994]. Cultures were rinsed with PBS, fixed with 3.7% formaldehyde in PBS and permeabilized with methanol at −20°C. After rinsing, selected colonies were surrounded with a ring of wax to form a well; solutions were pipetted into each for labeling.

Colonies in dishes were incubated for 1 h at room temperature with appropriate dilutions of primary antibodies in 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 2 mM $CaCl_2$, 3% BSA buffer (anti-osteocalcin and anti-BSP antisera at 1:200 dilutions; RBM211.13 at 1:100 dilution of purified ascites fluids; MPIIIB10_1 and WVID1 antibodies at 1:800 and 1:400 dilutions, respectively, of purified ascites fluids; anti-BrdU antibody at full strength). Control antibodies were used either at 1:200 in buffer (nonimmune rabbit serum) or at 40 µg/ml in buffer (nonspecific mouse immunoglobulin). Nodules were rinsed in buffer without bovine serum albumin (BSA) and incubated for 30 min at room temperature with anti-mouse or anti-rabbit secondary antibodies conjugated with either fluorescein (DuPont, Boston, MA; 1:50 dilution) or CY-3 (Jackson Immunoresearch Labortories, West Grove, PA; 1:300 final dilution). Fluorescent labeling of chromatin was done on some colonies by including the DNA-specific dye HD33258 (Hoechst Ltd, Montreal, PQ, Canada; final concentration: 1 µg/ml) in the secondary antibody solution. For double labeling, the samples were incubated successively with each primary antibody, and then with a mixture of the secondary antibodies. After rinsing, samples were mounted in Moviol (Hoechst Ltd, Montreal, PQ, Canada) and observed by epifluorescence microscopy on a Zeiss Photomicroscope III (Zeiss, Oberkochen, Germany). For photography and printing, equal exposure times were used for specifically labeled and control cultures. For all results reported, control cultures labeled with nonimmune serum or nonspecific IgG and second antibodies gave a weak or no fluorescence signal under the same conditions used for specific immunolabeling (not shown).

Statistical Analysis

The distribution of colony sizes was analyzed with the Statworks (Cricket Software, Philadelphia, PA) and Prism (Graphpad, San Diego, CA) sofware.

RESULTS

Osteoprogenitor Cell Proliferation and Bone Colony Growth

By plating fetal RC cells at very low densities (10–35 cells/cm^2), one obtains single isolated colonies that have arisen from individual cells that have attached and proliferated. As noted earlier [Aubin et al., 1982; Liu et al., 1994], the plating efficiency of primary RC cell populations is approximately 10% and a few of the resultant colonies (~10%) represent osteoprogenitor cells and their progeny; on differentiation, the latter yield cuboidal osteogenic cells, which deposit copious amounts of extracellular matrix that ultimately mineralizes, forming a bone nodule [Bellows and Aubin, 1989; Liu et al., 1994] (Fig. 1a). Other colonies comprise pleiomorphic or fibroblastic cells that do not undergo osteogenic differentiation under the conditions em-

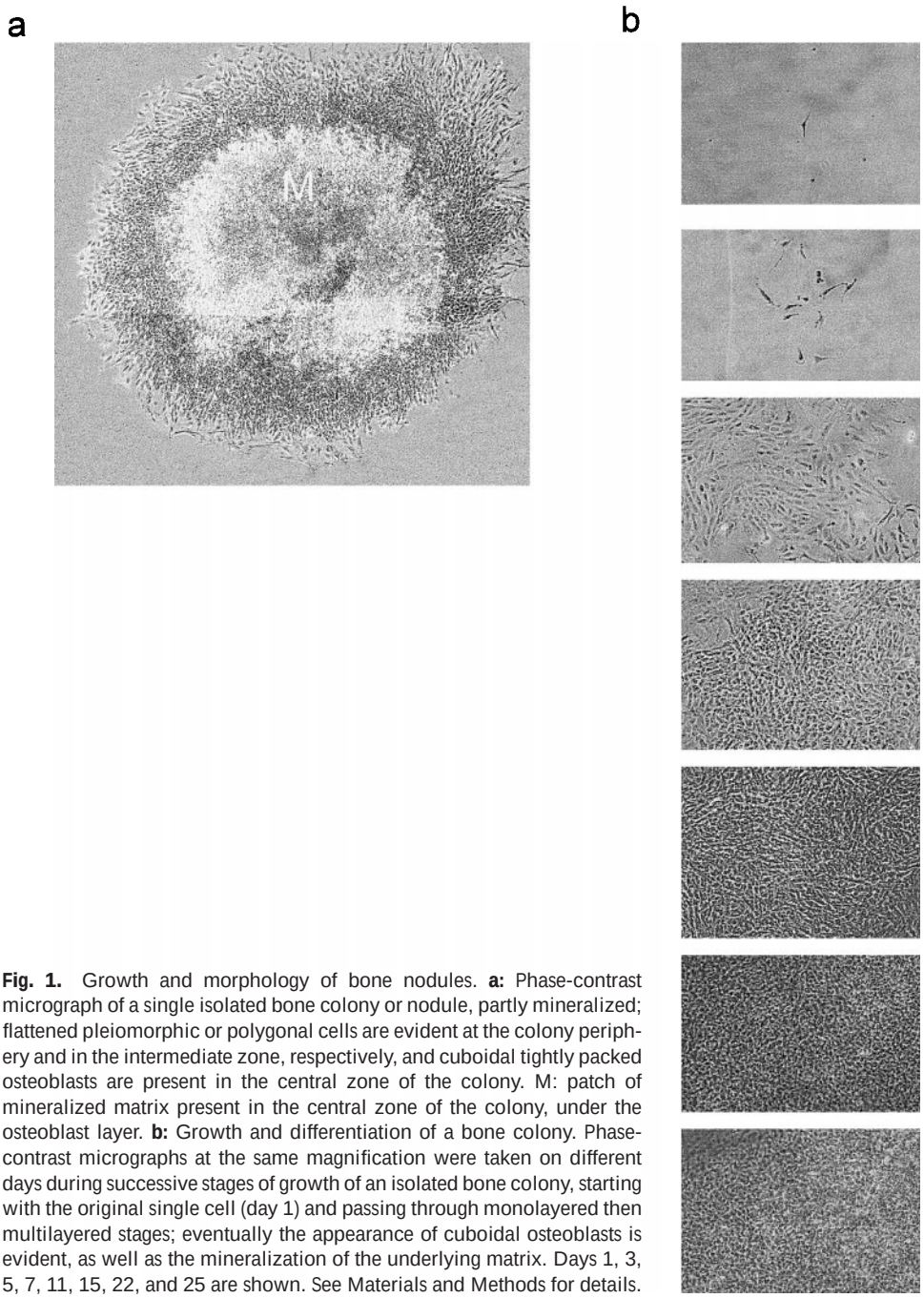

Fig. 1. Growth and morphology of bone nodules. **a:** Phase-contrast micrograph of a single isolated bone colony or nodule, partly mineralized; flattened pleiomorphic or polygonal cells are evident at the colony periphery and in the intermediate zone, respectively, and cuboidal tightly packed osteoblasts are present in the central zone of the colony. M: patch of mineralized matrix present in the central zone of the colony, under the osteoblast layer. **b:** Growth and differentiation of a bone colony. Phase-contrast micrographs at the same magnification were taken on different days during successive stages of growth of an isolated bone colony, starting with the original single cell (day 1) and passing through monolayered then multilayered stages; eventually the appearance of cuboidal osteoblasts is evident, as well as the mineralization of the underlying matrix. Days 1, 3, 5, 7, 11, 15, 22, and 25 are shown. See Materials and Methods for details.

ployed here. Within growing colonies (Fig. 1a), the morphologically most mature and nondividing cells in the colony reside in the central zone, followed by an intermediate zone and less differentiated cells at the colony periphery, where cell division persists for some days [see also Liu et al., 1994]. At late maturational stages of bone colony development, cell division ceases throughout the colony and cuboidal osteoblast morphology and mineralized matrix extends to the colony periphery.

We first asked how many cell divisions an osteoprogenitor cell undergoes before there are at least some progeny cells differentiated to the point of being morphologically recognizable (cuboidal cells surrounded by refractile matrix) within osteoblast colonies. From four separate experiments with independent cell isolates, a total of approximately 1,000 colonies formed and, of these, 103 colonies were followed from the single-cell colony starts by photomicrogra-

TABLE I. Progenitor Cell Doubling Analysis[a]

Progenitor/ colony no.	RC2	RC3	RC4	RC5	RC7	RC9
Day of culture	Estimated population doublings at the days indicated					
1	1	0	1	0	1	1
4	5	3.5	5	5	4.5	4
7	—	4.5	8.5	8	7	7.5
11	10.5	7	11.5	10	9	9.5
14	—	10	F[d]	OB	—	—
20	OB[b]	OB	F	OB	OB	OB
25	OB/MIN[c]	OB/MIN	F	OB/MIN	OB/MIN	OB/MIN

[a]Cell number was counted from photomicrographs taken of each Colony at each of the days indicated and the population doubling level calculated.
[b]OB: cuboidal cells surrounded by refractile matrix evident at the colony center.
[c]OB/MIN: cuboidal cells with mineralized matrix.
[d]F: cells remained fibroblastic/spindle-shaped throughout the experiment.

phy. Of these, 14 bone colonies that met the criteria for analysis (see Materials and Methods) were identified, cell number was counted, and population doublings calculated from the micrographs taken at daily intervals (Fig. 1b). Notably, in all cases, cuboidal osteoblastic cells were present only after a minimum of 9–10 population doublings (i.e., colonies had reached cell numbers of ~500–1,000); the colony kinetics of five of these and one fibroblast colony for comparison are summarized in Table I. Bone colonies comprising cuboidal cells and ultimately containing mineralized matrix continued to increase in size up to population doubling levels of >14, by which time cells became impossible to count visually as a result of extensive cell packing, multilayering, and matrix accumulation. However, bone colonies were found not to increase in size indefinitely; as cell division stopped, colonies ceased increasing in diameter, and the matrix became heavily mineralized. After determining the end-stage diameter size distribution attained by bone colonies, we found that the size of colonies followed a unimodal distribution, with a peak value of 4.5 mm, and was skewed to the right (larger colony sizes) (Fig. 2). The lower limit of bone colony size detected was approximately 2.5–3.0 mm (Fig. 2), corresponding to approximately 500–1,000 cells, respectively, consistent with the 9–10 doublings calculated from the original single progenitor cell giving rise to the colony (Table I). Most colonies ranged between 3 and 8 mm, but a few were measured at ≤13.5 mm (Fig. 2).

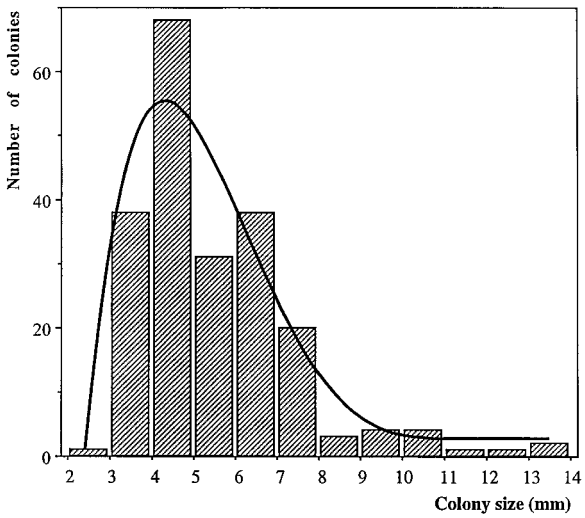

Fig. 2. The size distribution of fully grown bone nodules in low density cultures is unimodal but skewed. The diameters of 211 fully grown bone colonies were measured after 25 days of culture, and the values distributed in size classes of 1 mm. The best-fit curve (5th degree polynomial) is shown in black (r = 0.95).

Cellular Proliferation Within Bone Colonies

BrdU incorporation (Fig. 3a,b,e), as well as staining of chromatin with the fluorescent DNA stain HD33258 (not shown) showed that, in early stages, cycling/dividing cells were present throughout developing nodules (Fig. 3a), while in more mature nodules with multilayered cells and partly mineralized matrix, only peripheral cells were still dividing (Fig. 3b,e), and the inner cells were quiescent (Fig. 3e,g). In nodules kept for longer periods (30 days) in which

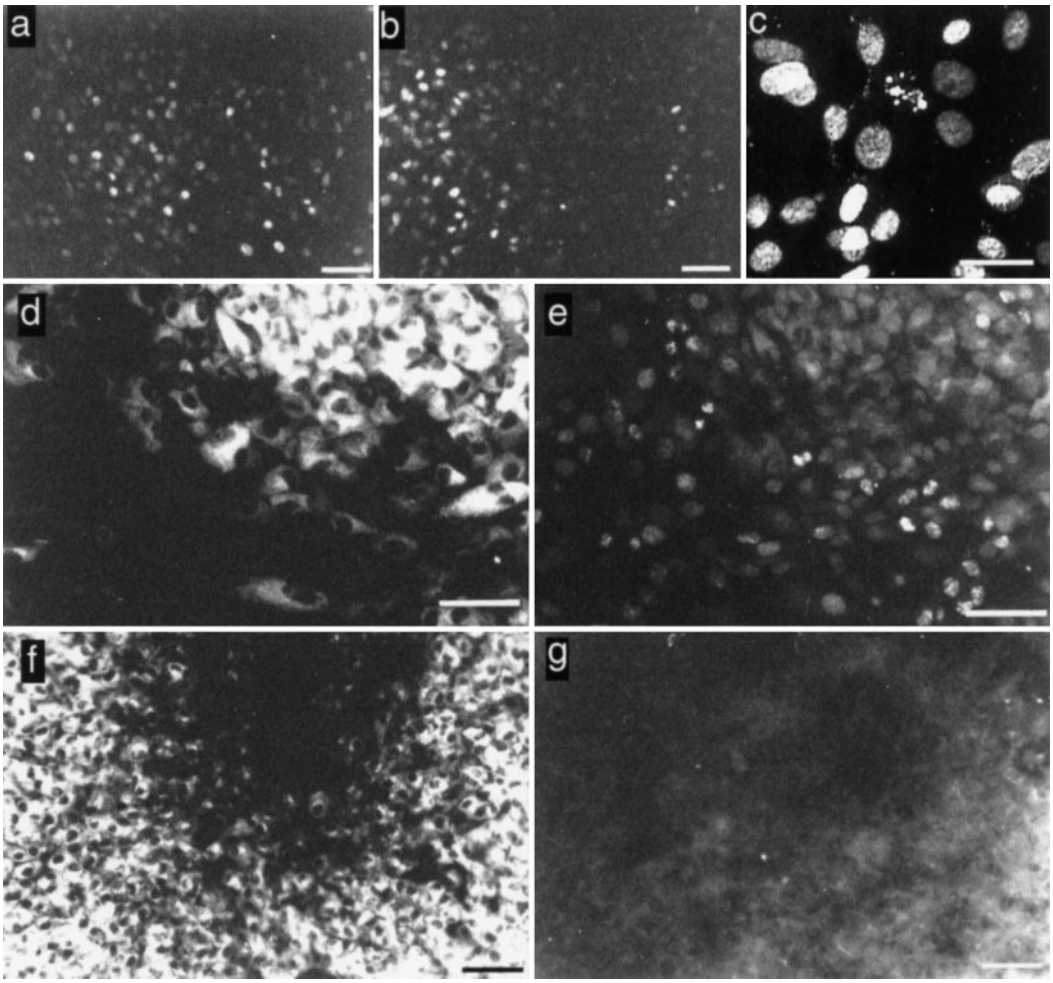

Fig. 3. Kinetics of cell proliferation and differentiation within bone nodules. **a,b:** BrdU labeling of dividing cells in monolayered (a) and early multilayered (b) bone colonies, showing progressive restriction of cell proliferation to the periphery of the nodules. **c:** HD33258 labeling of nuclei in the center of a mineralized nodule, showing nuclear fragmentation characteristic of apoptosis. **d,e:** Margin of the cuboidal cell layer of an early mineralized nodule double stained for osteocalcin (d) and BrdU (e); the center of the nodule is in the upper right corner; note the absence of BrdU incorporation within the inner mass of cuboidal osteocalcin-positive cells. **f,g:** Central part of a partly mineralized nodule stained for osteocalcin (f) and BrdU (g); note the absence of BrdU labeling in these oldest cells and the disappearance of osteocalcin labeling in the center of the nodule. Scale bars = 50 μm.

matrix becomes entirely mineralized, no BrdU incorporation could be detected in any zone (not shown). Staining with HD33258 revealed fragmented, apoptotic nuclei (Fig. 3c), first evident among the quiescent cells of multilayered colonies, and more abundant in late, fully mineralized nodules.

Expression of Osteoblast Markers

Immunolabeling for BRdU and differentiation markers was performed on developing bone nodules. The results, described below, are summarized in Figure 5. Control experiments (not shown) confirmed that primary and secondary antibodies penetrated the bone colonies down to at least the mineralization front [see Bhargava et al., 1988; Liu et al., 1994, 1997].

Osteocalcin. Given the suggestion that expression of osteocalcin appears concomitantly with growth arrest [Stein et al., 1990] in RC populations, we first analyzed the relationship between osteocalcin expression and BrdU incorporation. Cellular staining for osteocalcin was cytoplasmic and concentrated in the Golgi area [see also Liu et al., 1994; Malaval et al., 1994]. At all stages of nodule development—nascent

and maturing—cells staining for osteocalcin were present in the more central areas of nodules and were normally not found to be stained for BrdU after 24-h incorporation (cf. Fig. 3d and e). In very advanced nodules, both some flattened and cuboidal cells in the peripheral zone showed significant staining for osteocalcin, but staining could no longer be detected in the central zone, i.e., in the "oldest" cells of the colony (Fig. 3f), which were nondividing (Fig. 3g). These cells were not inactive or artifactually negative due to poor antibody permeation, since they expressed at least some other markers by antibody labeling, e.g., ALP and osteopontin.

BSP and ALP. We have described in detail elsewhere [Liu et al., 1994; Malaval et al., 1994; Turksen et al., 1992] the labeling of bone nodules with the antibody against ALP (RBM211.13). Briefly, labeled cells displayed a membrane-associated staining, and labeling was relatively homogeneous from cell to cell throughout well-developed nodules, with the exception that most, but not all, of the flattened cells of the colony periphery stained (not shown; see references above). Labeling of early/nascent nodules showed faint ALP staining, localized to only a minority of fibroblast-like cells radially oriented around the central zone of colonies (Fig. 4b).

We used two different BSP antibodies to localize cells expressing this macromolecule. Staining for BSP with the rat polyclonal antiserum was prominent in Golgi and intense in the whole cytoplasm (Fig. 4a,c,e,g). By contrast, the mouse anti-rat BSP monoclonal stained essentially the Golgi, with faint punctiform staining of the rest of the cytoplasm and, in some cases, the cell membrane (Fig. 4i). Strikingly, some cells labeling for BSP, but not for ALP, could be observed in the periphery of very early colonies in which some dividing cells were also seen

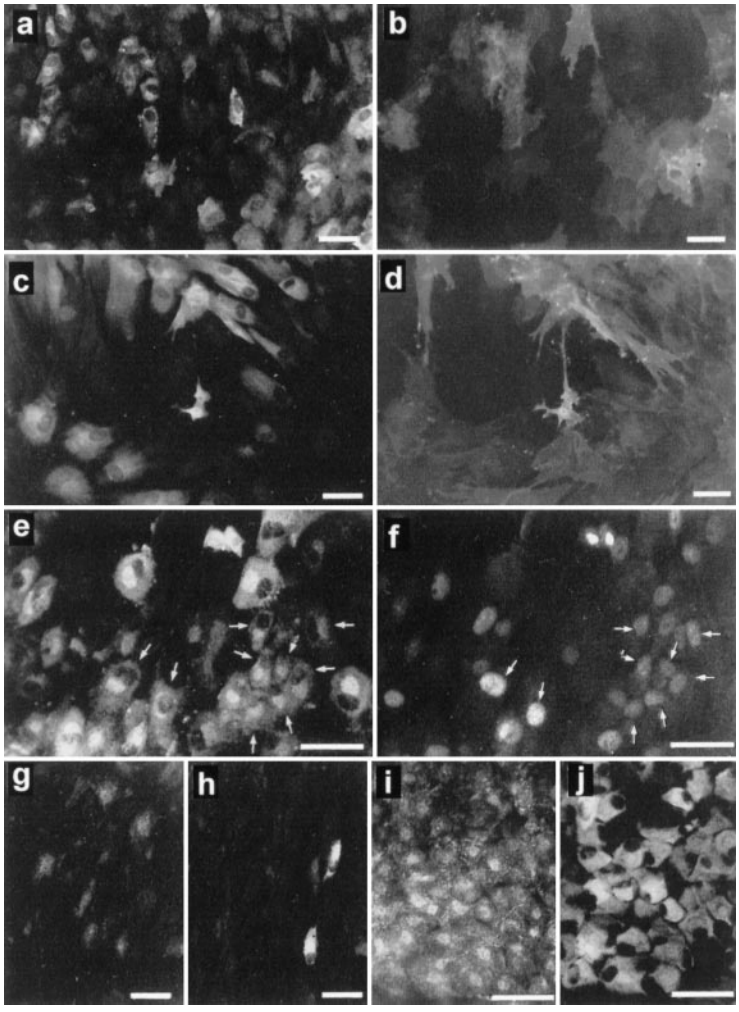

Fig. 4. Marker expression by proliferating cells of bone nodules. **a,b/c,d:** Peripheral (a,b) and more central (c,d) area of an early bone colony double immunolabeled for BSP (a,c) and ALP (b,d), showing that BSP and ALP staining do not always coincide, and in particular that some peripheral cells express BSP but not ALP. **e,f:** Proliferative zone of an early multilayered nodule, showing a high number of cells staining for both BSP (e) and BrdU (f). **g,h/i,j:** The few osteocalcin-positive cells in the central part of an early nodule (h) are surrounded by less advanced osteoblasts that do not yet express osteocalcin, but that already stain for BSP (g). In a more advanced colony (i,j), late, postmitotic osteoblasts stain for both BSP (i) and osteocalcin (j), with cell-to-cell variations in staining intensity. Scale bars = 50 μm.

labeled for both markers (Fig. 4a,b). Advancing centrally into the colony, but still in the zone of cycling cells, ALP positive-BSP negative cells were present, as well as cells expressing both markers (Fig. 4c,d). In more advanced colonies, most of the central cells expressed ALP, and a subset of these also stained for BSP. A few cells staining for BSP could still be spotted in the thin outer rim of ALP-negative cells at this stage (not shown).

In early multilayered colonies, the BSP antibody-labeled cells in the central zone and extended to many cells well out from it in the intermediate zone; most of the latter did not label with osteocalcin (Fig. 4g,h). Up to multilayered, but nonmineralized nodule stages, there was a significant overlap of BSP and BrdU staining, with groups of cells staining for both (Fig. 4e,f). Osteocalcin-positive, BrdU-negative cuboidal osteoblasts also stained for BSP (Fig. 4i, j). BSP labeling intensity, like that for osteocalcin, varied from cell to cell in these mature cells, with no direct correlation between the intensities of the two markers (Fig. 4i,j). Like that for osteocalcin, BSP staining intensity was also reduced in the central, oldest cells of late mineralized colonies (not shown).

Osteopontin. We have previously reported and confirmed here widespread osteopontin expression in bone nodules, i.e., the presence of osteopontin in both mature postproliferative cuboidal osteoblasts and in immature proliferative fibroblastic cells at the nodule periphery (not shown) [see Liu et al., 1994], consistent with other observations on this molecule [Owen et al., 1990; Pockwinse et al., 1992; Smith and Denhardt, 1989].

DISCUSSION

Although no marker has been found for rat osteoprogenitor cells [Aubin and Liu, 1996], the development of bone nodules in vitro permits their retrospective identification and analysis of the proliferation/differentiation sequence leading from progenitor to fully differentiated osteoblasts. Previously, we showed that proliferation is required for a bone nodule to form in vitro [McCulloch et al., 1991]; colony size would be expected to reflect the proliferation history, and perhaps the potential, of the progenitor cells. In this study, we found the size distribution of bone colonies, allowed to grow at low cell densities where they are unimpeded by growth restraints that might occur at higher densities, to be unimodal. One vision of lineage specification is that commitment of progenitor cells to terminal differentiation would occur with equal probability at any given time of their life span, i.e., after any number of divisions. Our results suggest instead that there may be an optimal "age," i.e., number of divisions, at which the probability is highest for terminal differentiation of the osteoprogenitor, with stochastic variation around this point. At this time, we cannot address explicitly how, or whether, the in vivo proliferative history of different osteoprogenitors contributes to the distribution observed, although the primitive progenitor population is generally considered relatively quiescent in vivo [Owen, 1998]. However, because our cultures were maintained in the presence of glucocorticoids, colonies formed would comprise those resulting from both the more mature glucocorticoid-independent progenitors and the immature glucocorticoid-requiring progenitors that we reported earlier [Turksen and Aubin, 1991].

The number of population doublings that precedes the appearance of the first morphologically recognizable osteoblasts is a different parameter, reflecting the minimum number of mitoses through which the descendents of an osteoprogenitor must transit before reaching terminal differentiation (marked by the acquisition of a cuboidal shape and arrest of proliferation, see below). This figure is high (~9–10 doublings), and concurs with the colony size analysis; i.e., bone colonies were not found with diameters smaller than approximately 2.5 mm, consistent with the view that they arise from progenitors that must undergo cell divisions and not from precursors of more limited division capacity, e.g., preosteoblasts. This was confirmed by immunolabeling studies, which showed clearly that most cells in early colonies, and the most peripheral cells (i.e., the most recently divided, and still dividing) in all other stages of nodule development, except in those that are fully mineralized, express no marker apart from osteopontin (and sometimes BSP; see below), confirming their undifferentiated/early differentiated nature. By contrast, cells located more centrally in colonies but still dividing display a sequence of differentiation, from proliferative BrdU-incorporating, ALP-positive/osteocalcin-negative cells (peripheral), through young postmitotic, osteocalcin-positive, to late postmitotic, BSP- and osteocalcin-negative cells

(colony centers; summarized in Fig. 5). Thus, during the last 9–10 divisions before terminal stage, the progeny of the original cell encompass the whole sequence of osteoblast differentiation. This minimum number of mitoses, which is remarkably constant, may represent a biological time or clock that progenitors require to undergo the successive steps of the lineage. Alternatively, it may be needed to reach a cell density and/or matrix amount necessary to trigger terminal differentiation, which has been shown to be dependent on collagenous matrix deposition [Aronow et al., 1990; Owen et al., 1990; Stein and Lian, 1993]. In accordance with these data, bone colonies that stopped growing after ~10 doublings (i.e., ~2.5 mm; 500–1,000 cells) are at the lowest end of the colony size distribution, while larger colonies are generated by progenitor cells expressing a higher proliferative potential. Cells committed to the osteoblast lineage, but expressing a lower proliferative potential (<9–10 doublings), would generate smaller colonies (<2.5mm) in which cells may never reach the late, cuboidal stage. Such colonies would not be recognized in the analyses reported here, and their absence may contribute to the unsymmetric shape of the size frequency curve. Another contribution to the skewness is the few very large colonies (>9 mm) that form. Although the number of divisions completed by cells in such large colonies may still be within the range of stochastic variation, they may, on the other hand, reflect the presence of a small subset of progenitors with a significantly longer life span (i.e., higher division potential) than average, e.g., are at earlier stages of the lineage than most other progenitors in the population. It should be noted that osteoprogenitors may exist that do not attach [Scutt et al., 1995] or divide under our culture conditions; they are not analyzed here. Our data also do not address what role symmetric versus asymmetric divisions may play in the osteoblast developmental process.

Osteocalcin expression has been reported to be acquired concomitantly with the production of a mineralizing matrix [Aronow et al., 1990], but we found it present already in incipient nodules with no detectable mineral [see also Malaval et al., 1994]. Osteocalcin was expressed in a pattern more restricted than that for ALP, in early postmitotic and later populations. The division time of RC cell populations in culture is approximately 20 h [Pockwinse et al., 1992; our unpublished observations]; thus, cycling cells labeled with BrdU for 24 h should have gone through one S phase; unlabeled cells had at least a lengthened cycle or were quies-

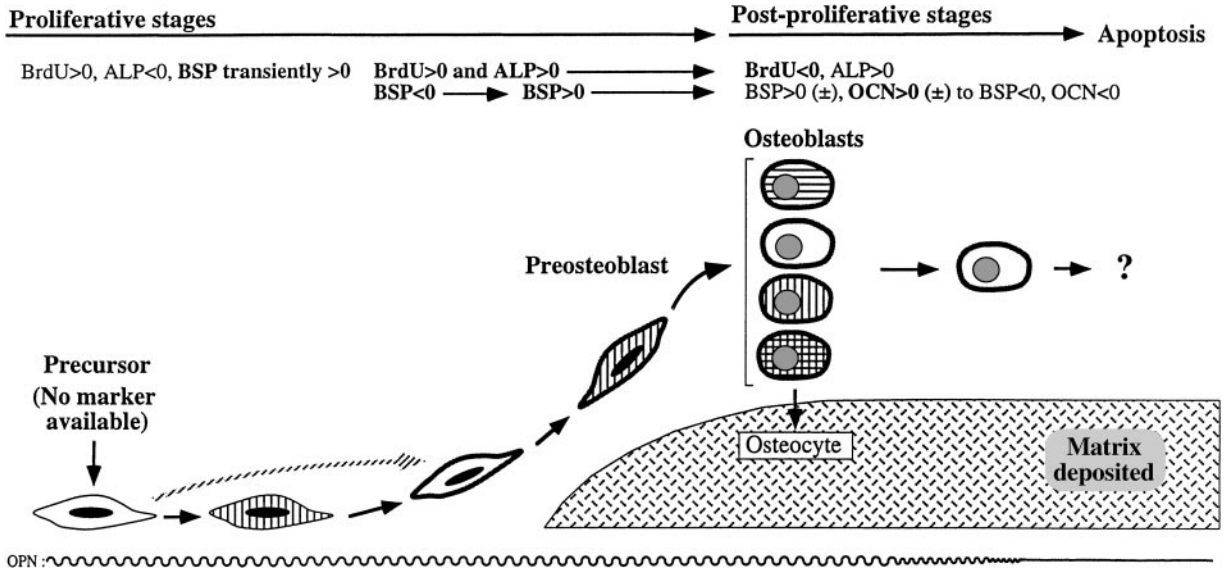

Fig. 5. Schematic summarizing the patterns of bone related protein expression that define successive stages of bone cell maturation in bone nodules from fetal rat calvaria cell cultures. Nuclei: ●, BrdU incorporation; ◍, no BrdU incorporation. Cytoplasm: ▥, BSP staining; ▤, OCN staining. Membrane: ⬭, ALP staining; ᗰ, relative intensity of OPN staining along the sequence of differentiation (results not shown) [see Liu et al., 1994]. The "stack" of osteoblasts on top of the mineralized matrix displays the diversity of phenotypic expression in early postmitotic osteoblasts. Dashed arrow represents lack of evidence that all cells go through the ALP < 0, BSP > 0 stage; boldface, marker diagnostic for the phenotype of the zone.

cent. As the cuboidal cells in the center of nodules were all unlabeled, it is likely that the cells that did not incorporate BrdU, but did express osteocalcin, were indeed noncycling. This result at the single cell level is consistent with the fact that in total populations of rat calvaria cells the onset of osteocalcin production coincides with confluence and exit from log phase growth [reviewed in Stein and Lian, 1993]. The use of the bone nodule model allowed us to compare the expression pattern of osteocalcin with another very specific marker of mature osteoblasts, BSP, for which conflicting data have been reported. On the one hand, BSP has been suggested to belong to the postmitotic mature osteoblast program [Bianco et al., 1993]; on the other hand, although a late marker of osteoblast differentiation, it is expressed earlier than osteocalcin [Chen et al., 1992; Liu et al., 1994; Malaval et al., 1994; Yao et al., 1994]. Our observations suggest two distinct phases of BSP expression. BSP stains prominently cells that are cycling and ALP-positive/osteocalcin-negative, i.e., "late preosteoblasts," as well as osteocalcin-positive, fully differentiated, postmitotic osteoblasts. Moreover, in very early nodules, BSP staining was also observed in some ALP-negative cells. Although expression of ALP is thought to be acquired relatively early in the osteoblast differentiation pathway, committed osteoprogenitor cells that are ALP-negative have been identified [Turksen and Aubin, 1991]. As mentioned above, the absence of staining for this marker in early nodules and/or the periphery of advanced nodules is consistent with these cells being very early in the differentiation sequence. Together with the observation that, in intermediate and central zones of nodules, no BSP labeling is observed among cells that are homogeneously ALP-positive, this early (pre-ALP) expression of BSP appears to be transient. This interpretation concurs with recent data that we have acquired by replica plating of progenitors in vitro and global amplification poly(A)-PCR [Liu et al., submitted], and by in situ hybridization of the 21-day fetal rat calvaria [Candeliere et al., 1997; submitted], the latter showing primitive BSP-positive/ALP-negative cells in the suture areas of the 21-day fetal rat calvaria close to the osteogenic front, suggesting that such a developmental stage occurs in the normal tissue environment in vivo. Further experimental work is needed to determine whether all bone cells go through an early transient stage of BSP expression (cf. Fig. 5) and to assess the biological significance of such early expression.

The remarkable heterogeneity of marker expression that we observed by immunocytochemistry in mature, noncycling osteoblasts confirms our recent analysis of the gene repertoires expressed in postmitotic osteoblastic cells using global amplification poly(A)-polymerase chain reaction (PCR) [Liu et al., 1997]. These data raise the intriguing possibility that the mature osteoblast phenotype is not a single unique phenotype, but rather that it encompasses biochemical plasticity that might also reflect functional plasticity [Aubin et al., 1982, 1995; Liu et al., 1997]. In recent studies on calvaria of the same developmental age (21-day fetal) as used for isolation of the cells used in this study, we found evidence to support the concept that osteoblast heterogeneity is regulated at various levels, i.e., by maturational age, by environmental (e.g., endocranial versus ectocranial tissues) and microenvironmental (adjacent/juxtaposed osteoblasts) conditions, and at both transcriptional and posttranscriptional levels [Candeliere et al., 1997; submitted], with considerable implications for osteoblast function in vivo. Whether processes such as apoptosis play a role in the heterogeneity observed is unknown, but our observations indicate that at least some cells in the central part of nodules undergo apoptotic death, as shown previously in nodules in vitro [Lynch et al., 1998], and consistent with recent observations that apoptosis is the fate of many osteoblasts in vivo [Jilka et al., 1998]. While expression of some osteoblast-associated genes has been reported to decline before apoptosis in one model [Ihbe et al., 1998], the issue of whether at least some late-stage osteoblasts may be induced to re-enter a proliferative phase has also been raised [see Rodan and Rodan, 1992]. Further studies are needed to assess whether the osteoblast quiescence is an irreversible phenomenon or whether these cells can resume proliferation after appropriate stimuli.

Although bone nodule formation is increasingly used as an assay of osteoblast differentiation in various cell systems, considerable discussion remains in the literature about their nature, as reflected in references to nodules forming by "cell aggregation" or "cell migration." As described elsewhere [Bellows and Aubin, 1989; Bellows et al., 1986, 1990; Bhar-

gava et al., 1988; Liu et al., 1994, 1997; Turksen and Aubin, 1991] and clearly established in this report, the bone nodules that form in RC cell cultures are colonies, generated by the clonogenic proliferation and differentiation of single progenitor cells. In cultures grown under the conditions described here, osteoblast differentiation occurs only within bone colonies, and not in other cells in the populations [see also Malaval et al., 1994]. Also, we document that multiple transitional stages in the osteoblast differentiation program can be seen in different parts of a single colony while it is forming. Osteogenesis in RC cell cultures has been described to encompass three stages: proliferation, matrix synthesis, and matrix maturation and mineralization [Owen et al., 1990; Stein and Lian, 1993]. Analysis of cellular events in isolated nodules shows, however, that proliferation and differentiation occur simultaneously in different areas of a given colony, indicating that results obtained with whole cultures are "averages" of expression of all cells present, and that there is not a strict inverse relationship between proliferation and differentiation in this system. It remains possible that aggregation or migration may influence osteogenic events in populations from other sources or grown under other conditions, or that osteogenesis itself may follow accelerated kinetics when cells are plated at higher densities and may involve less primitive cells than those followed here. Therefore, we believe that distinctions should be made in what kinds of processes contribute to the culture endpoint of mineralized matrix formation under various conditions.

In summary, in vitro analysis of the proliferation/differentiation kinetics within osteoblastic colonies formed in low cell density cultures demonstrates that they originate from a population of progenitors with a high proliferative potential, whose progeny transit successive stages of the osteoblast differentiation pathway in the last 9–10 divisions before terminal differentiation.

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