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Identification of osteocytes in osteoblast-like cell cultures using a monoclonal antibody specifically directed against osteocytes

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Summary. The development of a monoclonal antibody, OB 7.3, directed against a cell surface antigenic site on osteocytes is described.

Osteoblast-like cells were enzymatically isolated from calvaria of chicken embryos after removal of the periostea. The cells were cultured for 6 days, harvested and used to immunize mice. One of the monoclonal antibodies obtained, OB 7.3, reacted specifically with the cell surface of osteocytes. In frozen sections of bone only osteocytes were stained, all other cells present, including mature osteoblasts, were negative. Liver, kidney, spleen, intestine, bloodvessel and skin were also completely negative. Using the monoclonal OB 7.3, positive cells could be demonstrated in sparse osteoblast-like cell cultures. The OB 7.3 positive cells had a stellate morphology and were therefore identified as osteocytes. They behaved in culture as osteocytes in bone tissue in that they formed a network of cell processes connecting osteocytes with each other or with other neighbouring cells. Monoclonal OB 7.3 offers the possibility of isolating osteocytes thereby providing the means for a detailed study of their biochemical properties.

Introduction

Our knowledge on the metabolism of bone has been considerably increased by the use of isolated bone cells (Peck et al. 1964; Smith et al. 1973; Wong and Cohn 1975; Nijweide et al. 1981).

However, the diversity of cell types, present in bone tissue and therefore in cell isolates from bone tissue, has not always been fully appreciated (Rifkin et al. 1980). Some attempts have been made to separate the different cell types by sequential enzymatic digestion (Wong and Cohn 1975; Luben et al. 1976), by selective dissection (Smith et al. 1973; Peck et al. 1977; Yagiela and Woodbury 1977; Nijweide et al. 1981) or by differential serum additions to culture media (Wong and Kocour 1983). These procedures have proven to be insufficient. Recent investigations have shown sequential digestion procedures to be not appropriate for separate isolation of osteoblasts and osteoclasts or their precursors from fetal mouse calvaria (Burger et al. 1985). Although osteoblast populations isolated from periosteum-free chicken calvaria appeared to be relatively free of osteoclast precursors (Burger et al. 1985), contamination of these populations by fibroblasts can not be excluded (Nijweide et al. 1982). Furthermore none of the rather crude isolation and separation procedures mentioned above can be expected to differentiate between the various differentiation stages of any of the major cell types present in bone. Yet, for the understanding of bone metabolism and its hormonal regulation, the study of the separate differentiation stages of e.g. the osteoblast and osteoclast is crucial. We have therefore started the production of monoclonal antibodies against cell surface antigens specific for the various differentiation stages of the osteoclastic and osteoblastic cell lines. These specific monoclonal antibodies will enable us to selectively recognize and isolate osteoblasts (Tsuru et al. 1984), osteoclasts, their progenitors and precursors. In a recent publication (Nijweide et al. 1985) we have reported on the production of monoclonal antibodies against cell surface markers on osteoclasts. In this paper we describe the generation, production and use of a monoclonal antibody highly specific for the cell surface of the terminal differentiation stage of the osteoblastic cell line: the osteocyte.

Materials and methods

Bone cell isolation and culture. Osteoblast- and fibroblast-enriched populations (OB and PF populations) were obtained as described previously (Nijweide et al. 1981, 1982). In short: the ectocranial and endocranial periostea of calvaria of 18-day-old chicken embryos were removed by dissection; PF and OB populations were isolated by separate collagenase treatment of the periostea and the periosteum-free calvaria respectively; the cells were washed and seeded into plastic Petri dishes (Costar tissue culture cluster 6, 35 mm) or culture flasks (Costar, 150 cm^2); culture was performed in MEM (Gibco) to which 10% cock serum, 5% embryonic extract of 10 day-old chicken embryos, 50 µg/ml vitamin C, 200 µg/ml glutamin, 1 mg/ml glucose and 50 µg/ml gentamicin was added; when cultures were prolonged for more than 6 days embryonic extract was omitted and the serum concentration reduced to 2,5%; every 3 or 4 days the culture medium was refreshed for 50%.

Immunization and monoclonal antibody production. The immunization procedure used has been described previously (Nijweide et al. 1985). In short: BALB/c mice were injected i.p. with 10^7 cells isolated with collagenase from 6 day-old OB cultures; the cells were resuspended in 0.5 ml Hank's balanced salt solution (BSS), mixed

In honour of Prof. P. van Duijn

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ultrasonically with 0.5 ml complete Freund's adjuvant (Gibco); two weeks later the procedure was repeated using incomplete adjuvant; again two weeks later the mice were boostered i.v. on three successive days with 10^7 OB-cells in 0.2 ml BSS; on the fourth day the spleen was removed, spleen cells isolated and fused with Sp 2/0 myeloma cells using PEG (MW 4000; Merck) as fusing agent (Köhler and Milstein 1975; Fazekas de St. Groth and Scheidegger 1980); the fusion products were plated out in hypoxanthine-aminopterin-thymidine (HAT) supplemented RPMI 1640 medium (Boehringer) also containing 10% fetal calf serum (FCS; Flow Laboratories) and 10% human endothelial supernatant (HECS) (Astaldi et al. 1980). The supernatants of growing hybridoma colonies were screened on frozen sections of the phalange of 18-day-old chicken embryos using an indirect technique (see section immunocytochemical staining). Positive hybridoma colonies were subcloned. Positive subclones producing monoclonal antibodies were propagated and injected i.p. in pristan-treated BALB/c mice. The obtained ascites fluid was used for the experiments described, generally diluted 250 times.

Immunocytochemical staining. The antigenic determinant of OB 7.3 was proven to be extreemly sensitive to most fixatives, which limited the possibilities of tissue processing procedures.

Three types of cell or tissue preparations were used:

- frozen sections (5 µm) of tissues or cell cultures, air dried at room temperature.
- cytocentrifuge preparations of isolated cells, air dried, sometimes fixed in acetone for 10 min and kept at −80° C if the preparations had to be stored.
- living cell cultures, grown on glass coverslips.

Immunofluorescence analysis was performed using an indirect technique. Frozen sections or cytocentrifuge preparations were rehydrated for 5 min in phosphate buffered balanced salt solution (PBS) containing 1% bovine serum albumin (BSA). Cell cultures were washed with ice-cold PBS 1% BSA. Hybridoma colony or clone supernatant, or diluted ascites fluid was applied for 60 min. The preparations were washed three times for 5 min with PBS and again incubated for 60 min, this time with rabbit anti-mouse IgG-FITC conjugate (Miles-Yeda), 40 times diluted. They were washed again three times for 5 min with PBS and embedded in glycerol-PBS (9:1). All procedures took place at room temperature for the frozen sections and cytocentrifuge preparations but at 4° C for the (living) cell cultures.

Results

Specificity of OB 7.3

Most of the monoclonal antibodies obtained, were not specific for one cell type only. They reacted in frozen sections of embryonic chicken phalanges with osteoblasts, osteocytes, periosteum cells, chondrocytes and marrow cells or a selection out of these cell types, with different intensities. Only one of the monoclonals we obtained so far, OB 7.3, was highly cell specific. It reacted only with osteocytes (Fig. 1 A–C).

In order to analyse further its specificity, OB 7.3 was also applied to frozen sections of chicken embryonic liver, spleen, small intestine, skin, blood vessel (aorta) and kidney. All these tissues were completely negative for OB 7.3 (not shown).

Cross-reactivity with other species

OB 7.3 cross-reacted strongly with osteocytes in the phalange of embryonic quails and in the medullary bone of adult, egg-laying female quails (not shown). No reaction was found in embryonic rat tissues (not shown).

OB and PF cultures

The fact that we obtained a monoclonal antibody specific for osteocytes by immunizing mice with 6-day-old OB cultures, obviously meant that osteocytes were present in these cultures in reasonable numbers, a finding we had up to then not expected.

Indeed, when 3-day-cultures, unfixed, without any pretreatment, were incubated with monoclonal OB 7.3 and afterwards with rabbit anti-mouse FITC, two things were demonstrated. In the first place, OB 7.3 positive cells are present in OB cultures (Fig. 1 D); secondly, the antigenic site reacting with OB 7.3 is situated on the outside of the cell membrane. OB cultures older than 3 days were less suitable for immunocytochemistry without sectioning (see following chapter) because of overgrowth by rapidly proliferating osteoblast-like cells. However, when 6-day-cultures were treated with collagenase and cytocentrifuge preparations were made, these preparations clearly showed OB 7.3 positive cells among a large number of negative cells (Fig. 1 E). OB 7.3 positive cells appeared already to be present in OB-suspensions immediately after isolation from the tissue (not shown). In contrast PF populations, cultured or non-cultured, virtually never contained OB 7.3 positive cells (Fig. 1 E).

Cells, possessing the OB 7.3 cell surface marker adopted in culture a morphology remarkably similar to that of osteocytes in situ (compare Fig. 1 B with Figs. 1 D, 3 A and B). They could therefore be identified as true osteocytes. Their morphology was so exceptional that even without the help of monoclonal OB 7.3, osteocytes could easily be recognized in sparse cell cultures (Fig. 2 C and D). Osteocytes in culture behaved as osteocytes in situ not withstanding the absence of extra-cellular matrix. They sought or kept contact via slender, elongated cytoplasmic processes with each other (Fig. 3 A), or with other, OB 7.3 negative, cells present in the culture (Fig. 3 B).

After seeding of isolated OB cells some of the osteocytes present in the OB population attached themselves to the glass surface as solitary cells but most of them clinged together and attached to the surface in small clots. In the experiment of Fig. 2 A–F and other similar experiments we isolated such clots of osteocytes from other seeded cells by making a deep furrow in the plastic of the Petri dish around the clot. The furrow inhibited surrounding cells to overgrow the insulated osteocytes and marked the clot making longitudinal study of the osteocytes in culture possible. During culture the osteocytes migrated away from each other slowly, keeping however contact through numerous cytoplasmic processes (Fig. 2 A–E). Generally osteocytes kept their stellate form throughout the culture period and did not proliferate. We have never observed cell division in insulated, solitary osteocytes. It is, however, difficult to ascertain whether proliferation takes place within a clot or not. Sometimes an osteocyte with two or three nuclei were seen, formed either by fusion or more probably by nuclear division (Fig. 2 F). When OB cell populations were cultured for longer periods (1–4 weeks) the number of osteocytes per culture appeared to increase (Fig. 3 C) suggesting differentiation of precursor cells (osteoblasts) into osteocytes. PF cultures remained devoid of osteocytes even after prolonged culture (1–4 weeks, Fig. 3 D), confirming the earlier findings that PF cultures are free of osteoblasts (Nijweide et al. 1982).

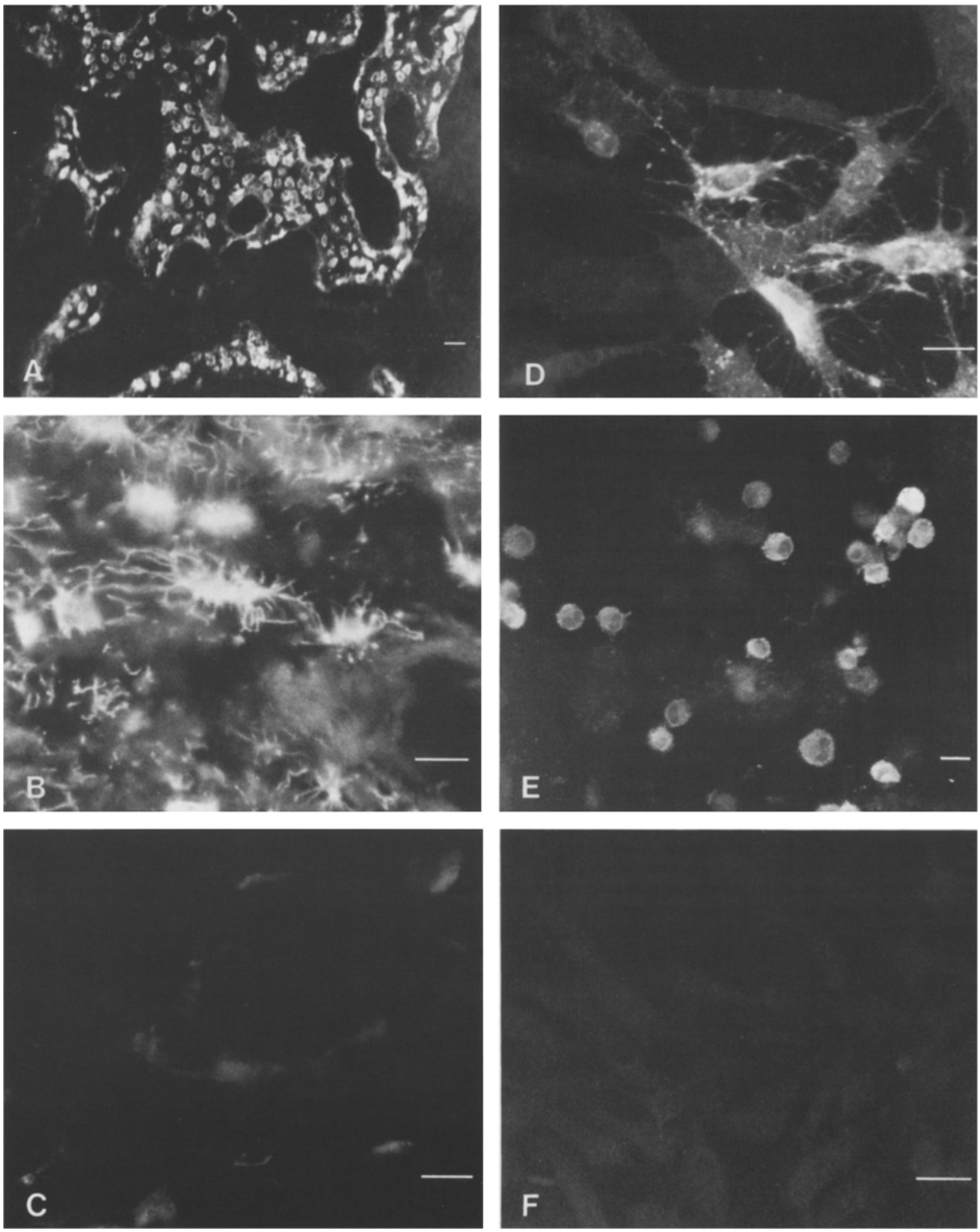

Fig. 1A. Frozen section of trabecular bone of the metaphysis of the first phalange of an 18 day-old chicken embryo stained with monoclonal OB 7.3 followed by rabbit anti-mouse IgG-FITC; only osteocytes are immunoreactive. **B** Higher magnification showing a large number of cell processes. **C** Control section; PBS was used instead of OB 7.3. **D** Three day-old culture of OB cells stained with OB 7.3; stellate cells are positive, other cells present in the culture (osteoblasts) are negative, **E** Cytocentrifuge preparation of a 6-day-old OB culture; a number of OB 7.3 positive cells is present, the majority is negative; note that the intensity of the fluorescence is variable. **F** Three day-old PF culture stained with OB 7.3; all cells are negative. *Bar*: 10 μm

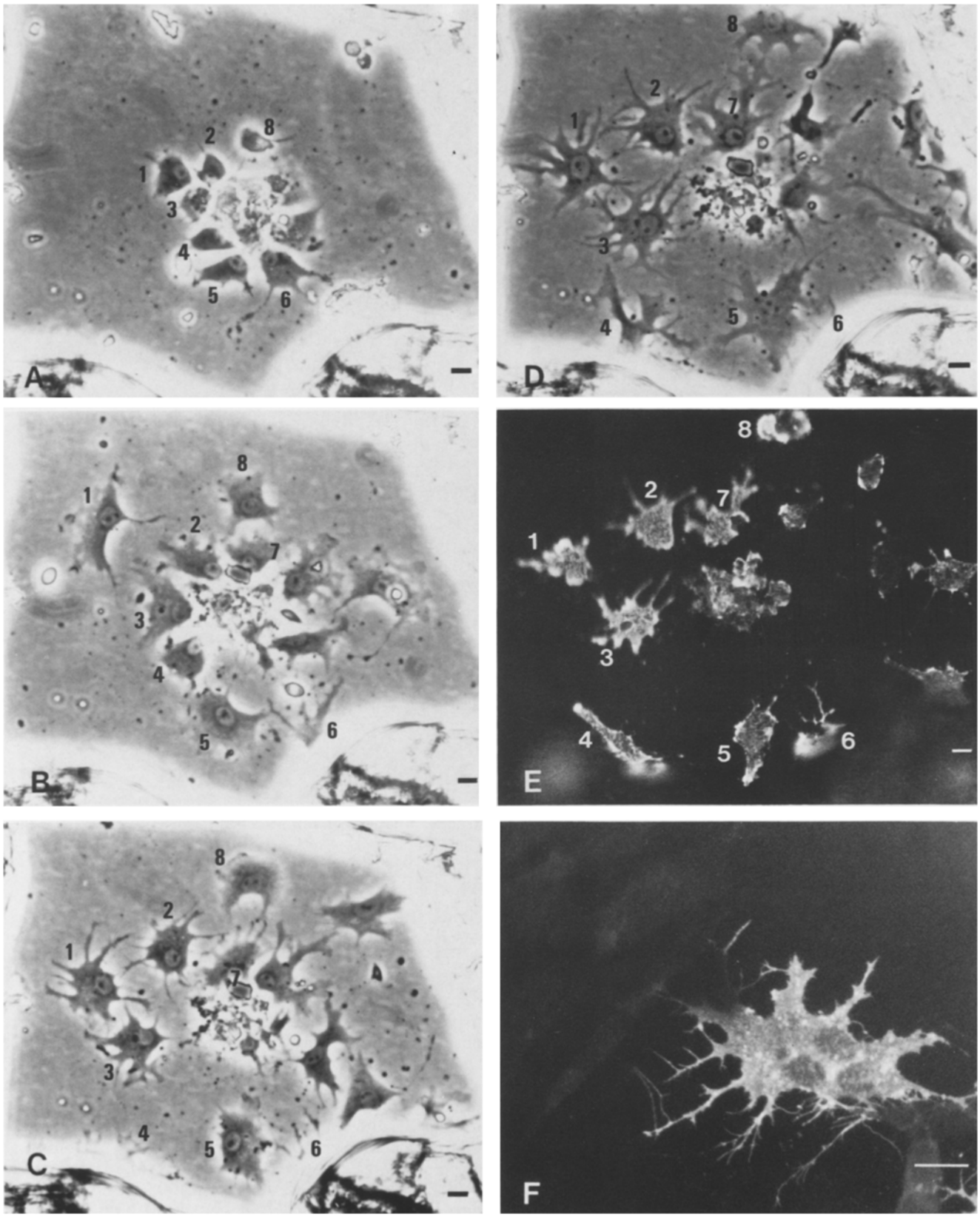

Fig. 2 A–D. Clot of osteocytes in an OB culture insulated by a furrow in the bottom of the Petri dish from other cells present in the culture, observed with phase contrast after 1 (**A**), 2 (**B**), 3 (**C**), and 4 (**D**) days of culture. **E** Same group of cells stained with monoclonal OB 7.3 after 4 days of culture; cells are numbered to facilitate the identification of the individual cells. **F** Osteocyte with at least three nuclei in culture. **Bar**: 10 μm

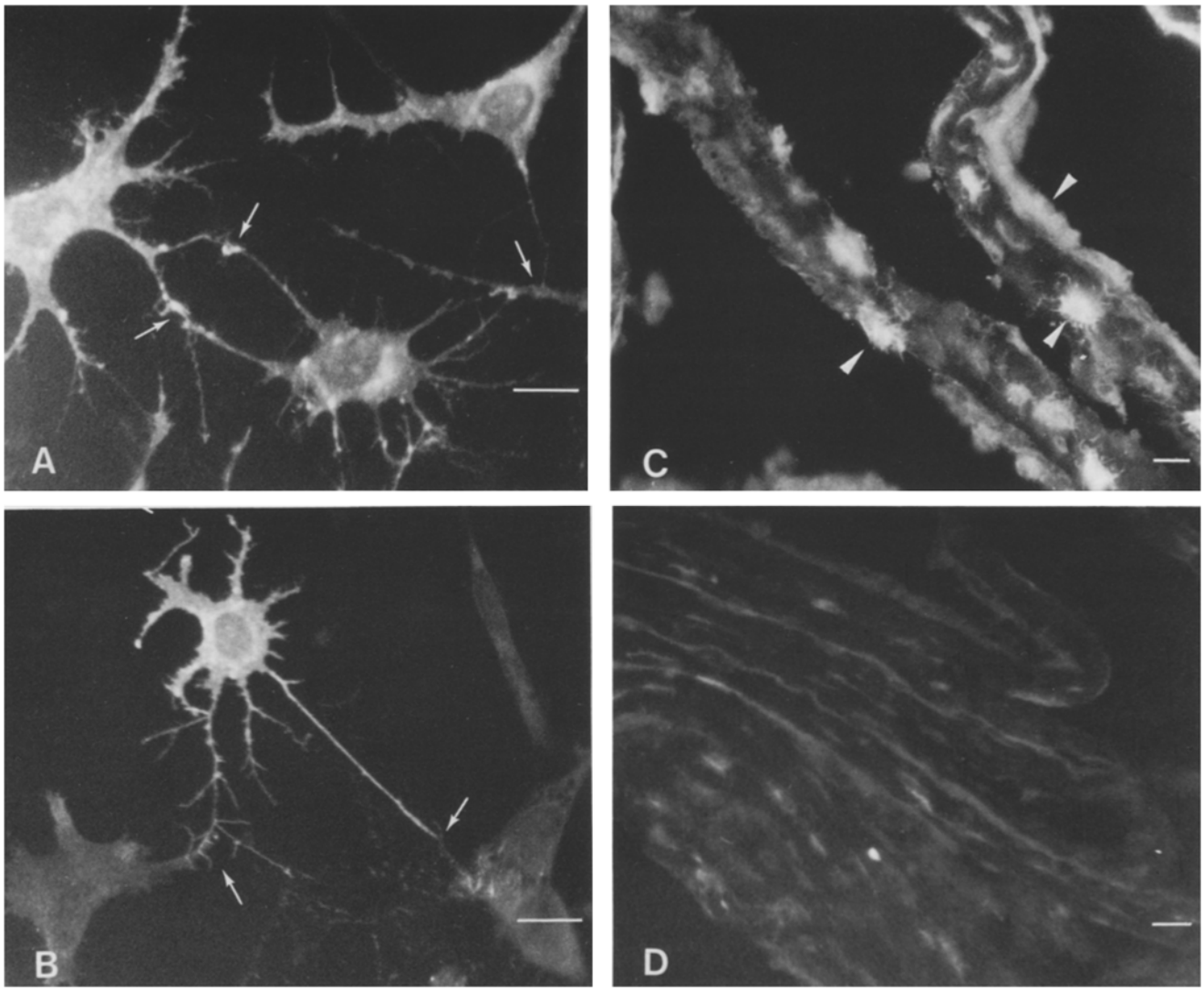

Fig. 3A and B. Three day-old OB culture showing OB 7.3 positive osteocytes attached via cell processes (arrows) to other osteocytes (**A**) or OB 7.3 negative neighbouring cells (**B**). Frozen sections of 3 week-old OB (**C**) and PF (**D**) cultures stained with monoclonal OB 7.3; note the presence of many positive cells in the OB culture (*arrow heads*) while the PF culture is negative. *Bar*: 10 μm

Discussion

The presence of osteocytes or rather cells with an osteocytic morphology in isolated bone cell cultures has been reported before. Miller et al. (1977) described the occurrence of a transient transformation of bone cells during maturation in monolayer culture. A small percentage of the surface area of cultures of bone cells isolated from embryonic rat calvaria transformed into a lacelike network. The stellate cells reverted, however, to the spherical shape within 6 h. The transformation could be stimulated by several hormones (e.g. parathyroid hormone) and by the removal of serum from the medium. Bindermann et al. (1974) observed clusters of osteocyte-like cells developing on top of a layer of periosteal cells in 20-day-old cultures. Later, they extented their observations with a scanning electron microscopical study (Boyde et al. 1976). Finally Rodan and Rodan (1984) have described the transformation of the osteoblast-like ROS 17/2.8 cells into stellate shaped cells in response to the addition of 1.25-$(OH)_2$ vitamin D_3.

In this paper we report on the development of a monoclonal antibody, OB 7.3, specifically reacting with a cell surface marker on the osteocyte. Up to now we have not found any other cell, including the mature osteoblast, expressing the same antigenicity. The presence of this OB 7.3 cell surface marker, in combination with the osteocyte-like morphology, proves beyond doubt that the stellate cells observed in OB cultures are indeed osteocytes. In contrast with earlier observations of others (Binderman et al. 1974; Miller et al. 1977) our results show that osteocytes can be isolated from embryonic calvaria and do not have to be formed by differentiation in long term cultures. Furthermore chicken osteocytes appear to preserve their typical morphology in culture for at least 4 days (Fig. 2). It is possible that also in the chicken system osteoblasts may differentiate into osteocytes. The number of OB 7.3 positive cells in cytocentrifuge preparations and frozen sections of long term chicken OB cultures appeared to increase with culture time (1–4 weeks). A rigorous quantitation proved, however, difficult, in the first place because we had to use frozen sections which makes serial sectioning almost impossible; secondly because in the cytocentrifuge preparations the flu-

orescence of the cells was variable. The absolute prove of osteoblast-osteocyte differentiation in vitro can be supplied when the generation of an osteoblast specific monoclonal antibody has succeeded.

As Binderman et al. (1974) already stated, it is intriguing that osteocytes resume their stellate shape in culture. Immediately after enzymatic isolation, when still in suspension, the OB 7.3 positive cells were of rounded shape (Fig. 1E). Soon after adhering to the bottom of the culture dish, the cells started with the formation of cytoplasmic processes. After a few days groups of osteocytes had formed a network of processes connecting osteocytes with each other and with other neighbouring cells. In the mean time almost no extracellular matrix had been layed down between the cells. In other words the phenotypic expression of the osteocyte did not depend on the presence of extracellular matrix. It will be interesting to investigate in the near future gap junction formation between the cell processes of osteocytes in vitro, the existence of which has been established in vivo (Weinger and Holtrop 1974). If intercellular junctions are formed, osteocytes in culture may represent a beautiful model system to study the role of intercellular communication via gap junctions in general (Sheridan and Atkinson 1985) and the importance of this communication system for the physiology of bone in particular.

Generally, osteocytes are assumed to be postmitotic cells. The assumption is corroborated by this investigation in that we have never observed cell division in cells with a fully expressed osteocytic morphology. On the other hand the experiment of Fig. 2 shows a clot of cell from which in time more and more osteocytes migrate outwards. It is impossible to say whether all OB 7.3 positive cells in Fig. 2E were already present in the cell clot from the beginning or whether some were formed by cell division. Careful microcinematography of OB cell cultures may furnish definite answers in the future. In any case even if osteocytes do divide in culture the physiological importance is doubtful. If occurring, it probably represents a culture induced effect as does the formation of two or three nuclei containing osteocytes (Fig. 2F).

In sum, the generation of an osteocyte specific monoclonal antibody has made it possible to identify osteocytes in mixed populations of isolated cells in culture. In the future it will enable us to isolate osteocytes and investigate morphological and biochemical changes in osteocytes in answer to the addition of hormones and drugs. Isolated osteocytes in culture may offer an excellent in vitro system to study intercellular communication via cytoplasmic processes and cellular junctions.

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Reference

Astaldi GCB, Janssen MC, Lansdorp PM, Willems C, Zeylemaker WP, Oosterhof F (1980) Human endothelial culture supernatant (HECS): a growth factor for hybridomas. J Immunol 125:1411–1414

Binderman I, Duksin D, Harell A, Katzir E, Sachs L (1974) Formation of bone tissue in culture from isolated bone cells. J Cell Biol 61:427–439

Boyde A, Jones SJ, Binderman I, Harell A (1976) Scanning electron microscopy of bone cells in culture. Cell Tissue Res 166:65–70

Burger EH, Boonekamp PM, Nijweide PJ (1985) Osteoblast and osteoclast precursors in primary cultures of calvarial bone cells. Anat Rec (in press)

Fazekas de St. Growth S, Scheidegger D (1980) Production of monoclonal antibodies: strategy and tactics. J Immunol Methods 35:1–21

Köhler G, Milstein G (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497

Luben RA, Wong GL, Cohn DV (1976) Biochemical characterization with parathormone and calcitonin of isolated bone cells: provisional identification of osteoclasts and osteoblasts. Endocrinology 99:526–534

Miller SS, Wolf AM, Arnaud CD (1977) Bone cells in culture: morphological transformation by hormones. Science 192:1340–1343

Nijweide PJ, van der Plas A, Scherft JP (1981) Biochemical and histological studies on various bone cell preparations. Calcif Tissue Int 33:529–540

Nijweide PJ, van Iperen-van Gent AS, Kawilarang-de Haas EWM, van der Plas A, Wassenaar AM (1982) Bone formation and calcification by isolated osteoblastlike cells. J Cell Biol 93:318–323

Nijweide PJ, Vrijheid-Lammers T, Mulder RJP, Blok J (1985) Cell surface antigens on osteoclasts and related cells in the quail studied with monoclonal antibodies. Histochemistry 83:315–324

Peck WA, Birge SJ, Fedak SA (1964) Bone cells: biochemical and biological studies after enzymatic isolation. Science 146:1476–1477

Peck WA, Burks JK, Wilkins J, Rodan SB, Rodan GA (1977) Evidence for preferential effects of parathyroid hormone, calcitonin and adenosine on bone and periosteum. Endocrinology 100:1357–1364

Rifkin BR, Brand JS, Cushing JE, Coleman SJ, Sanavi F (1980) Fine structure of fetal rat calvarium; provisional identification of preosteoclasts. Calcif Tissue Int 31:21–28

Rodan GA, Rodan SB (1984) Expression of the osteoblastic phenotype. In: Peck, WA (ed) Bone and mineral research, vol 2. Elsevier, Amsterdam New York Oxford, pp 244–285

Sheridan JD, Atkinson MM (1985) Physiological role of permeable junctions: some possibilities. Annu Rev Physiol 47:337–353

Smith DM, Johnston CC, Severson AR (1973) Studies on the metabolism of separated bone cells. I. Techniques of separation and identification. Calcif Tissue Res 11:56–69

Tsuru S, Kitani H, Oguchi M, Mashiko M, Zinnaka Y, Shimoura Y (1984) Separation of osteoblast-like cells from bone marrow by fluorescence-activated cell sorting. J Histochem Cytochem 32:43–48

Weinger JM, Holtrop ME (1974) An ultrastructural study of bone cells: the occurence of microtubules, microfilaments and tight junctions. Calcif Tissue Res 14:15–29

Wong GL, Cohn DV (1975) Target cells for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. Proc Natl Acad Sci USA 72:3167–3171

Wong GL, Kocour BAS (1983) Differential serum dependence of cultured osteoclastic and osteoblastic bone cells. Calcif Tissue Int 35:778–782

Yagiela JA, Woodbury DM (1977) Enzymatic isolation of osteoblasts from fetal rat calvaria. Anat Rec 188:287–306