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Experimental Study of Changes in Osteoblastic Shape Induced by Calcitonin and Parathyroid Extract in an Organ Culture System

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Summary. Neonate rat endocranial osteoblasts were cultured on their bone surfaces in control medium (CC) or medium to which either parathyroid extract (PTE) or calcitonin (CT) had been added for 2, 4, 8 or 24 h. Some were cultured for 24 h in CC, then for 2, 4, 8 or 24 h in either CT or PTE medium; or for 24 h in PTE, then for 2, 4, 8 or 24 h in either CC or CT; or 24 h in CT and 2, 4, 8 or 24 h in CC. The dorsal ruffling of the cells in CC was found to be suppressed by later culturing with PTE and the disoriented cells reorganized to form arrays of parallel cells. The effects of PTE were also reversed by CC or CT: the osteoblasts in the second culture (CC) lost elongation and order, and proceeded through a proliferative phase before exhibiting the ruffling form similar to a single CC 24 h culture. PTE-cultured osteoblasts showed an increase in cell overlap and contact so that a more competent barrier was formed separating the bone from the medium. In control or CT medium, however, intercellular gaps were greater than in vivo.

Key words: Bone — Osteoblasts — Cell surface — Cell shape — Calcitonin — Parathyroid extract — Scanning electron microscopy.

Introduction

In a previous report we described the changes that the cell surfaces of osteoblasts undergo when maintained in organ culture and the influence of parathyroid extract upon both the cell surface configuration and the arrangement of the cells on the bone surface (Jones and Boyde, 1976). This work has now been extended to determine the effect of both parathyroid hormone and calcitonin on the cell surface activity of osteoblasts previously cultured in control medium and consequently exhibiting dorsal ruffling, compared with their effect on cells not previously cultured.

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The surface changes shown by the osteoblasts cultured firstly in medium with added hormones and secondly in control medium alone have also been studied.

Material and Methods

A coronal incision was made along the cranial vault of anaesthetized neonate Albino Wistar rats and the two half calvaria were excised and placed immediately in Eagle's modification of Dulbecco's medium to which 10% foetal calf serum had been added. The periosteum and dura mater were removed

Table 1

No. of rats	1/2 calvarium from each rat	Other 1/2 of calvarium
2	2 h CC	2 h PTE
2	4 h CC	4 h PTE
2	8 h CC	8 h PTE
2	24 h CC	24 h PTE
1	2 h CC	2 h CT
1	4 h CC	4 h CT
1	8 h CC	8 h CT
1	24 h CC	24 h CT
1	2 h CT	24 h CT + 2 h CC
1	4 h CT	24 h CT + 4 h CC
1	8 h CT	24 h CT + 8 h CC
1	24 h CT	24 h CT +24 h CC
2	24 h CC + 2 h PTE	24 h CC + 2 h CT
2	24 h CC + 4 h PTE	24 h CC + 4 h CT
2	24 h CC + 8 h PTE	24 h CC + 8 h CT
2	24 h CC +24 h PTE	24 h CC +24 h CT
2	24 h PTE + 2 h CC	24 h PTE + 2 h CT
2	24 h PTE + 4 h CC	24 h PTE + 4 h CT
2	24 h PTE + 8 h CC	24 h PTE + 8 h CT
2	24 h PTE +24 h CC	24 h PTE +24 h CT
1	48 h CC	48 h CC
2	Fixed immediately: no culturing	1 h CC

CC = Control medium: Dulbecco's modification of Eagle's medium with 10% foetal calf serum added
PTE = 0.5 U/ml Parathyroid Extract (Lilley) in control medium
CT = 4 mU/ml Salmon calcitonin (Calbiochem, 2300 MRC units per mg) in control medium
Incubation was at 37° C, each piece of bone being cultured, with its endocranial concave surface uppermost, in 5 mls of medium in a plastic petri dish
In addition, one rat from each litter was sacrificed and the two half calvaria removed, as described above, and fixed immediately in 3% glutaraldehyde in 0.2 M cacodylate buffer

from the ectocranial and endocranial surfaces of the bones, leaving a layer of osteoblasts upon the osteoid. The experimental regimen following was then instituted, the control medium being that mentioned above (Table 1).

In those specimens that had a change of medium the piece of bone was lifted from the first dish and quickly placed in the prepared second medium, excess medium from the first culture period being tipped from the endocranial concavity.

At the completion of the culture periods, the pieces of bone were removed and fixed in 3% glutaraldehyde in 0.2 M cacodylate buffer at 37° C. Specimens were dehydrated through graded alcohols to 100% ethanol, for which Freon 113 was substituted gradually. The specimens were then critical point dried after the substitution of the Freon 113 by liquid carbon dioxide. Conducting coats of carbon and gold were applied by vacuum evaporation on to the endocranial aspect. The osteoblasts were then examined in a Cambridge Stereoscan S4–10 operated at 10 kV.

Observations

Growth Pattern

The endocranial surface of the parietal bone is mainly appositional in the neonate rat as, indeed, is most of the inner aspect of the cranial vault. The major growth site is at the sutural edges of the bone: the bones are carried outwards and apart above a rapidly expanding brain and increase in thickness by deposition of new bone on both surfaces. Remodelling resorption can be seen mainly at the peripheral areas of the bony vault and as small islands elsewhere on the surface.

Control Osteoblasts

The osteoblasts that were fixed immediately upon the removal of the bone were retained as a single sheet of cells upon the osteoid they had formed. They resembled those we have described previously (Jones, 1974; Jones and Boyde, 1976). There was sometimes little cell body overlap, and this presented problems in drying, for the cells tended to part, leaving artefactual spaces. The uppermost surfaces of the osteoblasts were slightly convex, and short, fine cell processes projected from the surfaces (Fig. 1). These may have contacted cells in the adjacent layers or may have been free ending in vivo. Fine microvillous processes also interconnected the cell bodies of neighbouring cells. The cells varied in shape and disposition from those with elongated rectangular or triangular outlines which were often aligned parallel or nearly parallel to the adjacent cells, to those of irregular triangular, quadrilateral or polygonal outline which were approximately equidiametrical and lay in areas in which no packing order could be readily discerned within the cell layer (Fig. 2).

Osteoblasts Cultured in Control Medium

The results obtained were similar to those we obtained in previous experiments which we reported in our earlier paper (Jones and Boyde, 1976). They are recorded here, together with those for culturing in control medium with added PTE (see

below) for completeness as it was necessary to have experimental controls to confirm the status of the cells at the end of the first culture period.

"Dorsal" ruffles were seen in many of the osteoblasts cultured for even the briefest time interval (one hour) used in this study. After brief culturing they were small, simple structures but, with increase in culture time, the dorsal ruffling activity was seen on more of the cell population, the ruffles had become larger and their structure was much more complex (Fig. 3). The cells had also become less elongated, parallel alignment was reduced and the cells became disoriented (Fig. 4). The fine cell processes noticed on the control specimens were no longer present but much longer and larger cell processes were seen. Some cells possessed small, rounded protuberances on their surfaces – these features did not appear to be associated with the surface folds and appeared on cells which also had both ruffles and long cell processes (Fig. 5). Removing the cells after 24 h from the first control culture medium to fresh control culture medium did not change the character of the appearance of the cell surfaces.

Osteoblasts Cultured with Added Parathyroid Extract

The osteoblasts cultured in control medium to which 0.5 U/ml of parathyroid extract had been added were initially similar in appearance to those in control cultures of the same length of time. Small ruffles appeared on the dorsal surfaces of the cells and fine cell processes were replaced by much longer, thicker ones which often contacted neighbouring cells. However, this dorsal ruffling activity did not become more frequent or extensive with increase in the length of culture period. Where dorsal ruffles persisted, they remained simple and short: in many cases such ruffling was suppressed altogether and the upper cell surface appeared to be smooth or to show faint, longitudinal ridging (this may have been due to a slight shrinkage so that subsurface features were evident). Cell processes became few or absent, and mainly confined to the periphery of the cells.

Fig. 1. Short microvillous processes on freed (dorsal) surfaces of control osteoblasts. Processes from the cells contact the cell bodies of adjacent cells. Field width 44 μm

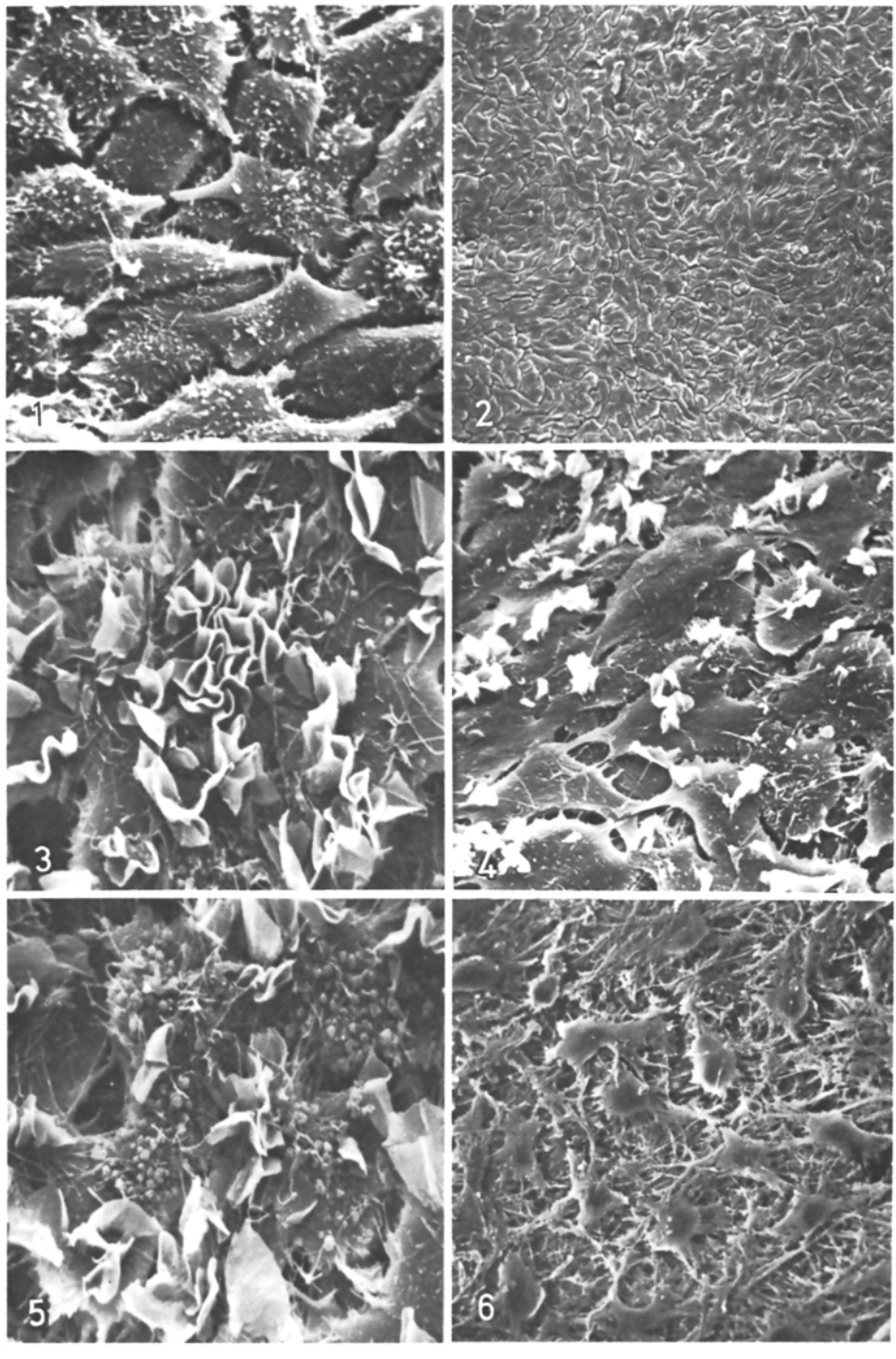
Fig. 2. Mosaic of osteoblasts on endocranial aspect of parietal bone of control rat. Field width 550 μm

Fig. 3. Complex dorsal ruffles and long cell processes on osteoblasts cultured for 24 h in control medium. Field width 19 μm

Fig. 4. The shapes of the osteoblasts are irregular and intercellular spaces have increased. 24 h control culture. Field width 58 μm

Fig. 5. Small round surface blebs were present on some of the ruffling osteoblasts. 24 h control culture. Field width 19 μm

Fig. 6. Fibroblasts on the surface of the fibrous periosteum cultured in control medium for 24 h show few surface features. Field width 220 μm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Furthermore, instead of a loss of cell elongation and alignment, these parameters increased so that by 8 h elongated cells were present in zones made distinctive by the nearly parallel alignment of the long axes of the cells (Fig. 7). Osteoblasts at the peripheries of such parallel arrays or domains were sometimes aligned markedly differently from those of the next array. In other cases, the cell alignment changed gradually and no abrupt demarcation could be observed between domains. By 24 h the pattern of cell arrays that the osteoblasts made was striking (Fig. 8). With the increase in elongation and alignment, peripheral ruffles developed, and the cells spread over one another both at the ends and laterally. The overlapping parts of the cells were often extremely thin and were always adapted closely to the surfaces of the adjacent cell bodies.

Osteoblasts Cultured with Added Calcitonin

Calcitonin added to the culture medium did not alter the configuration of the cell surface compared with that expected for control cultures. The same sequence of production of dorsal ruffling (Fig. 15) increase in ruffling complexity and incidence (Fig. 16) and loss of cell elongation and order was observed. No alteration in the character of the cell surface was observed when cells were maintained for twenty-four hours in medium in which calcitonin had been added (Fig. 17) and then for further periods in control medium (Fig. 18).

Osteoblasts Cultured for 24 Hours in Control Medium, Followed by Various Periods in Medium with Parathyroid Extract

A change was observed in the cell surface of osteoblasts when the second medium contained parathyroid extract, whereas none was noted when the cells were changed from the initial control medium to fresh control medium. The first alteration observed was suppression of ruffling activity on the dorsal surfaces of the osteoblasts.

Fig. 7. Thin lamellae of the elongated osteoblasts overlap adjacent cell bodies. 8 h PTE culture. Field width 16 μm

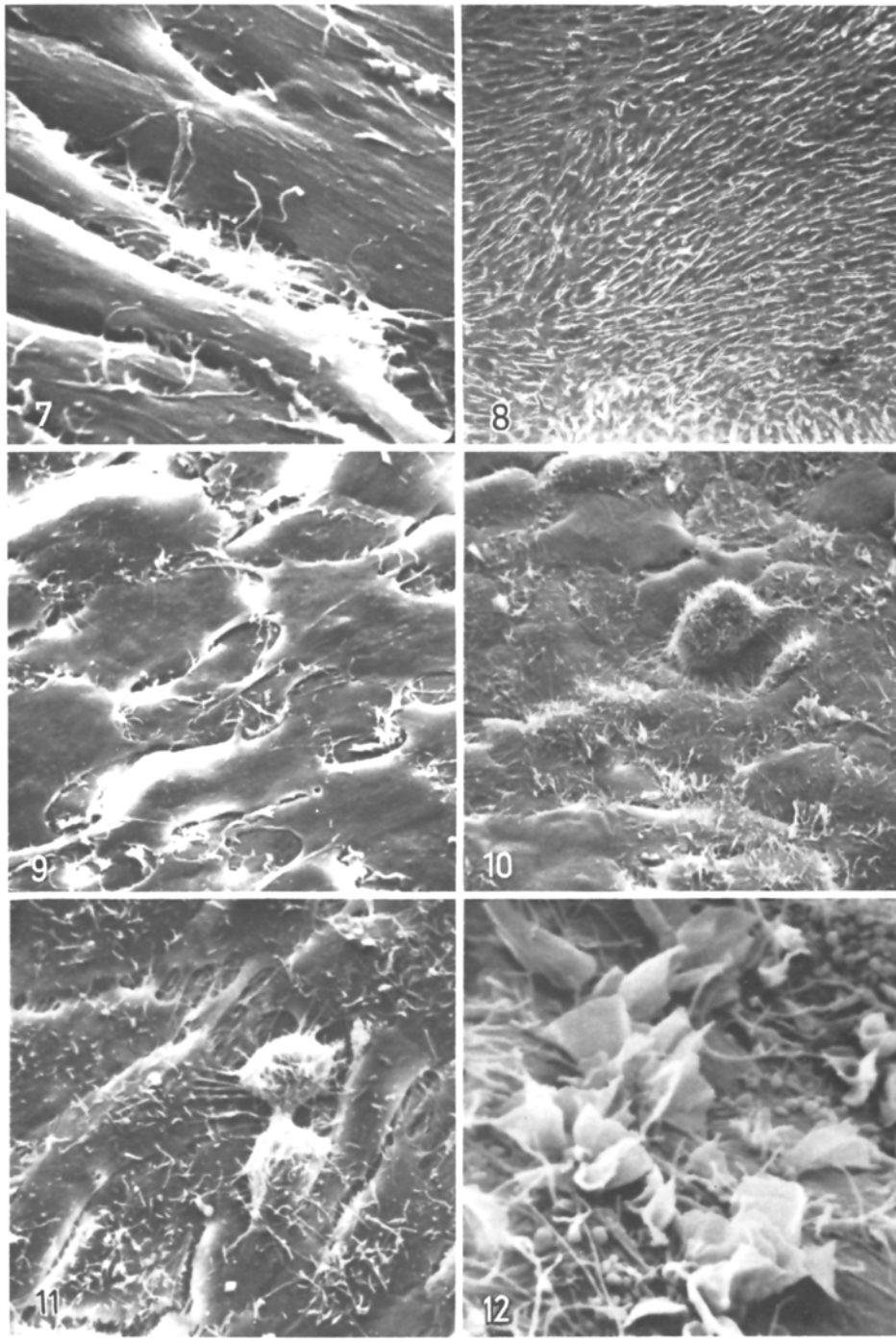
Fig. 8. Arrays of osteoblasts formed after 24 h culturing with PTE. Field width 550 μm

Fig. 9. Dorsal ruffling is no longer present, but the osteoblasts are spread to form an overlapping continuous sheet. 24 h control culture followed by 24 h PTE. Field width 55 μm

Fig. 10. Flattened, spread osteoblasts mingle with rounded "hairy" ones. Some cells have dorsal ruffles. 24 h PTE culture followed by 4 h control culture. Field width 95 μm

Fig. 11. Retraction fibrils surround the cell in telophase. 24 h PTE culture followed by 8 h control culture. Field width 55 μm

Fig. 12. Profuse dorsal ruffles, long cell processes and surface blebs on osteoblasts cultured for 24 h with PTE followed by 24 h in control medium. Field width 18 μm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These ruffles were finally either completely lost, or reduced in incidence and extent to those observed in the osteoblasts which had never been cultured in any medium other than that to which PTE had been added. Peripheral ruffling developed, however, and the cells began to elongate and align. The osteoblasts also showed a marked increase in the degree of overlap of their cell bodies as they spread out (Fig. 9). The extreme degree of cell elongation exhibited by the 24 h PTE cultures without prior control medium culturing was not seen by the end of the longest time these cultures were maintained, but orientation and the formation of cell arrays were obvious.

Osteoblasts Cultured for 24 Hours in Medium and Parathyroid Extract, followed by Various Periods in Control Medium

At the end of 24 h in medium with parathyroid extract added, the osteoblasts were elongated and aligned in domains and had spread with thin overriding and underriding lamellae. Any dorsal ruffles present were small and simple, and mostly peripherally positioned. Cell surface projections of microvillous form were also mostly confined to the peripheral regions of the cells.

After two hours in control medium following the 24 h of PTE treatment, the cells had remained elongated and in domains of aligned cells. Small ruffles were present on some cells, and long, thin extensions of the cell bodies were common. The osteoblasts could be broadly grouped into two morphological types: those that were flattened cells with thin extensions spread over or under adjacent cells and relatively few cell surface features; and those with a rounded upper surface with many long microvillous surface projections.

These two sets of cells, which were intermingled and not in discrete patches, were more evident in the specimens cultured for 4 h in control medium following the 24 h in PTE medium. The "hairy" cells were greater in number, however; their microvillous extensions were more numerous, and surface folds were common

Fig. 13. Spread and "hairy" rounded osteoblasts after culturing for 24 h with PTE and 8 h with CT. There has been a loss of cell order and elongation. Field width 52 μm

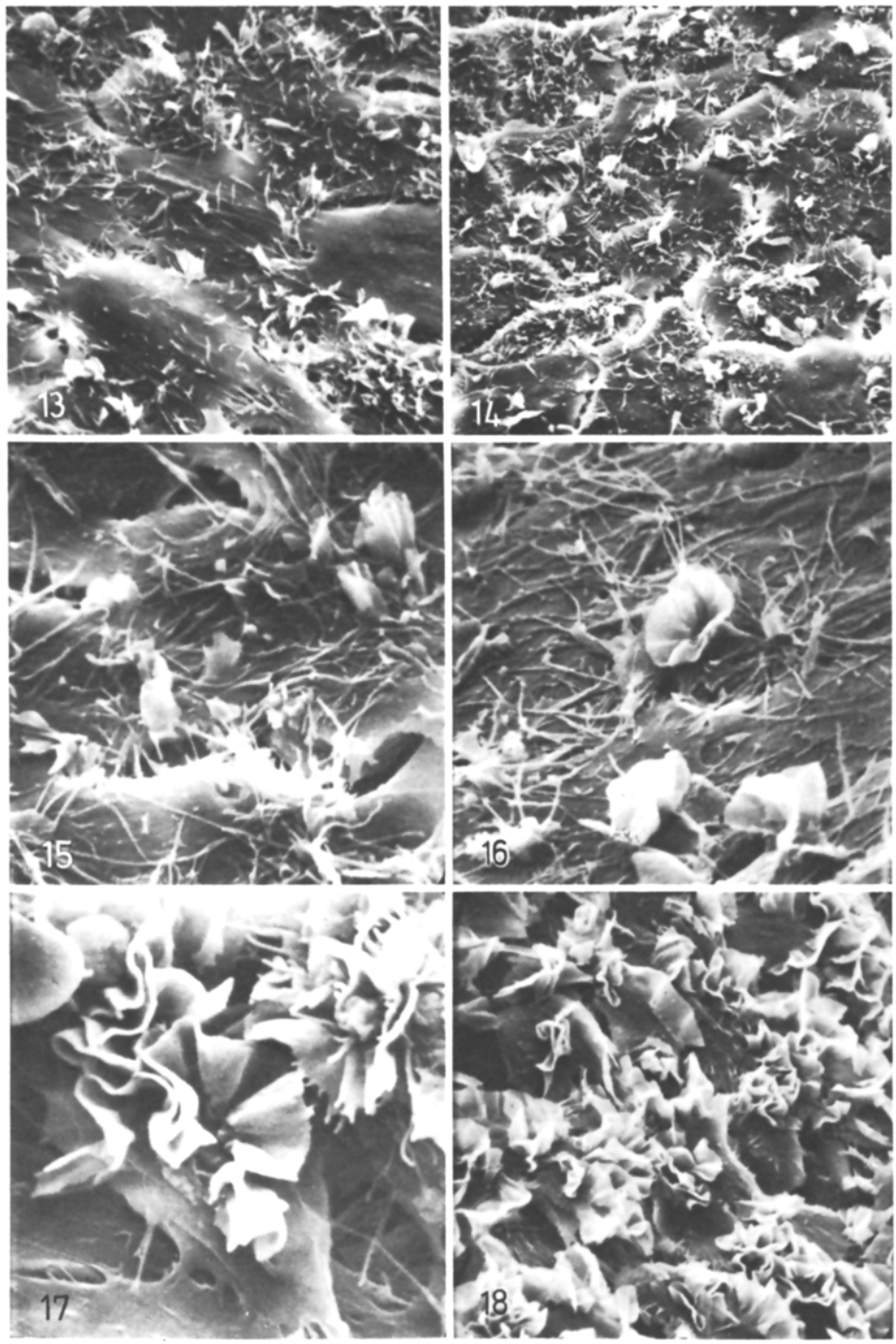
Fig. 14. The ruffling cells are not oriented but bear long cell processes. 24 h PTE culture followed by 24 h CT culture. Field width 92 μm

Fig. 15. Small ruffles on osteoblasts cultured 2 h with CT. Long processes interconnect the cells. Field width 20 μm

Fig. 16. Ruffles are more complex on osteoblasts cultured for 8 h with CT. These cells overlap and have long processes interconnecting the cells. Field width 19 μm

Fig. 17. Complex ruffles on osteoblasts cultured for 24 h with CT. The fimbricated edge of one may indicate a collapsing ruffle, the fingers showing the position of microfilaments. Gaps are present between the cells. Field width 15 μm

Fig. 18. Complex dorsal ruffles persisting after 24 h in CT medium followed by 2 h in control medium. The osteoid surface is visible in gaps between the cells. Field width 48 μm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(Fig. 10). Arrays of more elongated cells were still present, but areas in which the cells were much more equidiametrical and not obviously aligned were seen.

The two morphological cell types of smooth, extended flatter cells, and hairy, rounded cells were a striking feature of the specimens cultured for 8 h in control medium after 24 h in PTE medium. Thin, long extensions of cytoplasm which anchored the rounded "hairy" cells to surrounding cells were frequently noticed and assumed to be retraction fibrils. Numbers of cells were observed to be dumbell-shaped as one would expect of a cell in telophase (Fig. 11) and pairs of small cells were noted, and taken as evidence suggesting that many cells were undergoing, or had just undergone, mitosis.

The osteoblasts cultured for 24 h in control culture medium after 24 h in PTE medium resembled those cultured for 24 h in control medium along (Fig. 12). Cells were not aligned with one another in domains nor were they elongated. Complex and large ruffles were present on the dorsal surfaces of the cells and many cells were very "hairy". Some small blebs were seen on a few cells.

Osteoblasts Cultured for 24 Hours in Medium Plus Parathyroid Extract Followed by Various Periods in Medium with Calcitonin

In general, the alterations in cell surface configuration observed in the cells cultured in control medium after PTE medium were also seen in the osteoblasts of the specimens when CT had been added to the second medium (Fig. 13). The cells became "hairier", developed small dorsal ruffles, lost elongation and alignment, and finally exhibited complex and more profuse ruffles (Fig. 14). Smoother, flatter, more extended cells contrasted with hairier, more rounded cells, especially in the specimens with the 4 and 8 h culture times.

Although some very rounded cells surrounded by "retraction fibrils" were seen in the 8 h CT specimen, no telophase cells were observed at this time, so that evidence for cell division was inconclusive.

Discussion

Cell Division in Osteoblasts

The normal, fully-differentiated active osteoblasts which are producing bone matrix in vivo exist as a single continuous layer of cells with some cell body overlap and do not undergo mitotic division. Additional osteoblasts are gained from the overlying osteoprogenitor cells (Owen, 1963). Resting osteoblasts are flattened, less basophilic, cells that can been seen on bone surfaces that are not presently appositional. It has been suggested that these cells could be either dedifferentiated or quiescent osteoblasts, or undifferentiated osteoprogenitor cells (Pritchard, 1972), but whether formerly productive cells might retain the ability to divide before they become active once again had not been proven.

There could be little doubt as to the origin of the cells on the bone surface in vitro conditions of these experiments, as all overlying cells had been removed prior

to culturing. The fusiform, presumably resting cells seen following culturing with parathyroid extract had undoubtedly been previously productive osteoblasts. Similar cells released from the influence of PTE then proceeded to go through a proliferative phase (Fig. 10). The unusual frequency of obviously mitotic cells at the 8 h recovery period is suggestive of a retention of most of the cells in G1 phase of the cell cycle in the PTE medium and a precipitation into S phase with the introduction of control medium, culminating in mitosis some eight hours later (Fig. 11). The osteoblasts had, therefore, been enabled to regain an osteoprogenitor cell status by the alterations in their culture environments.

Deliberate synchronization of the cells in this manner might have clinical relevance in bone grafting. If the bone tissue for grafting were pre-treated by culturing in PTE medium before insertion, or even by culturing in PTE medium followed by, say, eight hours in control medium, the numbers of presumptive active osteoblasts would be artificially increased. Such culturing might also stimulate the osteoclasts already present on the bone surface to resorb bone, but as the bone would be isolated from its blood supply, the number of osteoclasts would be unlikely to increase significantly in this time. In a previous study of the effect of parathyroid extract in intact animals (Jones and Boyde, 1970), we found that the first increase in new osteoclastic activity, as indicated by Howship's lacunae, was noted at vascular openings, suggesting that blood-borne osteoclasts were responsible for an increase in the resorptive sites and cell numbers before the "resident" population began to increase. Under the culture conditions, only the "resident" osteoclasts would be present and the numbers of osteoblasts present on bone surfaces so far exceeds the osteoclastic population (Owen, 1971) that the pretreatment of cancellous bone might significantly enhance the success of the graft.

No evidence has yet been afforded under our experimental conditions to support the hypothesis that osteoblasts may become osteoclasts in response to parathyroid hormone – nor was evidence seen to lend credence to the idea of an amalgamation of cells to form multinucleate cells. This data suggests that osteoblasts enter a resting phase from which they can progress via a proliferative phase to bone production again once the PTE is removed, and that osteoclastic activity is the preserve of a different population of cells.

Dorsal Surface Ruffling

The osteoblasts reacted rapidly to the control culture conditions (Figs. 3–5, 12–18) and many cells developed ruffles within an hour. Ruffling activity of the cell surface involves the production of large areas of plasmalemma enveloping relatively little cytoplasm. Its significance is not understood. Peripheral ruffling is a feature of the leading edge of moving cells in culture (Abercrombie et al., 1970), but, although the leading lamella may contain both microtubules and microfilaments, only irregular filaments have been noted in the vertical ruffles, the lamellipodia (Abercrombie et al., 1971). Price (1972) also found no parallel filaments in the peripheral ruffles of cultured monkey kidney cells. These ruffles are moving, transitory structures which seem to be due to excess membrane production at the leading edge of the cell and have no inherent mobile capacity themselves. Peripheral ruffles were

present in the osteoblasts that had been cultured with PTE and were associated with a spreading and reordering of the cells.

Dorsal ruffling of cultured cells has been associated with the engulfing of medium (Lewis, 1931; Fawcett, 1965; Vesely and Boyde, 1973) and a heightened synthetic ability. Peripheral ruffles have also been shown to be endocytotic in kidney cells (Price, 1972). We could not tell from the SEM data whether endocytosis was occurring in the ruffling, control-culture osteoblasts because it was impossible to see if the ruffles were associated with vacuoles or not. However, although dorsal ruffling may be the means of producing large areas of surface membrane which could be used either for absorption of nutrients at submicroscopic level, or micropinocytosis or for engulfing medium at a grosser level of organization by pinocytosis, it may not always be dependent upon the level of protein synthesis in a cell (Ambros et al., 1975).

Under the present experimental conditions, dorsal ruffling was found to be a reversible phenomenon controlled by the addition of PTE to, or its subtraction from the medium. The suppression of the ruffling activity of osteoblasts by PTE was, moreover, correlated with the assumption of an elongated, fusiform shape normally indicative of a non-productive or resting osteoblast. Other workers have shown that the uptake of RNA precursor and tritiated thymidine (Owen, 1971) into osteoblasts is depressed by PTH. Moreover, calcitonin does not suppress such cell activity in osteoblasts and was not found to alter the ruffling ability of our cultured osteoblasts. It is apparent, therefore, that there is a correlation between synthetic ability and dorsal ruffling of osteoblasts, if not a dependence.

The suppression of ruffling activity by PTE could be a direct effect upon the cell surface; or secondary to alterations in the functional status of the cell; or due to a primary effect on the osteoid, causing some physicochemical alteration in their substrate; or due to a change in medium calcium or phosphate. It is not possible to distinguish between these alternatives from the present data. Culturing the cells isolated from their bony support would perhaps help to determine the steps involved, but would involve the prior destruction of the integrity of the cell layer.

Endocranial osteoblasts cultured with the overlying periosteum left intact do not show such ruffling activity (unpublished results) but whether their synthetic ability equals that of exposed, ruffling osteoblasts will have to await further investigation. The periosteum is probably acting as a barrier to nutrients in this case. It has been suggested that the intense ruffling activity of cells in culture is a response of the surface membrane of cells normally in contact with others attempting to reestablish that contact in the artificial environment (Revel, 1974). This hypothesis is relevant to the situation of osteoblasts which are normally overlaid by and in contact with osteoprogenitor cells. However, the PTE-treated osteoblasts do not exhibit such profuse dorsal ruffling activity in culture, nor have we found it to be a feature of periosteal fibroblasts of other calvaria when exposed to medium and cultured under identical conditions (Fig. 6). It would be most helpful to know whether the surfaces of the cells which are normally immediately adjacent to the osteoblasts would also show the same degree of ruffling activity when similarly freed and exposed to culture medium. These, the osteoprogenitor cells, have the same degree of contact with the osteoblasts as the osteoblasts do with them, and form nearly as continuous a cell layer. This information would allow us to decide if

there is any special property of the fully-differentiated and productive osteoblast which distinguishes it from relatively quiescent or immature cells of the same cell line in its response to culture conditions, or whether the ruffling is solely a factor of special relationships. We have gained the impression that elongated cells, which we assumed to be resting osteoblasts, seen near or adjacent to osteoblasts, less frequently bore dorsal ruffles: this, too, is indicative of the ruffling being associated with a functional phase.

The effects of both calcitonin and parathyroid extract upon bone in culture are modified by variations in the calcium and phosphate levels in the medium (Messer et al., 1975). The sensitivity of the response to parathyroid hormone is greater than that of calcitonin to such alterations, and as the osteoblasts readily and obviously reacted to the parathyroid extract, it is reasonable to assume that the effect of calcitonin was not affected by the calcium and phosphate levels either. This is important, for judged by the cell surface configuration alone, the effects of culturing the osteoblasts with calcitonin could not be distinguished from the appearance displayed by cells cultured in medium without this added hormone. (The potency of the calcitonin could not be doubted as the osteoclasts responded to the hormone.) The calcium (7.2 mg/100 ml) and phosphate (8.5 mg/100 ml) levels in Dulbecco's modification of Eagle's medium fall into the intermediate range of values where no effect on the bone should be experienced. The Ca^{++} and PO_4^{++} levels in the calf serum were not measured, nor were the hormone concentrations of the serum known.

The possibility also exists that variations in the Ca^{++} concentrating power of the osteoblasts, cultured under the various conditions, might have altered the medium content and thus affected the cells. Further studies are required to monitor this parameter in the cells, and in the medium before and after culturing.

Intercellular Gaps or Contacts

As the cells change shape and alter their spatial relationships, the cell surface area available for intercellular contact changes (Figs. 2, 4, 12, 17). An increase in cell overlap and underlap and spreading associated with PTE treatment lowered the incidence and extent of cell shrinkage such that gaps could not be seen between cells to the same extent and suggested an increase in cell adhesion or cohesion (Fig. 9). The cell surfaces remained close together, particularly where a thin flange of one cell could be seen to overlie another. As Davis et al. (1975) have suggested, the osteoblastic layer cóvering bone surfaces may constitute a cell membrane whose activity is controlled by, amongst other things, parathyroid hormone and calcitonin. These authors noted an increase in the obvious intercellular gaps in rat tibial endosteal osteoblasts from animals treated with calcitonin, although the size of the intercellular gaps shown in their control specimens suggests that their preparation procedure has given rise to excessive shrinkage of the osteoblasts vis á vis the bone matrix. The present results strongly support the concept of the osteoblastic sheet forming a membrane and suggest that the bone matrix compartment is more effectively isolated from the circum-capillary extracellular space as a result of the close contacts and overlapping relationships formed by cells in the osteoblastic sheet.

Incidentally, these results clearly show that there is an influence of parathyroid hormone on bone cells, although, as we discuss elsewhere, it is not clear whether this is a direct influence in the present model system. If PTE has the same effect in vivo, then we may surmise that it causes the osteoblastic sheet to control the egress of calcium and/or phosphate from plasma to bone matrix. It would seem that the rearrangement of the osteoblasts to form the continuous overlapping sheet considered here takes several hours. Thus the normal or calcitonin-induced incompetent state of the osteoblastic sheet membrane allows considerable and rapid egress of plasma calcium into unmineralized bone matrix as a short term effect when PTH is administered to the whole animal (Jones and Boyde, 1970).

Cell Shape and Arrays

We cannot surmise from our SEM data whether the incidence or extent of specialised cell contacts, such as the gap junctions or adhaerens junctions (Stanka, 1975) or spot-like tight junctions (Weinger and Holtrop, 1974) which have been described between osteoblasts in vivo alter under the various culture conditions. The amount and type of intercellular contact may be related to the transfer of information between osteoblasts and be crucial to the development of the patterns formed within the cell layer and its integrity. It is interesting that Cherny, Vasiliev and Gelfand (1975) found the transformed fibroblasts (M 22) that readily spread in culture had parallel contacts and specialized junctions and were electrically coupled whereas transformed mouse fibroblasts (L) that didn't spread but remained in an epithelioid monolayer had none of these features.

The contact between osteoblasts in vivo is limited (Cameron, 1972) and often confined to cell processes contacting adjacent cells. The formation of lamellar cytoplasm in the PTE-treated osteoblasts may indicate a reduction in contact inhibition during movement, or be due to underlapping of cells as they become less adherent to the osteoid. The different shapes of the osteoblast surfaces adjacent to the osteoid may well reflect alterations in the adhesion of the cells to their substrates in the different culture medium, as well as variations in the form of the matrix surfaces.

The patterns that the PTE-treated cells formed are similar in many respects to those of confluent fibroblastic cultures (Figs. 7, 8, 9). Several factors are known to affect the patterns that fibroblasts make in culture (Rovensky et al., 1971; Elsdale, 1973; Stoker and Piggott, 1974).

We have already discussed the likelihood that cell movement is important in the formation of groups of aligned osteoblasts (Jones and Boyde, 1976). The present results support this view, but introduce a new issue. If a more random collagen arrangement has been produced in any new osteoid formed immediately below the osteoblasts of the 24 h control cultures, as our preliminary investigations suggest, then the alignment of the osteoblasts in parallel arrays when PTE has been subsequently introduced would suggest that this ordering is not related to the underlying collagen order, but is solely a cell-cell reshuffling. This suggestion is dependent upon no new parallelly-oriented collagen being produced during the PTE culture period. However, as the suppression of dorsal ruffling and the spreading and re-

ordering of the cells takes time, one cannot assume that collagen synthesis is immediately stopped or even suppressed at the moment of introduction of the hormone. It may be that where parallel fibres of collagen exist as the immediate substrate, elongated osteoblasts will align parallel with them, but that, in the absence of such substrate, patterns of cells aligned in arrays will form more slowly. The degree of ordering and elongation that the cells achieved by the end of 24 h in PTE after the preliminary culturing in control medium was not as great as that found when the first culture stage was omitted. This could have been due solely to the time involved in suppressing the ruffling activity, but the possible role of the different form and nature of the substrate cannot be overlooked. Studies are now in progress to correlate the collagen and cell orders at all the experimental stages in the hope of clarifying this point.

The form of the control and experimental osteoblasts and their order within the cell layer may be related to both the extent and arrangement of their cytoskeletons.

The present results gave an indication of parallel microfibrous subsurface structures in the dorsal view of PTE-treated osteoblasts that was not apparent on control, control culture or CT cultured osteoblasts, suggesting that the density and order of the microfilaments in the osteoblasts alters during culturing in control or hormone-enriched media. Microfilaments are generally present in cells capable of movement (Pegrum and Heaysman, 1973). Bundles of microfilaments aligned parallel to the cell membrane occur in osteoblasts in vivo (Weinger and Holtrop, 1974). Cameron (1972) also observed that they occasionally ran in the same direction as the collagen below the cell. They are more common in the cytoplasm adjacent to the osteoid than elsewhere and extend into the cell processes. This cytoskeletal component is known to be important in the structural order of other cells, such as cultured fibroblasts. Abercrombie et al. (1971) suggested that the loss of microfilaments might result in disorder in cells, and an increase be associated with an increase in order and a development of parallel arrays and contact inhibition. Heavy bundles of microfilaments were also found to be a features of the sole plate of cultured BHK cells (Ravel and Wolken, 1973) and mostly to lie parallel with the long axis of the cells. They were usually absent in transformed BHK cells and the latter authors attributed the loss of shape, normal direction of movement and lack of parallel arrays of these cells to the absence of ordered microfilaments. Ambros et al. (1975) also suggested that a redistribution of actin and myosin-containing filaments might have occurred in their Rous sarcoma virus transformed fibroblasts, concomitant to their losing their normal fibroblastic form and rounding up.

Normal osteoblasts are reported to contain microtubules which appear to lie in no particular order (Weinger and Holtrop, 1974) in vivo. However, it has been reported that microtubules present in normally epitheliod, Chinese Hamster ovary cells which have been treated with dibutyryl cyclic AMP are aligned, and the cells are caused to elongate and form parallel arrays (Porter et al., 1974; Borman et al., 1975). The dbc AMP stabilizes the microtubules of Chinese Hamster ovary cells and may have no similarity in effect to that of PTE on osteoblasts: nevertheless, the end result of both treatments is the production of parallel arrays of elongated cells. It is possible that these dissimilar lines of cells might show cytoskeletal similarities, although both CT and PTH trigger the rise in 3′, 5′ AMP in bone (Hekkelman, Herrman-Erlee, Heersche and Gaillard, 1975). Revel, Hoch and Ho (1974) con-

cluded that, in their cultured cells, although the microfilaments represented a structural element, the microtubules were responsible for structural integrity.

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