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Involvement of Platelets in Stimulating Osteogenic Activity

Michael Slater, John Patava, Karen Kingham, and Rebecca S. Mason

Department of Physiology, University of Sydney, Sydney, Australia

Summary: Osteoblast-like cells have been shown to be sensitive to the proliferative action of a wide variety of growth factors. Many of these growth factors have been isolated from platelets and are thought to be released at local sites in response to injury. In this study, we tested whether human platelet concentrate, as a supplement to basic medium, would support the proliferative and functional activity of human fetal osteoblast-like cells in both short-term and long-term culture. In short-term studies, uptake of [^{3}H]thymidine was increased in platelet-treated cultures by more than 4-fold compared with 10% serum-supplemented controls. When cultured for prolonged periods on coverslips, the cells formed multilayers, with a collagen-based matrix separating the layers. Long-term cultures that were treated with 1.5% (vol/vol) platelets in serum-supplemented medium showed increases in the depth of the multilayers of as much as 36-fold at 30 days after confluence, compared with the 10% serum-supplemented controls; this difference persisted until day 50. Incorporation of growth factor in the matrix was examined with the use of colloidal gold immunoelectron microscopy. Immunogold labeling intensities for transforming growth factor-β1 were significantly lower in the platelet-treated cultures at 20 days and then increased to a maximum level of 2.1-fold more than in the controls at 40 days. Labeling intensities for insulin-like growth factor-I and basic fibroblast growth factor were significantly lower in the platelet-treated cultures than in the controls at all stages of culture. These results indicate that platelet-supplemented medium stimulates proliferation and maintains the differentiated function of human osteoblast-like cells. Platelets may play an important role in early healing of fractures and also may be useful as a cheap autologous source of multiple growth factors to enhance osteoblast proliferation *in vivo* and *in vitro*.

The importance of platelet-derived growth factors (PDGFs) in the repair of soft tissues has been widely documented (11,19,20). There has been relatively little interest in the role of platelets in fracture repair, however, despite their presence in the clot formed at the site of injury. In the healing fracture callus, platelets may act as an exogenous source of growth factors to stimulate the anabolic activity of bone cells (3,11).

Platelets contain a number of substances, some of which are released at wound sites (19,34) (Table 1). A number of these substances have particular relevance to bone formation, including osteocalcin (34), which, until recently, has been thought to be associated exclusively with osteoblasts. Osteoblasts also secrete many of the growth factors in platelets, such as transforming growth factor-β (TGF-β), insulin-like growth factors I and II (IGF-I and IGF-II) and PDGF (7,19,21). The fibroblast growth factors (FGFs) are secreted by osteoblasts but are not present in platelets. These growth factors may have an autocrine or a paracrine mode of action, or both (21). The functional and synergistic relationships between the exogenous and endogenous forms of these growth factors is not clear, however.

On degranulation at the fracture site, platelets release PDGF, IGF-I, IGF-II, TGF-β, and epidermal growth factor (EGF), providing an ideal system for delivery of growth factors to sites of injury (12,26). The reported actions and interactions of these growth factors vary among different cell types, the same cell types under different experimental conditions, and even with the stage of cell maturation (8,17). In relation to bone cells, IGF-I stimulates both proliferation and differentiated function in osteoblasts (4) and has a greater effect on osteogenic cell proliferation in combination with EGF, FGF, or TGF-β, or with all three, than it does in isolation (15,17). PDGF is a mitogen for cells of mesenchymal origin and also may promote the formation of extracellular matrix (20). The addition of PDGF to bone-derived cells in platelet-poor plasma significantly increased proliferation; this increase was greater when TGF-β and EGF also were added (26). The richest source of TGF-β is in platelets and bone. In platelets, it is in the forms of TGF-β1 and TGF-β2 (27,29). In human skeletal tissue, TGF-β may function alone, but more likely it acts in combination with a variety of other factors such as TGF-α or EGF, both of which are carried in platelets but not synthesised by osteoblasts (6). In human bone

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Address correspondence and reprint requests to M. Slater at Department of Physiology F13, University of Sydney, New South Wales 2006, Sydney, Australia.

TABLE 1. *Substances found in platelets*

Dense granules	α granules: Hudson-Goodman et al. (11)	α granules: Martin et al. (19)	α granules: Miyazono and Takaku (20)
Serotonin	Albumin	α-plasmin inhibitor	High molecular weight kininogen
Catecholamines	β-thromboglobulin	Fibrinogen	von Willebrand factor
ATP and ADP	Osteonectin	Proaccelerin	Thrombospondin
Calcium ions	Osteocalcin, platelet activating factor 4, PD-ECGF, EGF	Fibronectin, connective tissue-activating peptide III, TGF-β, IGFs	Phospholipid, C1-esterase inhibitor, hepatocyte growth factor, PDGF

ATP = adenosine triphosphate, ADP = adenosine diphosphate, PD-ECGF = platelet-derived endothelial cell growth factor (in platelet cytoplasm). EGF = epidermal growth factor, TGF-β = transforming growth factor-β, IGF = insulin-like growth factor, and PDGF = platelet-derived growth factor.

cells *in vitro* and *in situ*, as well as in rat calvariae and bovine fetal bone, TGF-β1 increases DNA synthesis, replication, and differentiated functions such as collagen synthesis (7,26).

The healing of a variety of wounds in animals has been enhanced by treatment with EGF, TGF-α, PDGF, and TGF-β (11,19), each of which is found in platelets. In the current study, we tested whether a relatively cheap and potentially autologous source of a combination of growth factors (human platelets) would enhance proliferation of human osteoblast-like cells and maintain or enhance their differentiated functions in long-term culture. If this hypothesis were correct, it would provide some *in vitro* evidence to support the view that platelet degranulation at the site of fracture may be involved in the healing process *in vivo*. Furthermore, previous studies in this laboratory had suggested that agents such as sex steroids, which increase production of growth factors by osteoblast-like cells, also increase incorporation of growth factors into the matrix (32). Therefore, we tested whether the probable increased concentration of exogenous growth factor in the presence of degranulating platelets also would result in increased incorporation of growth factor into the extracellular matrix of the cultures.

MATERIALS AND METHODS

All chemicals, including culture media, were obtained from Sigma Chemical (St. Louis, MO, U.S.A.) unless otherwise stated. [^{3}H]thymidine was obtained from Amersham Australia (Sydney, Australia). Salt solutions and fetal calf serum were obtained from Cytosystems (Sydney, Australia). Ethanol, acetone, sodium dihydrogen orthophosphate dihydrate, and disodium hydrogen orthophosphate dodecahydrate were obtained from Ajax Chemicals (Sydney, Australia). Cacodylate buffer, osmium tetroxide, Spurr's resin. LR-white resin, glutaraldehyde, and Beem capsules were obtained from Alltech (Sydney, Australia). 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) was a generous gift from Dr. D. Kingston (Roche Products, Dee Why, Australia).

Bone cells were cultured from the trabecular ends of long bones from human fetal tissue, estimated to be 17-20 weeks' gestation. (The experimental protocol conformed to the guidelines of the National Health and Medical Research Council of Australia for the use of human fetal tissue and was approved by the Sydney University Medical Ethics Review Committee.) The culture technique has been described previously (31). In brief, the bone was minced and the resulting fragments were washed extensively before plating onto 25 cm^2 flasks (Nunc-Inter Med, Roskilde, Denmark) in BGJ medium containing 10% (vol/vol) fetal calf serum and supplemented with penicillin (30 mg/ml) and streptomycin (40 mg/ml) (Commonwealth Serum Laboratories, Parkville, Australia). After 2-4 weeks of incubation, the cells growing out from the explanted bone chips reached confluence and were subcultured onto 24-well plates (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) for short-term studies and onto 24-well plates on Thermanox coverslips (Nunc, Naperville, IL, U.S.A.) for long-term studies. Cells were plated at a concentration of 100,000 cells per well. Long-term cultures were maintained in BGJ medium without antibiotics and supplemented with 10% (vol/vol) fetal calf serum with or without other additives such as platelets. Following incubation overnight, the medium was aspirated and experimental medium was added. The cells were maintained for the duration of the study, and the medium was changed every second day. During periods of rapid metabolism, as indicated by a yellow (acidic) colour of the medium, the medium was changed daily. This was particularly common in cultures treated with estradiol (32) or platelet concentrate. For the long-term cell cultures, the medium was supplemented with ascorbic acid (30 mg/ml) at every change.

The platelet concentrate was prepared by the New South Wales Red Cross transfusion service (Sydney, Australia) by subjecting whole blood to two centrifugation steps. The first step was at 700 g for 10 minutes, to deposit red and white cells, and the second was at 300 g for 20 minutes, to pellet the platelets. A 450 ml sample of whole blood produced 40-50 ml of platelet concentrate (1). At each change of medium, 5 μl (0.5%), 10 μl (1.0%), or 15 μl (1.5%) of platelet concentrate, as indicated, was added directly to each 1 ml of medium in each well, using polypropylene tips to minimise protein binding. The platelets diffused slowly, largely intact, down onto the multilayers of cells. The presence of relatively large amounts of protein in the medium would tend to minimise growth factor binding to the wells. As the platelets disintegrated slowly, their contents diffused to the multilayer immediately below.

Cells counts were performed, with use of a hemocytometer, on monolayer cultures after dispersion with trypsin, with trypan blue stain used to identify nonviable cells. Viability routinely exceeded 98%. The uptake of thymidine was measured as follows. The medium was aspirated from each culture and replaced with similar medium supplemented with [^{3}H]thymidine (100,000 cpm/ml). The cultures then were incubated for 4 hours, the labeled medium was removed, and the wells were washed three times with 1 ml of ice-cold phosphate buffered saline and then three times (5 minutes each) with ice-cold 10% trichloracetic acid. Next, the monolayer was washed three times with ethanol at room temperature and was

allowed to dry before solubilizing by incubation using 0.5 *M* NaOH with 1% Triton X-100 for 1 hour at 37°C. The solubilized monolayer was added with scintillant to scintillation vials before counting. Parallel wells were subjected to similar conditions and then counted. The uptake of thymidine was expressed per 10,000 cells (23). Alkaline phosphatase and osteocalcin activity as well as uptake of thymidine were measured as previously described (32).

Three sets of long-term cultures were processed for ultrastructural examination as follows. The coverslips were removed from the well, and the ones bearing the cell multilayers were cut into two equal strips and fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, for 1 hour. They then were washed three times (10 minutes each) in 0.1 *M* cacodylate buffer, postfixed in 2% aqueous osmium tetroxide for 2 hours, and washed three times (10 minutes each) in 0.1 *M* cacodylate buffer. Dehydration was carried out in 30, 50, 70, and 90% ethanol (20 minutes each), followed by water-free absolute ethanol (twice, 30 minutes each) and water-free absolute acetone (twice, 30 minutes each). A 1:1 mixture of acetone and Spurr's resin was prepared, and the tissue was transferred to it for 1 hour and then to a 9:1 mixture for 3 hours. The tissue was left in 100% Spurr's resin overnight. At this stage, the coverslips were placed in Beem capsules filled with Spurr's resin. Curing was carried out at 60°C for 18 hours. Electron dense areas in unstained multilayers were examined to determine ratios of calcium to phosphate in control and treated multilayers: electron dispersive spectroscopy was used, as previously described (32), at 120 kV with a Philips EM 12 (Philips Electronics, Eindhoven, the Netherlands) equipped with an EDAX 9900 system.

The depth of the multilayers was measured along the length of each multilayer by dividing the length visually into 10 subdivisions. To standardise the measurements, only one brand of 300-mesh copper grids (Alltech) was used. The interior aspect of each single grid square bar was 63 ± 2.0 μm (information supplied by manufacturer).

The ratio of cellular area to extracellular matrix area was measured by image analysis (TN-8502; Noran Instruments, Middleton, WI, U.S.A.). To determine the ratio of cellular to noncellular material, the plasma membrane of each cell was traced on each micrograph. This was deducted from the total area represented by the micrograph to provide a measurement, in two dimensions, of the cellular area in relation to the collagenous/extracellular matrix areas.

TABLE 2. *Osteoblast-like features of cell cultures*

Marker	Stimulator	Increase
Osteocalcin	10^{-8} *M* $1{,}25(OH)_2D_3$	15.4-fold
Alkaline phosphate	10^{-8} *M* $1{,}25(OH)_2D_3$	2.0-fold
Intracellular cyclic AMP	50 ng/ml parathyroid hormone	2.5-fold

AMP = adenosine monophosphate, and $1{,}25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3.

The coverslips for immunoelectron microscopic study were fixed in 1% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 20 minutes and then washed three times (10 minutes each) in phosphate buffered saline. Dehydration was carried out in a graded series of ethanols. The coverslips then were transferred to a 50:50 mixture of ethanol and LR-white resin for 1 hour and then embedded in 100% LR-white resin. Curing was carried out in gelatin capsules at 50°C overnight.

Staining for bone-relevant antigens by immunoelectron microscopy was carried out as previously described (31,32). In brief, 1 μm sections were stained with a routine toluidine blue stain. As the coverslips were embedded with their longest edges parallel to the sides of the gelatin capsule, the sections were transverse in orientation. Following verification of the presence and position of the bone cell multilayer, the block was trimmed and 120 nm sections were cut and then placed on 300-mesh nickel grids. To minimise nonspecific binding, the sections were floated on drops of 1% bovine serum albumin (radioimmunoassay grade; Sigma Chemical) and 0.1% sodium azide in Dulbecco's phosphate buffered

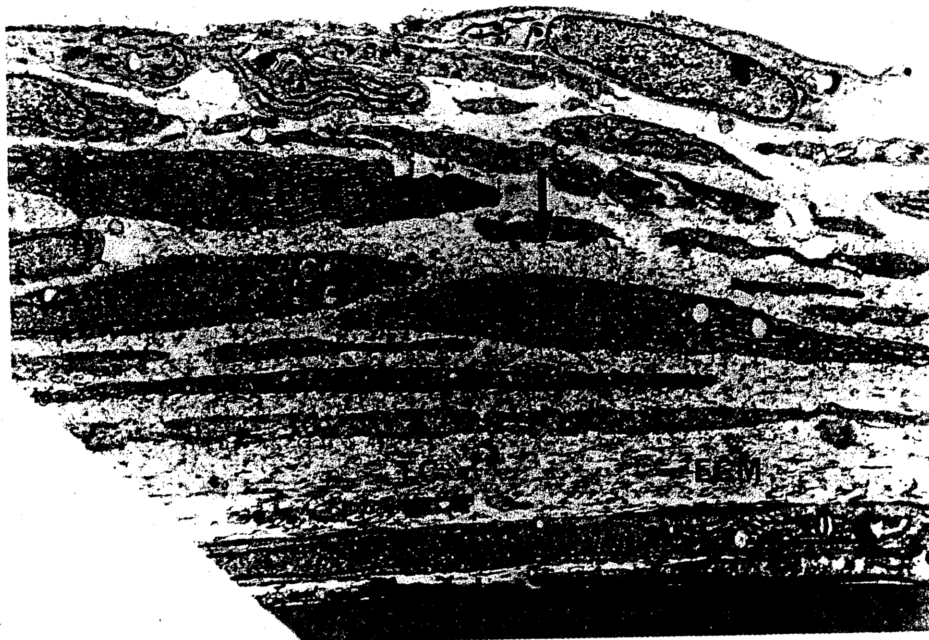

FIG. 1. Electron micrograph (original magnification ×7,000) of human fetal osteoblast-like cell multilayer at 20 days after confluence. T = Thermanox coverslip. TC = collagen fibres transverse to the plane of section, PC = collagen fibres perpendicular to the plane of section, ECM = extracellular matrix, N = cell nucleus, and RER = rough endoplasmic reticulum. (Fixation, 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.3; stain, uranyl acetate and lead.)

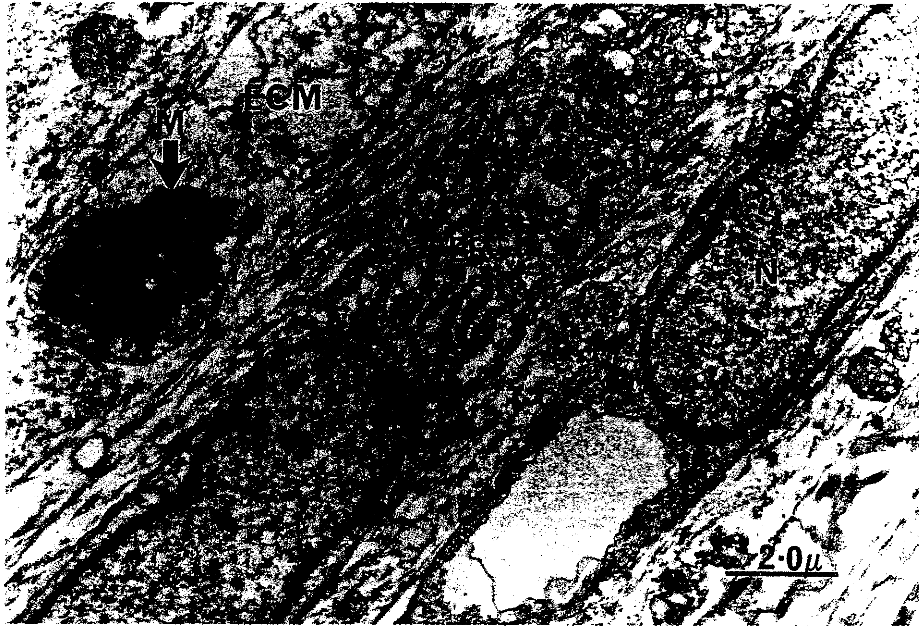

FIG. 2. Transmission electron micrograph (original magnification ×5,500) of osteoblast-like cell multilayer at 30 days. N = nucleus, ER = endoplasmic reticulum, ECM = extracellular matrix, and M = electron dense area. (Fixation, 2.5% glutaraldehyde; stain, uranyl acetate and lead citrate.)

saline for 45 minutes. The grids then were placed on the surface of a drop of the appropriate antibody diluted 1:100 with 1% bovine serum albumin, 1% Tween 20, and 0.1% sodium azide in Dulbecco's phosphate buffered saline for 45 minutes, followed by three 10-minute washes in phosphate buffered saline. For the monoclonal antibodies, each grid then was placed for 45 minutes on a drop of goat anti-mouse IgG and IgM conjugated to 10 nm colloidal gold, which was diluted 1:100 in 0.1% cold fish skin gelatin, 1% bovine serum albumin, 1% Tween 20, and 0.1% sodium azide in phosphate buffered saline. For the polyclonal antibodies, the grids were labeled with 10 nm protein A gold (diluted 1:100 in the same solution) for 45 minutes. Following labeling with the gold probe, the grids were washed again on three successive drops of phosphate buffered saline for 10 minutes each, followed by a rinse in ion-free water.

The monoclonal antibody to TGF-β was from British Biotechnologies (Oxford, England). It has no crossreactivity with acidic or basic FGF (bFGF), human PDGF, human IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, TNF-α, TNF-β, granulocyte-macrophage colony stimulating factor, or granulocyte colony stimulating factor, as determined by enzyme-linked immunosorbent assay. The polyclonal antibody to IGF-I was purchased from Chemicon International (Temecula, CA, U.S.A.) and showed only 8% crossreactivity with IGF-II. The polyclonal antibody to bFGF was purchased from Biomedical Technologies (Stoughton, MA, U.S.A.). It exhibits no crossreactions as determined by Western blot testing. In a separate

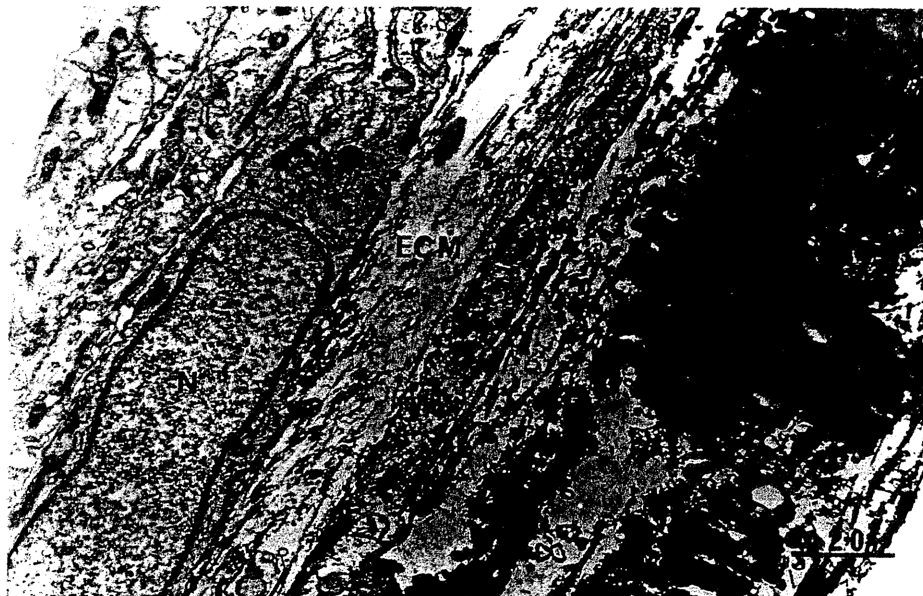

FIG. 3. Electron micrograph (original magnification ×5,500) of a platelet-treated culture at 30 days, showing an electron dense area in the extracellular matrix (ECM). N = nucleus. (Fixation, 2.5% glutaraldehyde; stain, uranyl acetate and lead citrate.)

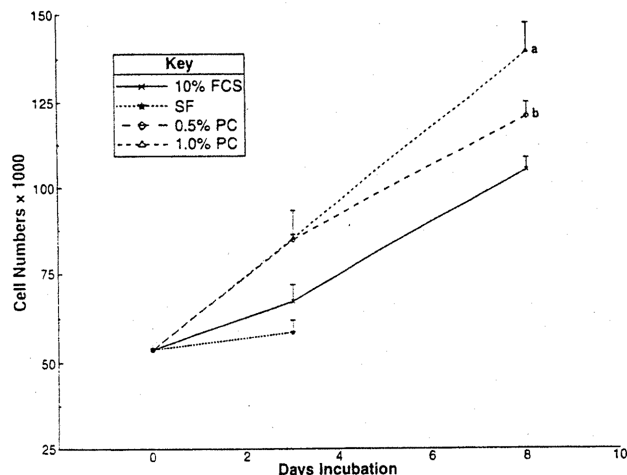

FIG. 4. Number of bone-derived cells after incubation in various media. Cells were plated at 6×10^4 per well and were maintained in BGJ (serum-free [SF] medium), BGJ with 10% fetal calf serum (FCS), or BGJ supplemented with platelets (PC) at a concentration of 0.5 or 1.0%. Each point indicates the mean ± SD for triplicate determinations. The results for the platelet-treated cultures (points a and b) are significantly different from those for the control cultures (cells in BGJ supplemented with 10% fetal calf serum) ($p < 0.005$) and from each other ($p < 0.005$).

set of experiments, the antibody to TGF-β1 used in this study neutralised the biological effect of TGF-β.

As previously described (30,32), positive and negative controls were established. Negative controls included substitution of specific antibody with normal rabbit serum (1:25 dilution), drops of bovine serum albumin-phosphate buffered saline, or omission of the primary antibody. These measures resulted in the presence of only occasional particles of gold across each entire ultrathin section. Any such background labeling was deducted from the gold probe count (per square micrometer). As an additional control, micrographs of the resin adjacent to the cell multilayers also were produced for each labeling parameter. Any labeling of the resin *per se* recorded in these micrographs was quantified and deducted from the data prior to statistical analysis. The sections also were incubated with a mouse monoclonal antibody of the IgM isotype (Silenus Laboratories, Melbourne, Australia), which does not react with any known human protein. IgM aggregates sometimes may cause nonspecific labeling. This procedure resulted in no apparent labeling. Immunoabsorption studies with osteocalcin, TGF-β, bFGF, and IGF-I were carried out as previously described (32). No immunogold labeling resulted.

For quantitation, the electron microscope was calibrated with use of a grating replica of known dimensions. The correct magnification value for each print was determined from these data. The quantitation protocol chosen was the direct particle counting method (30). For each print magnification value, a square corresponding to 1 μm^2 was cut in a piece of white card. Immunogold labeling probe counts per square micrometer (gpm^2) were made using these cards. At least five labeling intensity counts were made for each print. For each labeling parameter, at least 10 different prints were examined. The results were expressed as the mean ± SD. The statistical results were calculated using either Student's *t* test for unpaired samples or a one-way analysis of variance coupled with a Ryan's Q test.

RESULTS

Bone cultures were established from several donors, with similar results seen in each case—the explanted bone chips produced cells within 24 hours after seeding. The cells displayed several osteoblast-like features, including the production of the bone cell markers alkaline phosphatase and osteocalcin (Table 2).

Cells cultured on the coverslips for periods of as long as 50 days formed a relatively ordered structure, consisting of layers of cells with a well developed rough endoplasmic reticulum separated by extracellular matrix. The matrix was collagen-based, with fibres arranged in layers that tended to run alternatively parallel and perpendicular to the plane of the section (Fig. 1). At higher magnifications, the collagen fibres showed a regular banding pattern. The cell multilayer continued to increase in depth for 50 days after confluence (the latest time point examined). The cells did not form nodules where multilayering occurred (28,33); rather, the multilayer was fairly uniform over the whole well.

Electron dispersive spectroscopy was used to visualise electron dense areas in the control cultures at 30 days and in the platelet-treated cultures at 15 days.

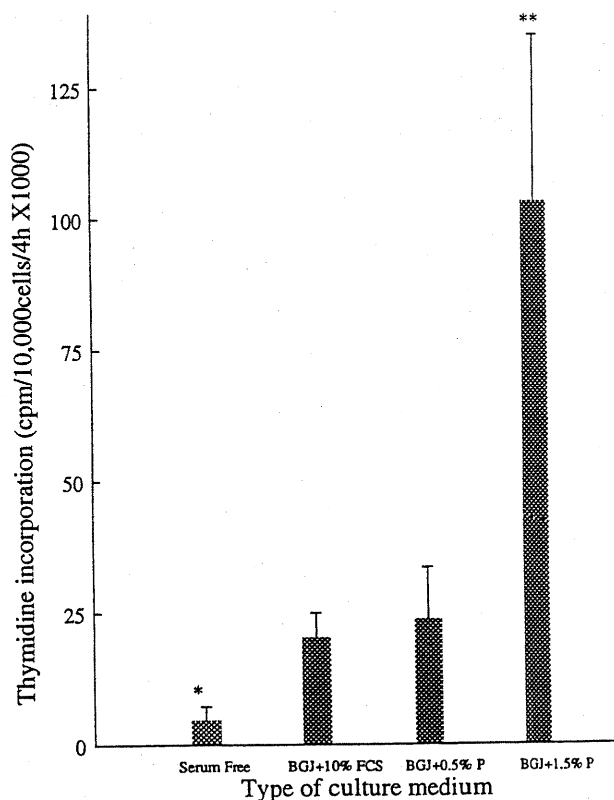

FIG. 5. Effect of platelet supplementation on incorporation of thymidine by bone-derived cells. Cells were plated at 6×10^4 per well in BGJ supplemented with 10% fetal calf serum. After incubation overnight, monolayers were washed with warm phosphate buffered saline before incubation with serum-free BGJ, BGJ with 10% fetal calf serum (FCS), or BGJ supplemented with platelets (P) at a concentration of 0.5 or 1.0%. The cells then were cultured for 6 days with three changes of the medium. The results represent the mean ± SD for triplicate determinations. The asterisks indicate a significant difference from cultures incubated in BGJ with 10% fetal calf serum (*$p < 0.01$ and **$p < 0.0025$).

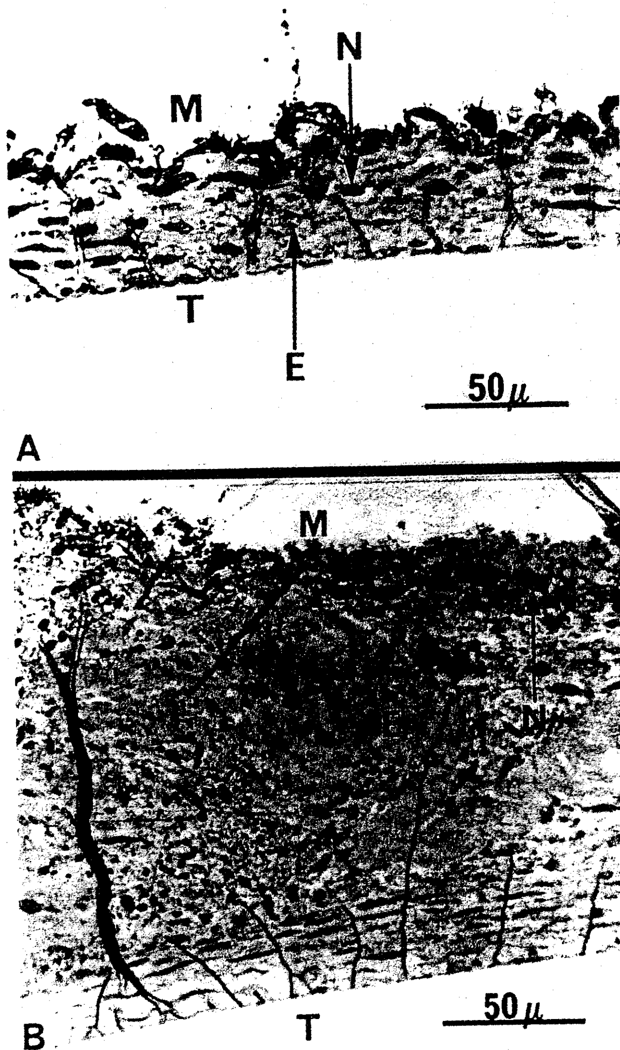

FIG. 6. A: Light micrograph (original magnification ×400) of control cell multilayer at 40 days. **B:** Light micrograph (original magnification ×400) of cell multilayer maintained in culture medium supplemented with 1.5% platelets (15 μl/ml) for 40 days. N = nucleus of osteoblast-like cell, M = medium side of multilayer, T = Thermanox coverslip side of multilayer, and E = extracellular matrix. (Embedding, Spurr's resin; fixation, 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.3; and stain, toluidine blue.)

Energy dispersive spectroscopy studies showed that the ratios of calcium to phosphate for these electron dense areas were 1:1.74 (synthetic hydroxyapatite, control), 1:1.62 (fully calcified fetal bone, control), 1:1.41 (control multilayer), and 1:1.43 (platelet-treated multilayer). This has been interpreted to mean that this material is a poorly crystallised form of hydroxyapatite, similar to that of mineralised fetal bone (10). Scattered areas of electron dense material, more than 2 μm^2, were seen in the control cultures from 30 days (Fig. 2) and in the platelet-treated cultures from 15 days. By 30 days, electron dense material occupied approximately 25% of the multilayer area in the platelet-treated cultures (Fig. 3).

Cell proliferation was affected by platelet supplementation; the number of bone-derived cells in platelet-treated medium was increased significantly compared with that in serum-free medium or medium supplemented with 10% fetal calf serum (Fig. 4). This effect was dose-dependent at 8 days. Short-term culture of the osteoblast-like cells in serum-free medium supplemented with platelet concentrate increased the incorporation of thymidine by the cells (Fig. 5). After 6 days in culture, the cells grown in serum-free BGJ medium incorporated very little thymidine compared with the cells grown in 10% fetal calf serum or 0.5% platelets (Fig. 5). In the cultures that had been treated with 1.5% platelets, there was a 23.5-fold increase in proliferation compared with the serum-free controls (Fig. 5).

The inclusion of platelets in BGJ medium with 10% fetal calf serum resulted in significant increases in the depth of the multilayers ($p < 0.005$) (Figs. 6-8). Because sections at several points along the multilayer showed that the multilayer was fairly uniform over the whole area of the coverslip, measurements of depth may be an index of the total volume of the cell-derived material in the well. In cultures treated with 10 μl (1.0%) and 15 μl (1.5%) of platelets, the cells appeared to be more pleomorphic than in the controls, with round cells predominating (Fig. 6). By 40 days,

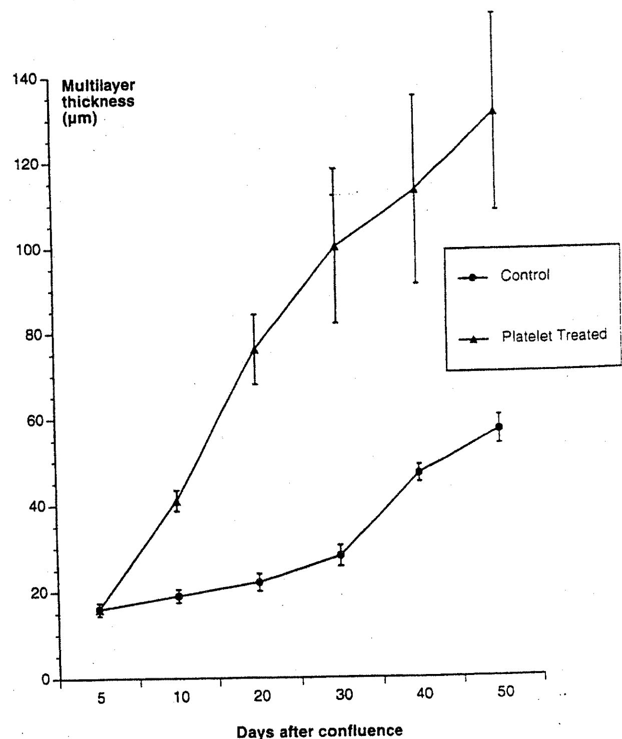

FIG. 7. Time course comparing the depth of the multilayer in cultures of osteoblast-like cells incubated with BGJ supplemented with 10% fetal calf serum (control) or BGJ (with 10% fetal calf serum) supplemented with 1.0% platelets. Each point indicates the mean ± SD for 10 determinations. All of the values for the platelet-treated cultures were significantly different from those for the controls ($p < 0.0005$).

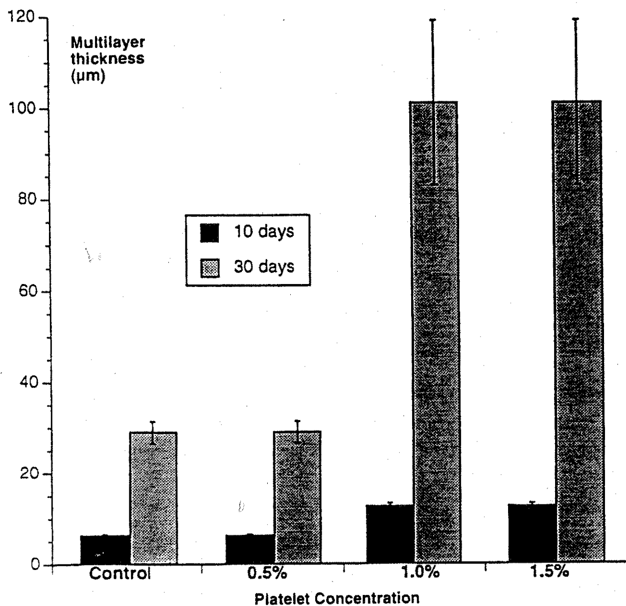

FIG. 8. Effect of platelet supplementation on the depth of the multilayer at 10 or 30 days. Osteoblast-like cells were cultured in BGJ supplemented with 10% fetal calf serum (control) or BGJ (with fetal calf serum) supplemented with 0.5, 1.0, or 1.5% (vol/vol) platelets. The results are expressed as the mean ± SD for 10 determinations. All of the values for the cultures treated with 1.0 or 1.5% platelets were significantly different from those for the controls and the cultures treated with 0.5% platelets ($p < 0.0005$).

about 50% of the medium surface of the platelet-treated multilayers was thrown into folds. At 10 or 30 days after confluence, there was no alteration in the multilayer depth in the cultures in BGJ medium with 10% fetal calf serum and 0.5% platelets compared with the control cultures (supplemented with serum only); the increases in depth were similar in cultures treated with 1.0 or 1.5% platelets compared with the controls (Fig. 8). At 10 days, control cultures supplemented with 10% fetal calf serum produced multilayers with a mean depth of 6.3 μm, similar to cultures supplemented with 10% fetal calf serum and 0.5% platelets, whereas the 1.0 and 1.5% platelet-treated cultures produced multilayers with a mean depth of 12.6 μm, a 2-fold increase (Fig. 8). Long-term cultures treated with 1.0 or 1.5% platelets in serum-supplemented medium also showed a 3-fold increase in the multilayer depth at 30 days compared with controls (Fig. 8); this difference persisted until 50 days after confluence. In cultures treated with 1.0% platelets, the depth increased from 20 to 75 μm ($p < 0.0005$) by day 20 and remained significantly greater than that for controls treated with 10% fetal calf serum until day 50 (Fig. 8).

The ratios of cellular area to extracellular matrix area were similar in the multilayers of the control cultures (1:5.7) and the platelet-treated cultures (1:5.5) at 30 days. These data were only two dimensional, as uniformity of cell shape could not be assumed.

The bone markers alkaline phosphatase and osteocalcin were distributed throughout the extracellular matrix, as determined by immunoelectron microscopy. Immunogold labeling for osteocalcin was increased ($p < 0.0001$) from 22.4 ± 8.4 gpm^2 in the controls to 80.2 ± 13.8 gpm^2 at day 30 in the cultures treated with 10^{-8} *M* 1,25$(OH)_2D_3$; this labeling was lower in platelet-treated cultures at 30 days (10.1 ± 6.2 gpm^2), but the difference disappeared by day 50. Immunogold labeling for alkaline phosphatase was increased ($p < 0.0005$) from 30.2 ± 11.4 gpm^2 in the controls to 70.2 ± 15.8 gpm^2 in the 30-day cultures treated with 10^{-8} *M* 1,25$(OH)_2D_3$, but there was no difference in labeling between the controls and the platelet-treated cultures.

Labeling for growth factors was examined from 20 days after confluence. Although labeling for TGF-β was decreased in platelet-treated cultures compared

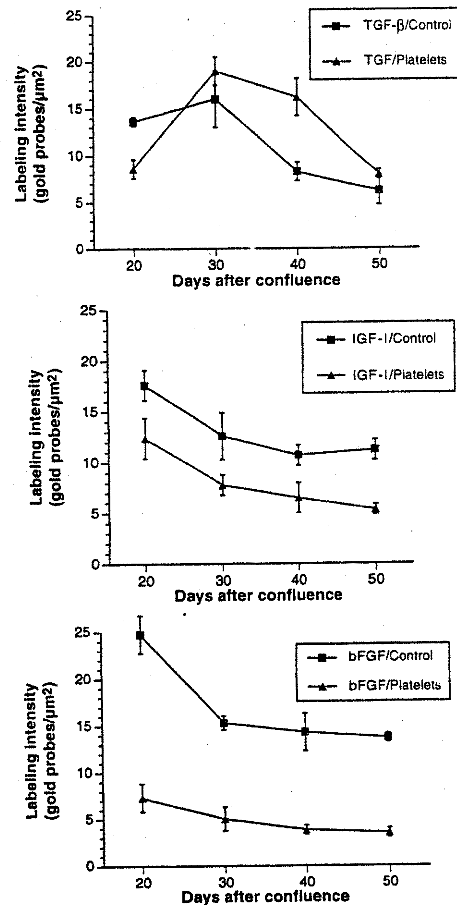

FIG. 9. Intensities of labeling for growth factors in the control and the 1.5% platelet-treated cultures from 20-50 days after confluence. Labeling for insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (bFGF) was significantly lower ($p < 0.0001$) in platelet-treated cultures than in control cultures at each time point. The intensity of labeling for transforming growth factor-β (TGF-β) in the platelet-treated cultures also was significantly lower than for the controls at 20 days, was greater at 30 days, but not significantly so, and was approximately twice the value for the controls at 40 days ($p < 0.0001$); this difference was reduced to insignificant levels by day 50. The error bars indicate the SD of the mean for 70 determinations.

with controls at 20 days, it subsequently increased and was significantly higher in platelet-treated cultures at 40 days (Fig. 9). Conversely, significant reductions in labeling for IGF-I (average, 38%) and bFGF (average, 70%) were observed in platelet-treated cultures relative to controls at all time points (Fig. 9). In cultures treated with 10^{-8} *M* $1{,}25(OH)_2D_3$, the pattern of labeling for TGF-β resembled that in platelet-treated cultures. Labeling was lower than in control cultures at 20 days (6 ± 2 gpm²), similar at 30 days, and significantly increased at 40 and 50 days (11.5 ± 3.1 and 13.2 ± 2.8 gpm²). Labeling for bFGF was decreased at all time points in cultures treated with 10^{-8} *M* $1{,}25(OH)_2D_3$; labeling for IGF-I, however, was increased 3 to 4-fold in these cultures.

DISCUSSION

In the current study, the addition of human platelets to culture medium stimulated the proliferation of human osteoblast-like cells and, in long-term cultures in the presence of fetal calf serum, increased the depth of the multilayer. The similarity between the ratios of cellular area to extracellular matrix area in control and platelet-treated cultures suggests that most of the increased material produced in culture may be due to a platelet-treatment-induced increase in cell numbers with maintenance of their functional activity. At the very least, this suggests that platelet-supplemented medium may therefore be useful as an inexpensive additive or partial alternative to serum, which is currently used in most osteoblast culture systems. This may be particularly relevant if osteoblast-like cells are to be cultured in relatively large numbers for subsequent clinical use. The marked increases in proliferative activity and the continued differentiated activities, including matrix formation and mineral deposition, of osteoblast-like cells in the platelet-supplemented cultures, provide some *in vitro* evidence to support the proposal that the substances released by platelets may play a role in fracture repair and potentially may have clinical applications in fracture healing. The very large biological effects of a small volume of platelet supplement may be a reflection of the interactive and possibly synergistic effects of the multiple platelet contents (16,18).

Using this culture model, we have shown previously that when multilayer depth was increased by treatment with 17β estradiol, an agent also shown to increase local production of growth factors TGF, IGF-I, and IGF-II (among others), incorporation of growth factors into the extracellular matrix also was increased (32). Finkelman et al. (9) reported reduced concentrations of TGF-β in the matrix of oophorectomised rats. It was not clear from the estradiol experiments alone, however, whether the increased incorporation in the matrix in the presence of estradiol was a particular effect of estradiol or a nonspecific result of locally increased concentrations of growth factor. Growth factors incorporated into the matrix may act as skeletal coupling factors (24), so this seemed an important point to test. Studies by other groups have clearly shown that the platelet growth factors TGF, IGF-I, IGF-II, PDGF, and EGF are released upon platelet activation, adhesion, or aggregation (12,14). In the current study, the presence of platelets clearly had marked biological activity in the bone cell culture model. Nevertheless, there was no increase in labeling for IGF-I in the matrix at any time, and the increased incorporation of TGF-β was detected only at later stages (from 40 days) of the culture. This suggests that the probable mere presence of increased concentrations of growth factor is not sufficient to result in their incorporation into the matrix.

The reason for the decrease in the labeling intensities for bFGF and IGF-I in the platelet-treated cultures is not clear. TGF-β has been shown to suppress the production of IGF-I in cultures of MC3T3-E1 cells (35), although it has been reported that TGF-β stimulated the release of IGF-I in murine osteoblast-like cells (11). PDGF is known to oppose the effects of IGF-I in fetal rat calvariae, possibly by suppressing production of IGF (5). In the current study, the increased levels of TGF-β and the PDGF supplied in the platelet supplement may have suppressed the endogenous production of IGF-I and bFGF. The increase in labeling for TGF-β in platelet-supplemented cultures, which was significant at 40 days, may be due to an effect of PDGF to increase synthesis of TGF-β, as PDGF has been shown to stimulate the production of TGF-β from granulation tissue fibroblasts (19).

These studies are consistent with the proposal that exogenous platelet growth factors are potential regulators of osteoblastic cellular proliferation, differentiation, and extracellular matrix synthesis during fracture repair (3). The problem of nonhealing fractures is one of some importance in the population at large. A number of strategies for dealing with this have been devised, and it seems that the use of a combination of approaches is likely to produce the best clinical outcome. In view of the many observations that a variety of growth factors have marked stimulatory effects on bone-forming cell proliferation or anabolic activity *in vitro* (3,15,17,18,21) and bone healing *in vivo* (2,4,13,22,25), growth factors may be useful in stimulating fracture repair when they are applied locally and preferably in combination. Unfortunately, the cost of genetically engineered growth factors is prohibitively high for routine laboratory or clinical use, whereas human platelets represent a relatively cheap and potentially autologous source of a combination of growth factors. The possibility arises that platelets may provide a readily accessible source of

growth factors that could supplement other forms of treatment for impaired fracture repair, including *in vitro* expansion of patients' own preosteoblasts before returning them to the fracture site (36).

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