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INFLUENCE OF CELL ISOLATION, CELL CULTURE DENSITY, AND CELL NUTRITION ON DIFFERENTIATION OF RAT CALVARIAL OSTEOBLAST-LIKE CELLS *IN VITRO*

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Abstract

The effects of various cell isolation procedures, growth media and the cell culture density on the *in vitro* differentiation of neonatal rat calvarial osteoblast-like cells were investigated.

Cells were isolated by enzymatic treatment, or after explant culture and inoculated as a monolayer or micromass in serum containing BGJ_b, or Dulbecco's Modified Eagle Medium (DMEM). The cells were kept for up to 3 weeks in culture and were then characterized, both morphologically and biochemically.

The isolation technique appeared to have no effect on the differentiation process. The calvaria could be used several times as explant cultures for a reliable source of differentiating osteoblast-like cells. The cultures kept in DMEM had a significantly higher DNA content, but significantly less alkaline phosphatase activity (ALP) per DNA and protein per DNA content than the BGJ_b cultures. Monolayer cultures had a significantly higher DNA content than the micromass cultures, in both growth media. Furthermore, the micromass culture had a significantly higher ALP per DNA than monolayer cultures at 1 week. The morphology of all cell cultures at 3 weeks reflected the biochemical results. Only the cells grown in BGJ_b formed abundant ALP positive and mineralized nodules in monolayer cultures. In contrast, cells grown as micromasses formed a dense calcified area, independently of the growth medium used.

DMEM promoted the proliferation, whereas BGJ_b stimulated the differentiation of osteoblast-like cells in monolayer cultures. Micromass cultures were less sensitive to nutritional conditions than monolayer cultures and promoted the differentiation of osteoblast-like cells.

Key Words: Cell isolation, cell differentiation, osteoblast-like cells, cell monolayer, micromass, growth media, cell density, biochemistry, calvaria.

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Introduction

The immediate micro-environment of osteoblast-like cells has a fundamental influence on the differentiation of these cells *in vitro*. It is therefore important to optimize culture conditions in order to recreate an *in vitro* environment as similar as possible to that found *in vivo*.

Explant cultures of endosteal bone fragments have been used successfully to obtain new bone formation since the beginning of the last century (Fell, 1932). The modern version of explant technique is based on the same ability of osteoblast-like cells to migrate off the bone onto a substrate such as nylon meshes, glass fragments or directly onto the culture dish surface. Subsequently the cells are mechanically scraped off or subcultivated by using enzymes (Jones and Boyde, 1977; Ecarot-Charrier *et al.,* 1983; Lomri *et al*., 1988; Masquelier *et al*., 1990). Enzymes such as trypsin (Fitton Jackson and Randall, 1956; Binderman *et al*., 1974), or crude collagenase (Peck *et al.*, 1964; Yagiela and Woodbury, 1977), or sequential collagenase/trypsin treatment (Wong and Cohn, 1974; McCarthy *et al.*, 1988) are also used to isolate osteoblast-like cells. The use of enzymes has some drawbacks. The cells become damaged by contaminating clostripain and other enzymes (Hefley *et al*., 1981). During subcultivation proliferation and differentiation related events are re-initiated in these cells (Owen *et al.,* 1990). The most commonly used growth media to culture osteoblast-like cells isolated from various species, anatomical sites and ages are Dulbecco's Modified Eagle Medium (DMEM) (Ecarot-Charrier *et al.,* 1983; Masquelier *et al.,* 1990; Whitson *et al.*, 1992; Modrowski *et al.,* 1993), BGJ_b Fitton -Jackson's modification (Binderman *et al.*, 1974; Williams *et al.*, 1980; Owen *et al.*, 1990; Aronow *et al*., 1990; Tuncay *et al*., 1994), and Minimal Essential medium (MEM) (Williams *et al*., 1980; Bellows *et al.*, 1986; Gerstenfeld *et al.*, 1987; Zimmermann *et al.*, 1988; Harris *et al.*, 1994). The growth media are usually supplemented with 10-20% fetal calf serum (FCS), and ascorbic acid. To promote mineralization of cultured osteoblast-like cells, additional external phosphate is added in the form of inorganic phosphate (Chung *et al.*, 1992; Bellows *et al.*, 1992; Marsh *et al.*, 1995), and in the form of organic phosphate such as b-glycerophosphate (Fell and Robison, 1934; Nefussi *et al.*, 1985; Zimmermann *et al.*, 1991; Lee *et al.*, 1992; Chung *et al.*, 1992; Bellows *et*

al., 1992). The addition of organic or inorganic phosphates has many drawbacks such as the induction of apoptosis (Adams *et al*., 2001) and cell death (Gronowicz *et al.*, 1989; Aronow *et al.*, 1990), calcium phosphate precipitation (Kaji *et al.*, 1990; Chung *et al.*, 1992; Marsh *et al.*, 1995) and a reduction in collagen synthesis (Tenenbaum *et al.*, 1992).

The cells, kept as a single cell suspension in growth medium, can be inoculated as a monolayer or as a micromass. Micromass cultures are mostly used to study the differentiation of limb mesenchymal cells (Solursh *et al*., 1978; Osdoby and Caplan, 1979; Boskey *et al*., 1992) and periosteal cells (Fang and Hall, 1996).

The number of mineralized nodules is correlated with the density of plated cells in both monolayer (Abe *et al.*, 1993) and micromass cultures of limb mesenchymal cells (Osdoby and Caplan, 1979; Boskey *et al*., 1992). When the cells are inoculated at 2-5x10^4/cm^2 the formation of mineralized nodules starts at 2 to 3 weeks (Owen *et al.*, 1990; Aronow *et al.,* 1990; Whitson *et al*., 1992). Micromass culture at 10^5 cells/100µl promotes the formation of a multilayer and a large mineralized area (Masquelier *et al*., 1990). At a higher density of 2x10^6/10µl fetal rat calvarial cells the produced matrix mineralizes at 7 days, and after 18 days most of the culture consists of mineralized tissue (Zimmermann *et al*., 1988).

As outlined extensively above, osteoblast-like cells are isolated by different methods, inoculated at variable cell densities and kept in various growth media. In the present study, we investigated the effect of various cell isolation procedures, the influence of different cell culture density conditions, and distinct growth media on the *in vitro* differentiation of neonatal rat calvaria osteoblast-like cells. We compared the effect of enzymatic cell isolation versus explant culture procedures. The effect of different cell culture density conditions was investigated by using monolayer versus micromass cultures, with the same amount of inoculated cells per dish. Distinct growth media such as BGJ_b Fitton Jackson modification, DMEM or DMEM without pyruvate were used to study the effect of nutrition on the *in vitro* differentiation of neonatal rat calvaria osteoblast-like cells. At 1, 2, and 3 weeks the alkaline phosphatase activity, DNA and total protein content of the cell cultures were analyzed. At 3 weeks cells were characterized at an ultrastructural and histochemical level, by mineral deposition, and by analyzing the collagen types produced. Furthermore, the qualitative differentiation potential of osteoblast-like cells was analyzed by using the same calvariae explant cultures as a source of cells, over a period of 18 weeks. At intervals of 3 weeks the migrated cells, along with the calvariae, were treated enzymatically to harvest the cells. The same calvariae were then used for further explant cultures. The isolated cells were inoculated as monolayer and micromass cultures. At 3 weeks, the cells were stained histochemically to detect the presence of the ALP activity and calcification was assessed by von Kossa staining.

Materials and Methods

Materials

All tissue culture disposable materials were purchased from Falcon (Becton Dickinson AG, Basel, Switzerland). All growth media and fetal calf serum were purchased from Gibco (Life Technologies, Basel, Switzerland). All chemicals were purchased from Fluka (Buchs, Switzerland), when not otherwise stated.

Methods

Enzymatic cell isolation: Parietal and frontal calvariae (4 per animal) were explanted aseptically from 6 day old IcoIbm rats. The calvariae were placed in Tyrode's buffered salt solution calcium- and magnesium-free (TBSS). The periosteum and endosteum were removed enzymatically by treatment in 0.05 % trypsin (1:250; Sigma, Buchs, Switzerland) and 0.02 % collagenase A (0.76U/mg; Roche Diagnostics, Rotkreuz, Switzerland) dissolved in TBSS (40 calvaria/20 ml). The calvariae were shaken for 70 minutes in a water bath at 37°C. They were washed with TBSS and then transferred to 60 mm culture dishes (40 calvariae/dish) containing 5 ml of 0.02 % collagenase A (0.76 U/mg) in culture medium BGJ_b Fitton Jackson modification or in DMEM or in DMEM without pyruvate (DMEM w/o) and placed in the incubator for 3 hours. After the second collagenase treatment the dishes containing the calvaria and cells were rinsed with serum containing media (BGJ_b, DMEM, DMEM w/o). The cells obtained were filtered through a 40 µm nylon mesh to remove bone debris and cell aggregates. The suspended cells were centrifuged at 600 g for 5 minutes. The cell pellet was resuspended in serum containing medium (BGJ_b, DMEM, DMEM w/o) and centrifuged. The viability of the resuspended cells was examined by the 'dye exclusion' of 0.4 % trypan blue and the vital cells counted using a haemocytometer. The inoculation densities were $2 \cdot 10^5$ cells/10 cm^2 for monolayer and $2 \cdot 10^5$/30 µl/10 cm^2 for micromasses. The micromass cultures were kept for 30 minutes in the incubator before 2 ml growth medium was added.

Explant cultures: The procedure follows the one described under the previous section. After the second digestion step, the remaining calvariae were washed with culture medium (BGJ_b, DMEM, DMEM w/o), supplemented with 10 % fetal calf serum (FCS). The calvariae were transferred into 60 mm culture dishes (4 frontal and 4 parietal/60 mm dish). The growth medium, supplemented with 10% FCS and 50µg/ml ascorbate, was changed completely every 48 h. The explant cultures were kept for 3 weeks.

After 3 weeks the migrated cells, along with the calvariae, were treated with enzymes to harvest the cells. The dish was washed with TBSS and 5 ml of TBSS containing 0.05 % trypsin and 0.02 % collagenase A (0.76 U/mg) was added. After 1 hour in the incubator the dish was washed with culture medium (BGJ_b, DMEM or DMEM w/o) supplemented with 10 % FCS. The cell suspension was treated as described under *'enzymatic cell isolation'*. For the repeated use of calvariae, the remaining calvariae were transferred into a fresh 60 mm culture dish and kept for another 3 weeks as explant cultures in

BGJ_b supplemented with 10% FCS and ascorbate. After 3 weeks the migrated cells along with the calvaria were treated as described above. This cycle of explant culture and subsequent passage was repeated 5 times. The cells isolated after the first 3 weeks of explant cultures were named 'transfer 1 cells', and then after the second explant culture 'transfer 2 cells', etc. up to 'transfer 6 cells'. In total, the calvariae from the same experiments were kept in culture over a time period of 18 weeks. At intervals of 3 weeks the migrated cells, along with the calvariae, were passaged and the cells inoculated as described above.

Cell culture conditions: All the cultures were kept at 37°C in a humidified atmosphere of 5 % CO_2, 95 % air. All culture media (BGJ_b, DMEM and DMEM w/o) were supplemented with 50 µg/ml ascorbate. To increase plating efficiency, during cell isolation and inoculation, no ascorbate was used (Burks and Peck, 1979). No antibiotics, no antimycotics and no β-glycerophosphate were added. The media were changed completely every 48 hours (60 mm culture dish: 5 ml.; 35 mm culture dish: 2 ml).

Light microscopy: After 3 weeks the cultures were stained histochemically for the alkaline phosphatase, using the Sigma Kit no. 85L. As a result, an insoluble, visible blue pigment is formed at sites of phosphatase activity. The deposition of calcium phosphate, at 3 weeks, was determined by the von Kossa stain.

Transmission electron microscopy (TEM): At 3 weeks the cultures were fixed with 2.5% glutataraldehyde in 0.1 M cacodylate buffer pH 7.4 for 20 min, postfixation in 1% OsO_4 in 0.1 M cacodylate buffer pH 7.4 for 1h at 4°C, then 2 % aqueous uranyl acetate for 1h at room temperature; dehydration in an ethanol series and embedding in LR White (London Resin Co, Basingstoke, U.K.). Ultrathin sections were cut with a Drukker Diamond knife (Drukker International, Cuijk, The Netherlands) on a LKB III Microtome (LKB, Bromma, Sweden) and stained with 2% uranyl acetate and lead citrate (Reynolds, 1963). Osmium rich regions, i.e., the nodules in the monolayer, and the centers of the micromass cultures, were selected respectively. The sections were examined using a JEOL (Tokyo, Japan) JEM 100CX transmission electron microscope operated at 100kV.

Biochemistry: Whole cultures were washed at 1, 2 and 3 weeks with phosphate buffered saline (PBS) and 500 µl 0.25 M sucrose/35 mm dish was added. The cells and the matrix were scrubbed off the dish and transferred into a cryotube and frozen at -80°C. Immediately before the assays, the cells were thawed and sonicated (3 x 20s) at 70 W and 20 kHz on ice. The samples were centrifuged at 600 g for 7 min. to remove cell debris and the volume of the supernatant was determined. The samples were analyzed in a Perkin Elmer UV/VIS Spectrophotometer Lambda 12 (PerkinElmer Instruments, Shelton, CT), except for the DNA determination, which was obtained using a Hoefer TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Alkaline phosphatase activity (ALP): We used Sigma Kit no. 245 (Sigma, Buchs, Switzerland) for the quantitative, kinetic determination of alkaline phosphatase activity.

Total protein: The Bio-Rad protein assay kit II (BioRad, Glattbrugg, Switzerland) was used with bovine serum albumin as standards.

DNA: A simple and rapid assay for quantitative DNA determination in crude homogenates was used (Labarca and Paigen, 1980). Each sample was diluted with 3 ml of phosphate buffered saline, pH 7.4 containing 2M NaCl (DPBS) and sonicated as described above. Then aliquots were mixed with 0.1 µg/ml Hoechst 33258 (Aventis, Strasbourg, France) in DPBS and the fluorescence was read in a Hoefer TKO 100 Mini-Fluorometer.

Collagen: During the culture period, the growth medium was supplemented with 60 mg/ml β-aminopropionitrile to prevent cross linking of the collagen fibrils. Whole cell layers were harvested and processed as previously described for chondrocyte cultures (Bruckner *et al.*, 1989). After 3 weeks in culture whole culture dishes were frozen (calvaria and the outgrown cell were separated), thawed, homogenized and treated with 25 ml of 0.5 M acetic acid, containing 0.2 M NaCl and 1 mg/ml of pepsin and stirred for 48 h at 4°C. To inactivate pepsin, 1/10 vol of 1 M unbuffered Tris was added and the samples were neutralized by the addition of 10 M NaOH, adjusted to 1 M in Na^+-ion concentration by the addition of solid NaCl, and extracted for 48 h at 4°C. The undigested residue was centrifuged at 27,000 g. The collagens were precipitated with 176 mg/ml $(NH_4)_2SO_4$. The pellet was dissolved in 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.4. Aliquots were precipitated with 3 vol ethanol, and dissolved in 0.1 M Tris HCl, 0.8 M urea, 2% SDS, 10% glycerine, pH 6.8. A first aliquot remained unreduced, a second aliquot was reduced with 2 % β-mercapto-ethanol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970), using a 4.5 % stacking gel and a 4.5 % - 15 % gradient running gel. Collagen standards were run in parallel with the samples. The electrophoresis was performed at 220 V, followed by staining with Coomassie blue.

Statistics: To compare the effect of the various growth media, contrast analyses of variance models were evaluated. Main effects and interaction effects were examined by F-Tests. 'Least Squares Means' (LS) were calculated to yield average means accounting for the other variables in the model. LS Means were compared by using Tukey's multiple range test. QQ-Plots of the residuals and Tukey-Anscombe plots (residuals x predicted) were analyzed to check for normal distribution assumption.

Results

Enzymatic cell isolation and explant cultures

The mean yield by enzymatic isolation was $4x10^4$ cells/calvaria when the collagenase was dissolved in BGJ_b, and $6x10^4$ cells/calvaria in DMEM or DMEM w/o, but these differences were non significant (data not shown). If the cells were isolated subsequently, after explant cultures, the mean yield was around $3x10^5$ cells/calvaria in all the transfer groups of the different growth media (data not shown).

During the subsequent cell culture period, the enzymatic isolated cells and the cells isolated after explant culture behaved in a similar way. The cells grown

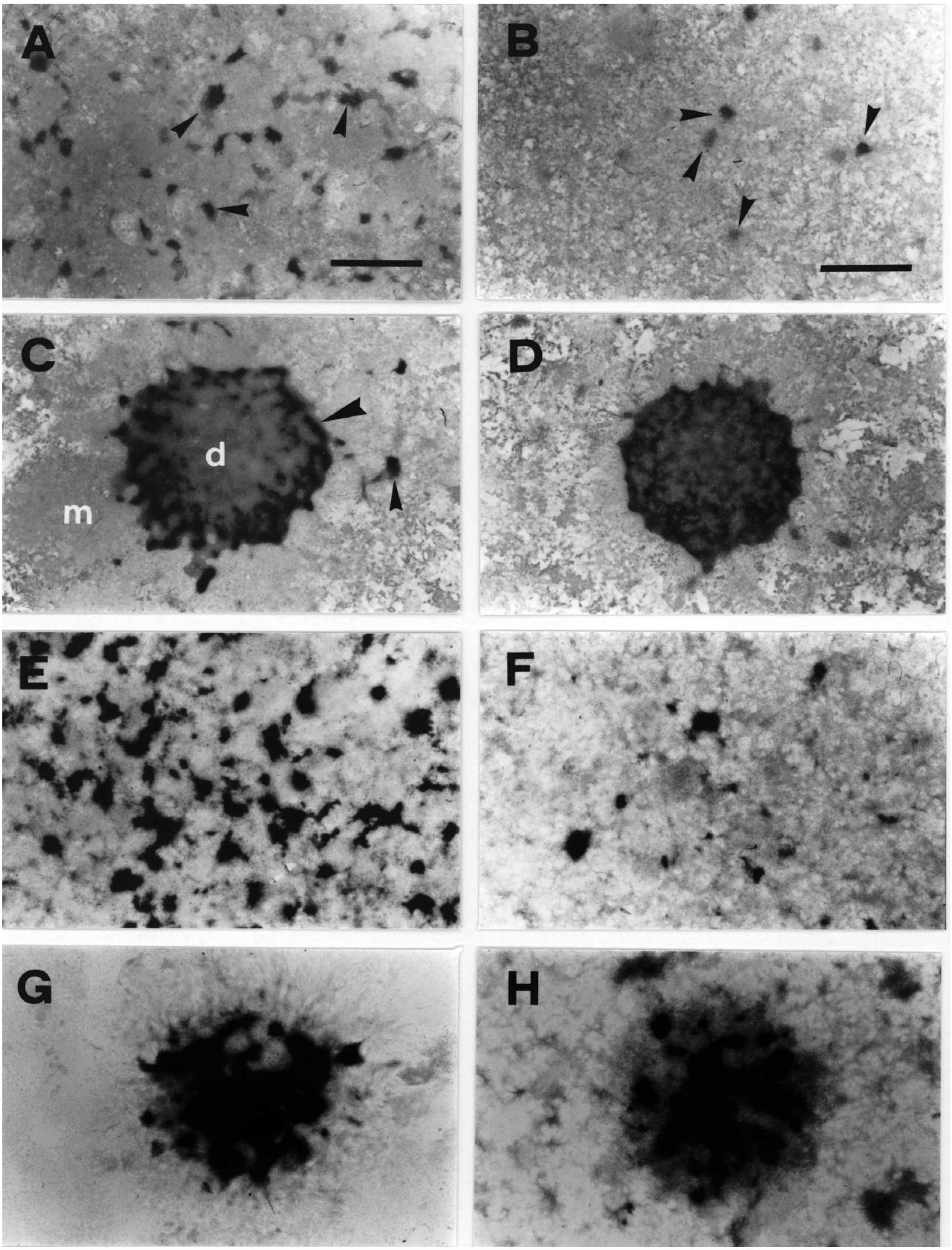

Figure 1. Cell cultures at 3 weeks. Same magnification in all images; scale bar 3mm. (**A**) Monolayer of transfer 1 in BGJ_b stained for ALP activity. Nodule formation (arrow head). (**B**) Monolayer of transfer 1 in DMEM stained for ALP activity. Nodule formation (arrow head), (**C**) Micromass of transfer 1 in BGJ_b stained for ALP activity. Originally inoculated drop of cells (d) and its margin (large arrow head); migrated cells (m), nodule formation (small arrow head), (**D**) Micromass of transfer 1 in DMEM stained for ALP activity, (**E**) Monolayer of transfer 1 in BGJ_b; von Kossa stained mineralization (black areas), (**F**) Monolayer of transfer 1 in DMEM; von Kossa stained mineralization (black areas), (**G**) Micromass of transfer 1 in BGJ_b; von Kossa stained mineralization (black areas), (**H**) Micromass of transfer 1 in DMEM; von Kossa stained mineralization (black areas).

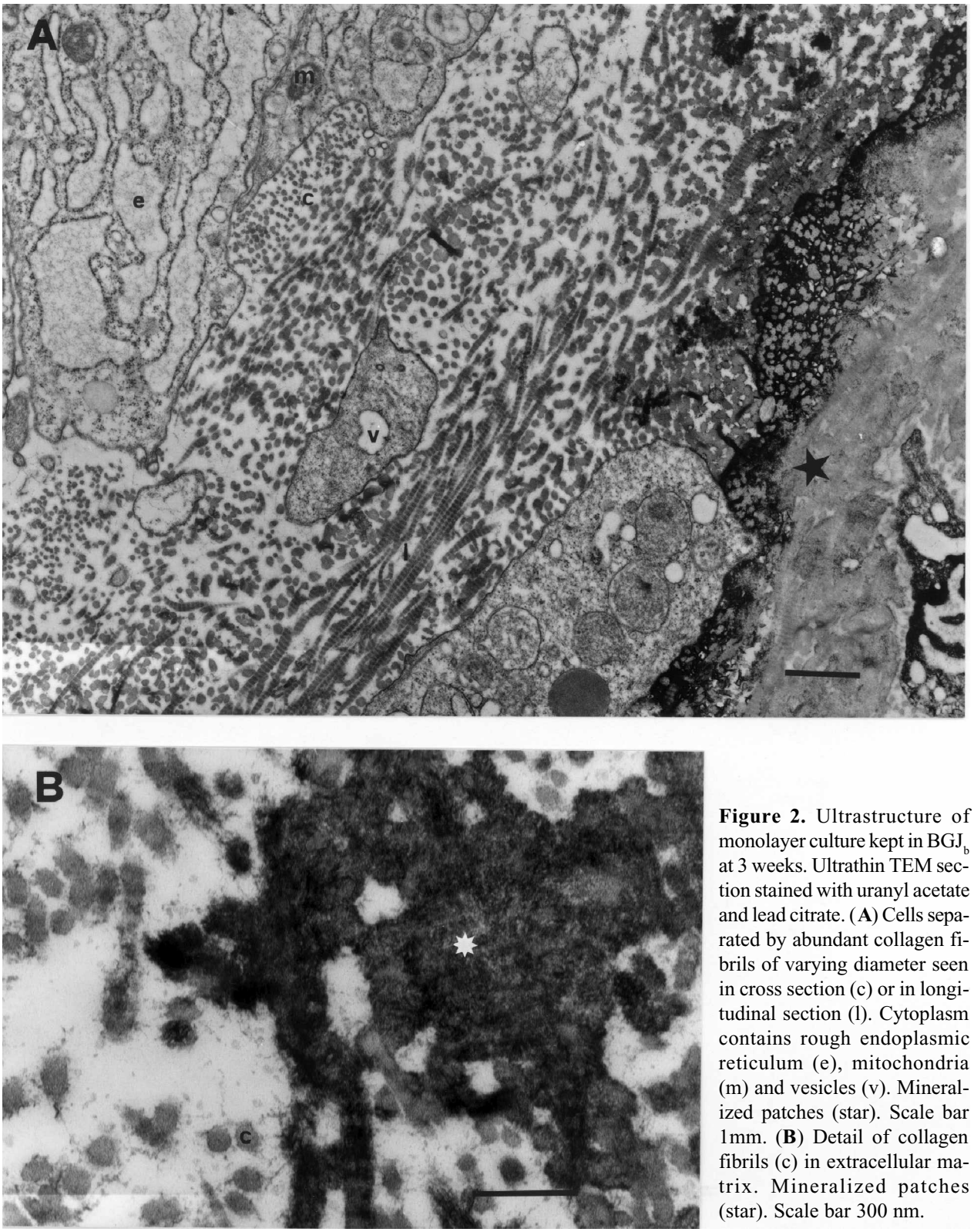

Figure 2. Ultrastructure of monolayer culture kept in BGJ_b at 3 weeks. Ultrathin TEM section stained with uranyl acetate and lead citrate. (**A**) Cells separated by abundant collagen fibrils of varying diameter seen in cross section (c) or in longitudinal section (l). Cytoplasm contains rough endoplasmic reticulum (e), mitochondria (m) and vesicles (v). Mineralized patches (star). Scale bar 1mm. (**B**) Detail of collagen fibrils (c) in extracellular matrix. Mineralized patches (star). Scale bar 300 nm.

as monolayer cultures in BGJ_b for 3 weeks formed dense alkaline phosphatase (ALP) positive, calcified nodules (Fig. 1A, 1E). The cells grown in DMEM formed ALP positive multilayers, while some areas displayed a nodular structure, where some mineralization could be observed (Fig. 1B, 1F). The cells kept in DMEM w/o did not have so many cells staining for ALP and they failed to produce the same calcified nodules (data not shown).

All the cells grown as micromasses behaved in a similar way. No effect which could be attributed to the various culture media was observed. At the edge of the micromass there were cells growing out. After 3 weeks, the culture dishes were confluently covered by ALP positive cells (Fig. 1C,D). The original drop of cell suspension formed a cal-

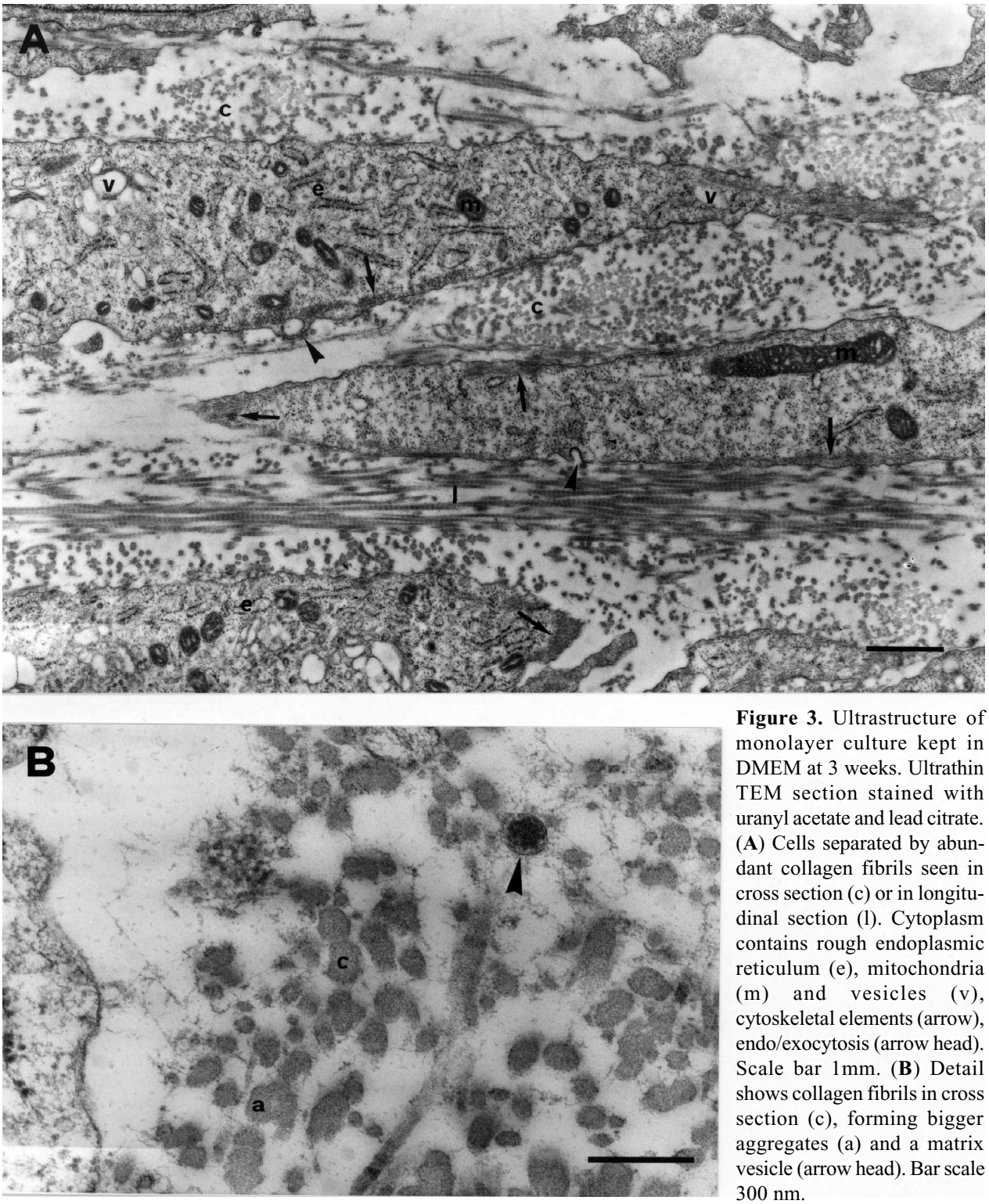

Figure 3. Ultrastructure of monolayer culture kept in DMEM at 3 weeks. Ultrathin TEM section stained with uranyl acetate and lead citrate. (**A**) Cells separated by abundant collagen fibrils seen in cross section (c) or in longitudinal section (l). Cytoplasm contains rough endoplasmic reticulum (e), mitochondria (m) and vesicles (v), cytoskeletal elements (arrow), endo/exocytosis (arrow head). Scale bar 1mm. (**B**) Detail shows collagen fibrils in cross section (c), forming bigger aggregates (a) and a matrix vesicle (arrow head). Bar scale 300 nm.

cified area (Fig. 1G,H).

The ultrastructure of cells under all conditions showed typical features of osteoblast-like cells, such as a well developed cytoplasm with abundant rough endoplasmic reticulum, Golgi vesicles, cytoskeleton and mitochondria (Fig. 2A, 3A). The cells kept in DMEM had the tendency to have a less dilated rough endoplasmic reticulum than the cell in BGJ_b. The matrix consisted of abundant orthogonally oriented collagen fibrils, with a wide range in diameters from 50 nm to more than 100 nm (Fig. 2, Fig. 3). Furthermore, more matrix vesicles were observed in the extracellular matrix of the cells cultured in DMEM than in BGJ_b. Intense mineralization was only observed in micromass cultures, under all conditions, and in cell monolayer cultures grown in BGJ_b (Fig. 2A, 2B), but not in cultures kept in DMEM (Fig. 3A, 3B).

Repeated use of the calvariae for cell isolation

The calvariae and all the isolated cells were cultured in BGJ_b, for optimal differentiation. The cultures were only characterized morphologically. The cells isolated from the

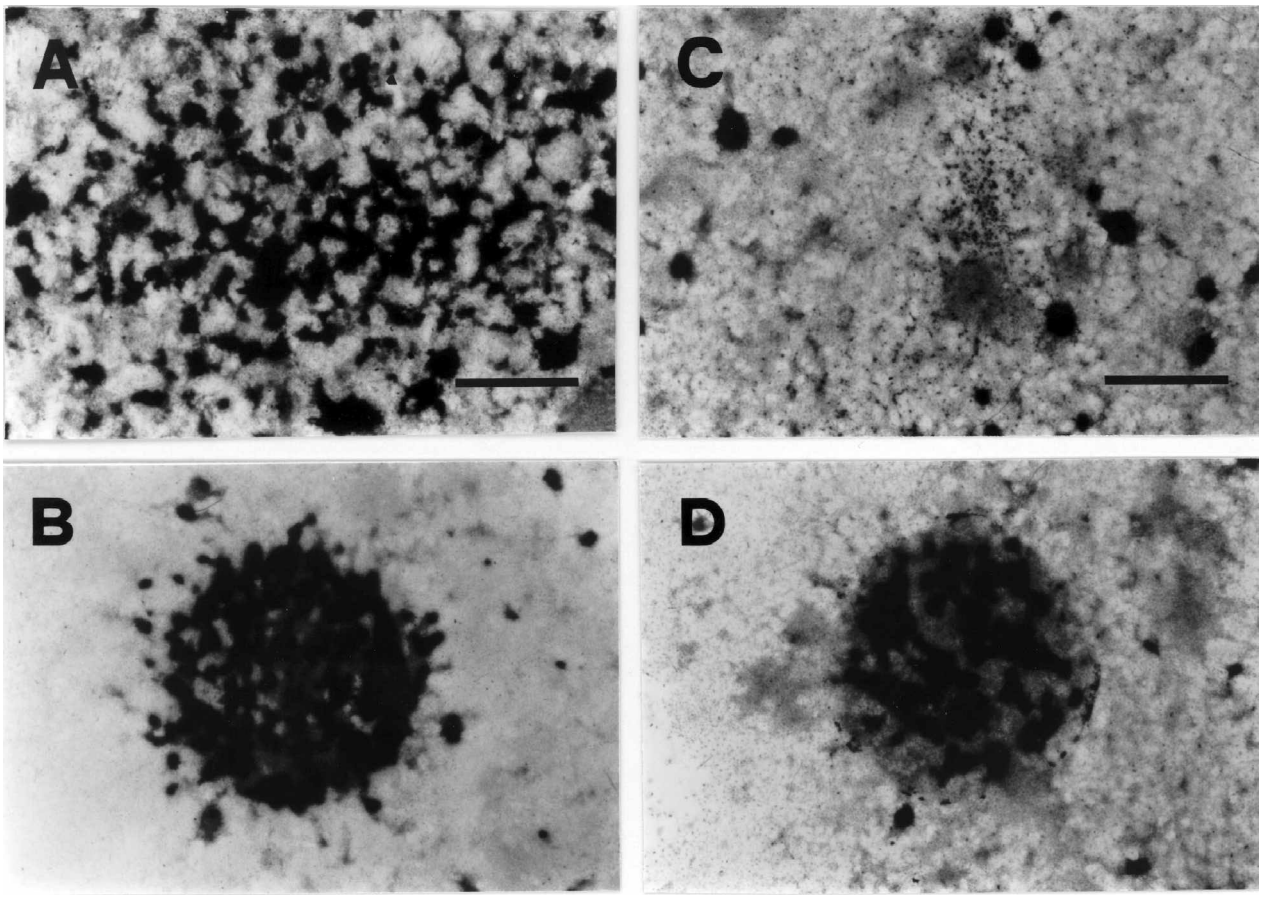

Figure 4. Transfer experiments. Mineralization assessed by von Kossa staining (black areas) at 3 weeks. Same magnification in all pictures; Scale bar 3 mm. (**A**) Monolayer of transfer 4 in BGJ_b, (**B**) Micromass of transfer 4 in BGJ_b, (**C**) Monolayer of transfer 6 in BGJ_b, (**D**) Micromass of transfer 6 in BGJ_b

first to the fourth cycle of explant culture and passage showed no qualitative differences in their differentiation potential. ALP positive and calcified nodules were formed when the cells were inoculated as monolayer cultures (Fig. 1A, 1E, Fig. 4A). The appearance of micromass cultures was also similar in the transfer group 1 to 4 (Fig. 1C, 1G, Fig 4B). Cells from the 6th cycle of explant culture and passage, when kept as monolayer cultures, had a qualitative reduction in the capacity of nodule formation but in micromass cultures this effect was less pronounced (Fig. 4). The ultrastructure of the nodules formed by all cells of all transfer groups was similar, and as described above.

Biochemistry

The increase in cell number was reflected by the DNA content, which increased significantly ($p<0.0005$) in both monolayer and micromass cultures kept in BGJ_b and DMEM, over the culture time (Fig. 5A). DMEM cultures had a significantly ($p<0.04$) higher DNA content than the cultures in BGJ_b. Furthermore monolayer cultures had a significantly ($p<0.001$) higher DNA content than the micromass cultures in both growth media. The protein content per DNA was significantly ($p<0.0002$) lower in the DMEM cultures than in BGJ_b, but there was no significant difference in either monolayer or micromass cultures over time (Fig. 5B). The ALP content per DNA was significantly ($p<0.0015$) higher in BGJ_b cultures than in DMEM (Fig. 5C). At 1 week the ALP per DNA content was significantly ($p<0.008$) higher in micromass than in monolayer cultures. In monolayer cultures there was a significantly ($p<0.0005$) higher ALP per DNA content at 3 weeks when compared to that at 1 week, whereas in micromass cultures no significant difference was observed over time.

The main collagen type synthesized by the calvariae, the migrated cells and the passaged cells under the different culture conditions was collagen type I along with some collagen type V (Fig. 6).

Discussion

The growth media used did not have an influence on the cell number isolated either by enzymes or after explant cultures. The calvariae were treated with collagenase/trypsin to remove the periosteum and then with collagenase to get the first cell population from periosteal and endosteal bone surfaces. Subsequently, the remaining calvariae were used as explant cultures. The number of cells isolated by enzymes was similar to that reported in the literature, which ranges from $4x10^4$ to $5x10^5$ cells/calvaria dependent on the age and species used (Binderman *et al.*, 1974; Yagiela and Woodbury, 1977; Ecarot-Charrier *et al.*, 1983; Gerstenfeld *et al.*, 1987; Whitson *et al.*, 1992). After explant cultures the cell yield per calvarium was about 5 times higher than after the enzymatic cell isolation.

Under all culture conditions the cells in monolayer,

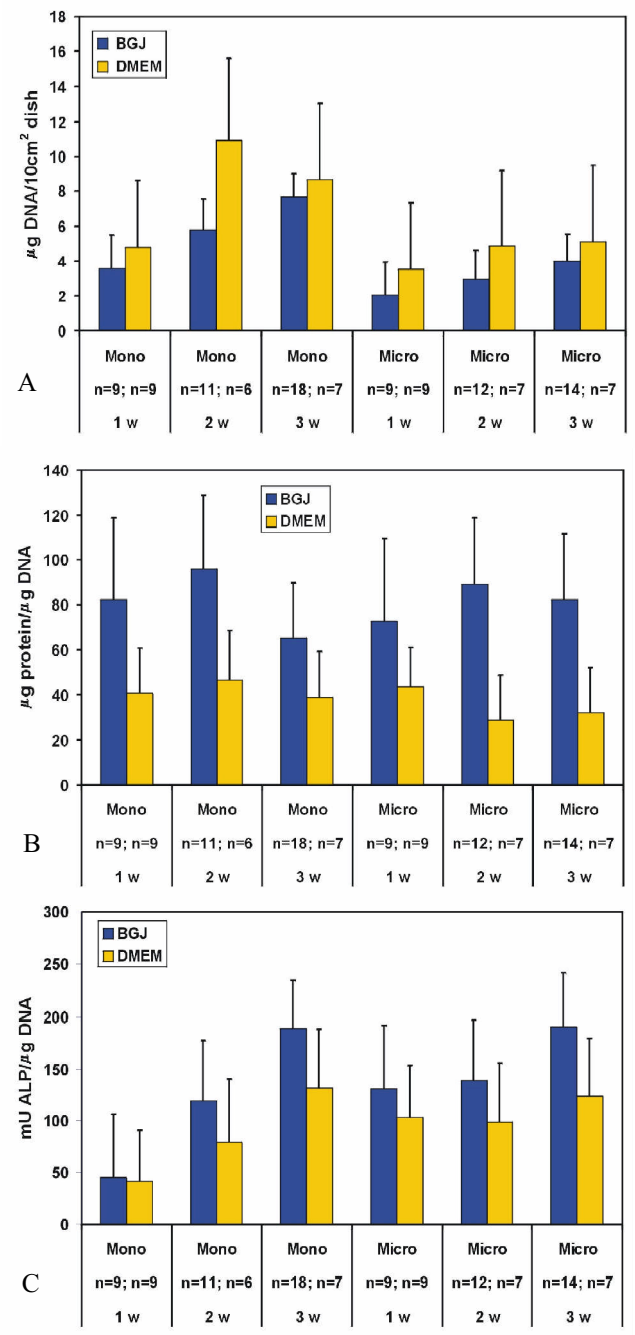

Figure 5. Biochemical analysis. DNA content, protein content and alkaline phosphatase activity of monolayer (Mono) and micromass (Micro) cultures kept in DMEM or in BGJb at 1, 2 and 3 weeks (1w, 2w, 3w). Least squares means ± standard error. n= number of experiments. (**A**) Total DNA content per 10 cm^2 culture dish, (**B**) Total protein content per mg DNA, (**C**) ALP activity per µg DNA**.**

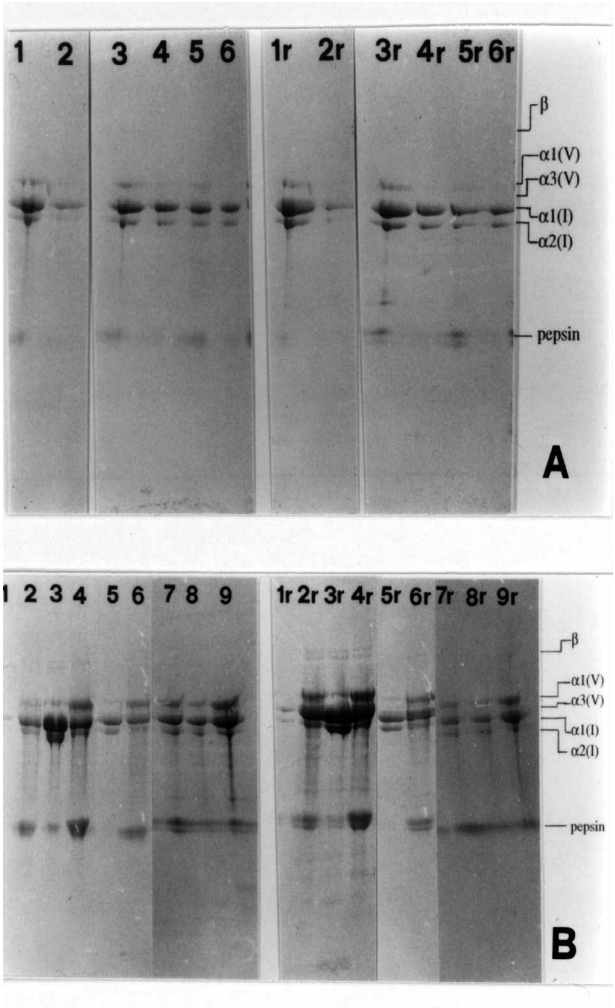

Figure 6. SDS-PAGE (4.5-15 %) of collagens extracted by pepsin digestion from whole cultures at 3 weeks stained with Coomassie blue. Samples were reduced with 2% 2-mercapto-ethanol before electrophoresis (r). (**A**) Enzymatic Isolation, Lanes 1, 1r: monolayer in DMEM; Lanes 2, 2r: micromass in DMEM; Lanes 3, 3r: monolayer in BGJ_b, Lanes 4; 4r: monolayer in DMEM w/o; Lanes 5, 5r: micromass in BGJ_b; Lanes 6, 6r: micromass in DMEM w/o, (**B**) Non-enzymatic isolation: Explant cultures (outgrown cells and calvariae) and passaged cells. Lanes 1, 1r outgrown cells in DMEM w/o; Lanes 2, 2r calvariae in DMEM w/o; Lanes 3, 3r outgrown cells in BGJ_b; Lanes 4, 4r calvariae BGJ_b; Lanes 5, 5r outgrown cells in DMEM; Lanes 6, 6r: calvariae in DMEM; Lanes 7, 7r: passaged cells in DMEM; Lanes 8, 8r: passaged cells in DMEM w/o; Lanes 9, 9r: passaged cells in BGJ_b.

micromass and during explant cultures produced mainly collagen type I and some type V, as shown by SDS-PAGE. This is in accordance with the literature (Wiestner *et al.*, 1981; Ecarot-Charrier *et al.*, 1983; Berry and Shuttleworth, 1989; Masquelier *et al.*, 1990; Collin *et al.*, 1992; Gerstenfeld *et al.*, 1993). The presence of collagen type III is controversial. Some authors found type III collagen in bone (Scott *et al.*, 1980; Ecarot-Charrier *et al.*, 1983; Whitson *et al.*, 1984; Masquelier *et al.*, 1990; Collin *et al.*, 1992; Stringa *et al.*, 1995), but others do not (Scott *et al.*, 1980; Gerstenfeld *et al.*, 1987; McCarthy *et al.*, 1988).

During the subsequent culture period the differentiation of the osteoblast-like cells was influenced by the choice of growth media used and the inoculation technique. The cells kept in DMEM had a significantly higher DNA content than the cells kept in BGJ_b. This indicated that the DMEM promoted the proliferation of the isolated cells both in micromass and monolayer cultures, when compared to the BGJ_b. In contrast, the BGJ_b growth medium

showed a significantly higher ALP activity per DNA and significantly more protein per DNA in both monolayer and micromass cultures, when compared with those grown in the DMEM. This provided evidence that the BGJ_b supported the differentiation of osteoblast-like cells *in vitro*. This observation was in agreement with that shown by others (Williams *et al*., 1980; Aronow *et al*., 1990). Mineralization was therefore possible without the external addition of inorganic or organic phosphates, when the cells were grown in BGJ_b. These results were in accordance with those of others (Binderman *et al*., 1974; Williams *et al*., 1980; Aronow *et al.*, 1990; Zimmermann *et al*., 1991), but were in contrast to other papers which claim that mineralization only takes place in media supplemented with either organic or inorganic phosphate (Ecarot-Charrier *et al.*, 1983; Nefussi *et al.*, 1985; Gerstenfeld *et al*., 1987; Masquelier *et al*., 1990; Bellows *et al*., 1992).

The reason why mineralization in BGJ_b was more pronounced than in DMEM has to be related to the composition of the growth media. BGJ_b was developed for embryonic tibiae organ culture (Biggers *et al.,* 1961) whereas DMEM is a widely used growth medium. BGJ_b has a higher concentration of vitamins, amino acids, glucose, calcium and inorganic phosphate than the DMEM. For example, DMEM is proline-free but BGJ_b contains 3.5 mM proline. The rate of proline incorporation into protein and collagen is directly proportional to the extracellular proline concentration up to 0.15 mM in fetal rat calvariae (Finerman *et al*., 1967). DMEM contains 25 mM glucose, 0.9 mM phosphate and 1.8 mM calcium, whereas BGJ_b has 55.5 mM glucose, 1.57 mM phosphate and 2.55 mM calcium. Bone cells generate their energy through mitochondrial and glycolytic pathways (Borle *et al.*, 1960; Cohn and Forscher, 1962), but glycolysis dominates (Borle *et al.*, 1960; Cohn and Forscher, 1962; Pollesello *et al.*, 1991; Vittur *et al.*, 1994). The possible effects of inorganic phosphate are various. It exerts a regulatory effect on glycolysis and glycogenolysis. Furthermore, inorganic phosphate acts as a buffer in the growth medium (Waymouth, 1978) and is incorporated into phospholipids (Dirksen *et al*., 1970).

Concerning the inoculation technique, micromass cultures were less sensitive to nutritional effects than the monolayer cultures, in such a way that mineralization was observed at 3 weeks – independent of the growth media used. Micromass cultures of limb mesenchymal cells, periosteal cells, and osteoblast-like cells grown as organoid culture produce a mineralized matrix (Osdoby and Caplan, 1979; Zimmermann *et al.*, 1988; Masquelier *et al*., 1990; Boskey *et al.*, 1992; Fang and Hall, 1996). The differentiation of osteoblast-like cells was promoted, but their proliferation was reduced in micromass cultures at 1 week because the ALP activity per DNA was significantly higher and the DNA content was significantly lower – as compared to the monolayer cultures. These results of the micromass cultures indicated that cell-cell contacts and cell-cell communication were important for the differentiation process. The micromass inoculation corresponds to the state of condensation of mesenchymal cells during membranous bone formation, which amplifies the number of pre-osteoblasts (Dunlop and Hall, 1995). The cells in the condensation have a dramatically increased cell-cell communication, increased cell-cell contact, attain a rounded morphology and increase the number of gap junctions (Thorogood and Hinchliffe, 1975; Coelho and Kosher, 1991).

Furthermore, this study showed that it was possible to use the same calvaria several times over, as explant cultures over a period of 18 weeks. The cells could be harvested at intervals of 3 weeks. The differentiation potential of the cells from the fourth cycle of explant culture and subsequent passage was similar to the first one. This was confirmed by morphological criteria such as ALP activity, mineralization and the ultrastructure of the cells. In the very late sixth transfer there was a reduction in the formation of mineralized nodules, but the ultrastructure was similar to that of the earlier transfers. This agreed with the literature, where it is observed that the morphology of later passage (transfer 16-20) is similar to primary passaged cells from explant cultures. Multilayers are formed, though the tendency to do so is somewhat reduced as compared to primary passaged cells from explant cultures (Williams *et al*., 1980).

Conclusions

The microclimate applied had a profound effect on cellular differentiation *in vitro*. The cell isolation technique was not important for the later differentiation, in contrast to the choice of inoculation technique and to the growth medium used. The combination of the enzymatic and explant cultures allowed the isolation of osteoblast-like cells from the same calvaria with a similar differentiation potential over 12 weeks. DMEM promoted the proliferation, whereas BGJ_b promoted the differentiation of rat osteoblast-like cells *in vitro*.

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