Enhancement of human osteoblast proliferation and phenotypic expression when cultured in human serum

David P Hankey[1], Ruth E McCabe[1], Mary-Jo Doherty[1], Paul C Nolan[2], M Gavan McAlinden[2], John Nelson[3] and David J Wilson[1]

Departments of [1]Anatomy, School of Clinical Medicine, The Queen's University of Belfast, Medical Biology Centre, 97, Lisburn Road, Belfast, BT9 7BL, U.K. E-mail: dj.wilson@qub.ac.uk; [2]Orthopaedic Surgery, The Queen's University of Belfast, Musgrave Park Hospital, Stockman's Lane, Belfast BT9 1NN, U.K., [3]Biology and Biochemistry, The Queen's University of Belfast, Medical Biology Centre, 97, Lisburn Road, Belfast BT9 7BL, U K. Correspondence: Dr. D. J. Wilson
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ABSTRACT – Traditionally, culture medium is supplemented with foetal bovine serum (FBS). However, in cultures of osteoblasts intended for human re-implantation, such serum presents potential risks of foreign protein contamination and transmission of viral or prion-related material, if used. We cultured human osteoblasts from 16 patients in 10% autologous human serum, 10% pooled human serum, 10% FBS or 2% Ultroser G. Non-synthetic sera were tested in both heat-treated and non-heat-treated forms. We determined cell growth and osteoblast phenotype. Cell proliferation in all types of human serum was significantly greater than in FBS. This was most marked in heat-treated autologous human serum. Cells cultured in Ultroser G had less proliferation than all other groups. The phenotypic tests showed that cells cultured in human and foetal bovine serum displayed an osteoblast phenotype, with greater protein expression in cells cultured in human serum.

We conclude that culture of human osteoblasts in autologous human serum enhances cell proliferation, while maintaining an osteoblast phenotype. These findings have implications for the use of cultured osteoblasts in self-cell therapy. Human osteoblast growth is supported by autologous human serum, which allows re-implantation of cultured cells, while avoiding the risk of foreign protein carry-over with enhancement of cell proliferation.

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It has been demonstrated that osteoblasts populate a wide variety of graft materials in vitro (Nakahara et al. 1991, Sautier et al. 1991, 1994, Begley et al. 1993, Doherty et al. 1994, Ducheyne 1994). It has also been suggested that a graft material, coated with a patient's cultured osteoblasts, could be implanted back into the patient, in a form of "self-cell therapy" (Caplan 1991, Nolan et al. 1994). It may be more appropriate to culture such cells in autologous rather than conventional bovine serum, because of the risk of foreign protein contamination (Nijweide and Burger 1990).

Serum contains factors, often species-specific, that modulate cell growth and function (Maurer 1992). Convenience, expense and availability usually dictate the use of serum from other species, most frequently new-born and foetal bovine sera. Homologous serum (from the same species), may have the disadvantages of lack of a defined composition, batch-to batch variability, and possible toxic components. In addition, serum contains growth factors which could promote growth of non-osteogenic progenitor cells (Aubin et al. 1982, Bellows et al. 1986). Ideally, the medium supplement should be defined, lead to sustained growth and expression of the desired phenotype. The synthetic serum substitute Ultroser G is said to be of "constant" composition, unlike other sera, whose chemical and hormonal composition is batch-dependent. Cellular growth, adhesion and metabolism have all been investigated using Ultroser G (Gibco, Paisley, Scotland) in many cell types (Ronot et al. 1984, Myara et al. 1986, Schmidt and Kulbe 1993) (Ultroser G, Product Information 1984). Studies using human osteoblasts have claimed that loss of the osteoblastic phenotype (as occurs in long-

term culture with FBS) could be avoided when 2% Ultroser G was used (Schmidt and Kulbe 1993). Human serum supplementation of bone cell cultures has been shown to enhance outgrowth of cells from bone explants, although this report did not comment on cell phenotype or morphology (Shigeno and Ashton 1995).

We compared the effects of FBS, autologous and human serum (AHS) and Ultroser G on human osteoblast proliferation and morphology. Cells from 8 patients selected at random were also studied to determine whether treatment of cells with autologous serum caused any significant changes in the osteoblast phenotype.

Material and methods

Bone and serum harvesting

The members of the study (aged 60–81 years, n 16) had degenerative osteoarthritis and were undergoing total knee arthroplasty. Patients with metabolic bone disease or on medications other than simple analgesia were excluded. Total knee replacement allows bone of high quality and standard size (triangular fragments known as "chamfers") to be obtained during the femoral cuts of the technique. The fragments retrieved had no soft tissue attachments and only minimal amounts of retained blood. The bone was placed in sterile bottles containing culture medium at 37 °C. Blood (60 mL) was taken from each patient 3–5 days after surgery, allowed to clot at 21 °C for 3 hours and stored intact at 4 °C overnight. The serum (25–30 mL) was separated from the clot by centrifugation at 3000 rpm for 10 minutes, passed once through a sterile filter (0.2 μm) (Millipore) and stored at –20 °C, pending use.

Human bone cell isolation

Human osteoblast cultures were established by an explant technique, a modification of the method described by Beresford et al. (1984) as detailed by Begley et al. (1993). Briefly, bone fragments had all soft tissue connection removed, were repeatedly washed in Hank's Balanced Salt Solution (HBSS), placed in a 25 cm^2 culture flask (Nunclon, U.K.) with Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemicals, Poole, U.K.) supplemented with 10% FBS (Gibco, Paisley, Scotland), L-glutamine, pyruvate, penicillin, streptomycin and amphotericin. The flasks were incubated at 37 °C in 95% air/5% CO_2. The medium was changed every 3 days. At confluence (mean 28 (24–33) days), the cells were given several washes in HBSS and then trypsinised from the flask by trypsin-EDTA digestion (0.05%, Sigma Chemicals, Poole, U.K.) and replated into two new flasks. The cells were phenotyped by 2D-PAGE using the method described by Hankey et al. (1993).

Serum treatment

At confluence, cells were trypsinised, counted using an improved Neubauer haemocytometer and plated at a seeding density of 2500 cells/cm^2 (750 cells/well) in eight 96 well plates. 6 replicates of each serum treatment were performed. After 24 hours (time taken for the cells to plate down), the medium was replaced with serum-free medium. After a further 24 hours, this was removed and replaced with medium containing one of the following serum supplements: autologous human serum (AHS), laboratory heat-treated (56 °C/30 minutes) or untreated; pooled human serum (PHS) laboratory heat treated (56 °C/30 minutes) or untreated; foetal bovine serum (FBS) (Gibco, Paisley, Scotland); untreated (Table 1). Heat inactivation has been said to remove complement from serum and reduce the cytotoxic action of immunoglobins without damaging polypeptide growth factors (Freshney 1987). Subsequently, medium changes were done with the appropriate serum supplementation every 3 days.

Crystal violet binding assay

After 10 days, the number of cells in each well was determined by a modification of the crystal violet colorimetric dye binding assay (Gillies et al. 1986). The medium was removed from the wells and the cells were fixed in 100 μL of 10% formaldehyde (pH 6.8 in Phosphate buffered saline (PBS)) for 1 hour. Cells were rinsed in distilled water and air dried before staining in 100 μL of 0.1% crystal violet (Sigma Chemicals, Poole, U.K.) in distilled water for 30 minutes. Stained monolayers were rinsed in distilled water and air-dried. The retained dye was eluted in 100 μL of

1% 0.1 M HCl in 100% ethanol and the absorbance was read at 540 nm on a microwell plate reader. A standard curve, constructed by serial dilutions of a known number of cells determined by a Coulter counter, was used to convert the absorbance reading into the number of cells present. Cell numbers were expressed as the sum of the number of cells in all six wells.

In addition, cells from 8 patients, selected at random, were studied to determine whether treatment of cells with autologous serum caused any significant changes in the osteoblast phenotypic expression when compared with osteoblasts cultured in FBS.

Scanning electron microscopy

Cells were seeded at a density of 12,500 cells/mL onto Thermanox (Nunclon, U.K.) coverslips placed in the wells of two 6-well plates. After 24 hours, the medium was removed, the cells rinsed with HBSS and fed with medium containing 10% untreated AHS, heat-treated 10% FBS or 2% Ultroser G. The medium was changed after 3 days and on the fifth day, the cells were rinsed in HBSS and fixed in 3% buffered glutaraldehyde in 0.1M cacodylate buffer (pH 7.3). 4 replicates of each treatment were performed.

The cells were kept at 4 °C for 24 hours before dehydration in a graded series of ethanols. The coverslips were air-dried before adhesion to stubs. Stubs were coated with 40–50 nm of gold and examined and photographed using a JEOL 840A scanning electron microscope.

Alkaline phosphatase expression

Osteoblast expression of alkaline phosphatase (ALP) was determined both quantitatively and qualitatively in cells from 3 patients (males 69 and 70 years, female 62 years). For spectrophotometric analysis of cellular ALP-activity, cells were plated in 24-well plates (Nunclon, U.K.) at 10,000 cells/cm^2 and cultured for 6 days in media supplemented with either FBS or AHS, after which the cells were washed in cold PBS. The cell layer was scraped into 2 mL of cold distilled water, sonicated, and the cell lysate was centrifuged. ALP activity in the supernatant was determined spectrophotometrically using a Beckman Synchron CX analyser by the conversion of p-nitrophenylphosphate to p-nitrophenol and phosphate and the results expressed in international units per mg of protein (IU/mg protein).

ALP expression was localised in cells grown for 6 days in 24-well tissue culture plates (Nunclon, U.K.), supplemented with either FBS or AHS using the histochemical semiquantitative demonstration procedure (Sigma Chemicals, Poole, U.K., Kit #86-R).

Osteocalcin production

Osteocalcin assay was carried out as previously described by Marie et al. (1989) on cells from 3 patients. Briefly, cells were plated in triplicate at 10,000 per cm^2 and grown in FBS or AHS supplemented media for 10 days. The cultures were then stimulated with 10^{-9} M 1,25 $(OH)_2D_3$ (kindly donated by Roche, Basel, Switzerland) for 48 hrs after which they were scraped into assay buffer and sonicated. Osteocalcin was measured with a radioimmunoassay kit using an antibody raised against bovine osteocalcin (CIS Bioindustries, France. Kit OSTK-PR). Results represent the mean of the readings. Sensitivity of the assay is 0.35 ng/mL.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cells from 2 patients (male 68 years, female 72 years) were used in this study. This procedure was based on a method previously described by O'Farrell (1975) with minor modifications (Hankey et al. 1993). Cells were plated at confluence (25,000 cell/cm^2) in 24-well plates. Medium supplementation with either FBS or AHS was made for 24 hours and (^{35}S)-methionine labelling (Amersham International, Bucks, U.K.) was done for the concurrent 24-hour period in 0.5% FBS methionine-free medium. The volume of medium loaded on the two-dimensional gels was related to the amount of (^{35}S)-methionine incorporated in the equivalent cellular sample. After electrophoresis, the gels were dried and autoradiographed with Hyperfilm Betamax (Amersham International, Bucks, U.K.).

Determination of proliferative capacity

The proliferative capacity of cells grown in media supplemented with 10% AHS was compared to that of cells grown in media supplemented with 10% FBS by two other methods.

The method of (^{3}H)-thymidine incorporation used by Marie et al. (1989) was applied. First passage cells from 3 patients (males 77 and 69 years, female 81 years) were seeded at 2,500 cells/cm^2 in 24-well plates and incubated for 24 hours to allow the cells to adhere to the base of the wells. The medium was then removed, the cells were washed several times with HBSS, serum-free medium (500 µL) was added and the cells were incubated for a further 24 hours to ensure the removal of residual FBS.

On removal of this medium, medium supplemented with 10% FBS (control) or 10% AHS (study) was added. The following day (day 0), the medium was replaced and 2 µCi of (^{3}H)-thymidine in 1 mL of medium was added to 6 wells (3 controls and 3 study). Labelling of the cells was carried out on days 0, 2, 4, 6 and 8. Thymidine incorporation was determined 24 hours later on days 1, 3, 5, 7 and 9. After 24 hours the medium was removed and the cell layer washed twice with HBSS to remove unincorporated thymidine. Trypsin (250 µL, 0.05%) was added and the cells were returned to the incubator for 30 minutes. The cells were washed three times with distilled water pooled with the cell extracts. Trypsin activity was stopped by adding 1 mL of PBS and then DNA was precipitated by adding 400 µL of 60% TCA. After centrifugation at 2000 rpm for 14 minutes at 4 °C, the supernatant was removed and the TCA-insoluble fraction dissolved in 0.5 µL M NaOH. The incorporation of thymidine into DNA was measured in aliquots using a scintillation counter (Romberg et al. 1986).

Total protein estimation

Cells from three patients (males 69 years and 70 years, female 62 years) were plated at 10,000 cells/cm^2 in duplicate in 24-well plates. After 24 hours, the serum-free media was changed. After a further 24 hours, medium supplemented with either AHS or FBS was added for 10 days. At this stage, cell layers were scraped into assay buffer (PBS and Tween) and sonicated for 15 seconds at a frequency of 20 KHz to solubilise completely the protein. A 100 mL sample was removed and the protein content determined, using a protein assay kit (Sigma Chemicals, Poole, U.K., Kit #P 5656).

Table 1. Concentration and details of treatment with serum supplements

Serum supplement	Conc. (%)	Treatment [a]
Autologous human serum (AHS)	10	–
Autologous human serum	10	Lht
Pooled human serum (PHS)	10	–
Pooled human serum	10	Lht
Foetal bovine serum (Gibco) (FBS)	10	–
Foetal bovine serum (Gibco)	10	Lht
Ultroser G (Gibco) [b]	2	–
Foetal bovine serum (Gibco)	10	Cht

Heat treatment—the serum was kept at 56 °C for 30 minutes. Raw FBS and laboratory heat-treated FBS were used as controls.
[a] Lht lab. heat-treated, Cht commercially heat-treated
[b] A concentration of 2% Ultroser G was specified as being equivalent to 10% FBS by the manufacturers

Statistics

The proliferative response was assessed using log-transformed data from the crystal violet dye binding assay. One way Analysis of Variance (repeated measures) was performed using Fisher's PLSD test, allowing intrapatient comparisons which were pooled to permit interpatient comparisons.

Results

Cell proliferation following serum supplementation

The proliferative response of osteoblast cultures from the 16 patients in the 8 serum supplement regimens (Table 1) showed wide interpatient variation, when assessed using the crystal violet dye-binding assay after 10 days in culture (Table 2): AHS ht (heat-treated) cell numbers ranged from 7,300 to 24,200 and FBS ht cell numbers ranged from 5,500 to 18,600. The values were log-transformed to allow comparison of the results between patients. The comparisons were expressed as a percentage of FBS ht supplemented cells. Cells supplemented with AHS ht demonstrated a mean response 134% that of FBS ht (n 13, 95% Confidence Interval (CI) 124–145). All the human serum treatments elicited an increase in cell proliferation over the FBS. Cells grown in Ultroser G showed the lowest proliferative response: just over half that obtained with FBS ht (61%, n 16, 95% CI 57–65).

Table 2. Response of serum treatment groups expressed as a percentage of growth of cells in foetal bovine serum (100%) after a culture period of 10 days

Treatment group	No. of patients	Log mean cell number	Std. dev	Std. error	Response (%)	CI 95%
FBS ht [a]	16	3.97	0.20	0.05	100	(–)
AHS [c]	13	4.03	0.10	0.06	116 [b]	107–125
AHS ht [c]	13	4.10	0.21	0.06	134 [b]	124–145
PHS	16	4.06	0.21	0.05	125 [b]	116–131
PHS ht	16	4.07	0.20	0.05	126 [b]	119–135
FBS	16	3.95	0.19	0.05	96	90–102
FBS ht [a]	16	3.96	0.19	0.05	97	91–103
Ultroser	16	3.75	0.16	0.04	61 [b]	57–65

FBS foetal bovine serum, AHS autologous human serum, PHS pooled human serum, Ultroser Ultroser G human supplement (Gibco), ht heat-treated serum (56 °C for 30 minutes).

[a] The difference between two batches (used as an internal control) was not significant.

[b] Denotes significant values using the Fisher PLSD at confidence interval of 95% (CI 95%).

[c] The difference in response between untreated AHS and AHS which is heat treated was significant using the same test. Heat treatment did not produce a significant difference between any of the other serum types.

No statistically significant difference was found between heat-treated and not heat-treated FBS.

The daily incorporation of thymidine by osteoblasts cultured in AHS and FBS (combination of 3 patients, male aged 77, male aged 69 and female aged 81) and the cumulative thymidine incorporation showed that cells cultured in AHS demonstrated a consistently higher level of DNA synthesis than those in FBS: approximately 2.4–6.7 fold greater increase in total thymidine incorporation (n 3, mean 4.6) (Figure 1). A peak in thymidine incorporation occurred on day 5 in cells cultured in AHS and after this time, the incorporation of thymidine decreased gradually. Although a similar peak occurred in cells cultured in FBS, thereafter thymidine incorporation remained constant. However, the overall trend was a continued cumulative increase in thymidine incorporation in both groups over the 10-day period. This was more marked in the AHS-treated group, particularly after day 3.

In addition to the dye-binding assay and to confirm that the increase observed in thymidine incorporation represented an increase in cell number, we measured total cellular protein.

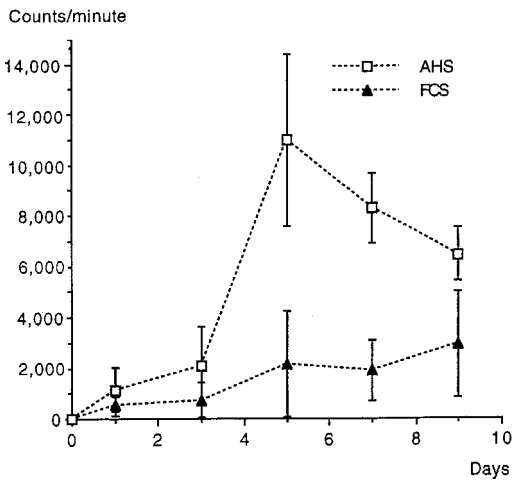
The quantity of cellular protein ranged from 65 to 118 µg/well for cells treated with FBS and from 75 to 242 µg/well for cells treated with AHS. Each patient's cells produced more cellular protein when grown in AHS than in FBS, ranging from a 15% to a 105% difference (n 3, mean 62%).

Phenotyping of cultured osteoblasts

The level of cellular ALP ranged from 89 to 740 IU/mg protein (mean = 456; s.e.m. 272) for cells grown in FBS, and from 103 to 950 IU/mg protein (mean = 780; s.e.m. 498) for cells grown in AHS. Although the differences in ALP between each individual in the same treatment group appeared large and no trend was apparent, the amount of ALP measured in the cells supplemented with 10% AHS was always greater (mean difference 71%) than with cells supplemented with 10% FBS. The largest difference occurred in the youngest patient (62 years).

To determine whether these results reflected a greater number of cells expressing ALP or an increase in synthesis per cell, histochemical staining of cultures was performed. Qualitative assessment for ALP involved examining the wells under phase microscopy to establish whether any significant staining differences were apparent: no difference in synthesis per cell between the groups was observed.

Osteocalcin was produced by cells cultured in FBS and AHS after stimulation with 10^{-9} M 1,25 $(OH)_2$ D_3. Values were 7.1–15 ng/mg protein (n 3,



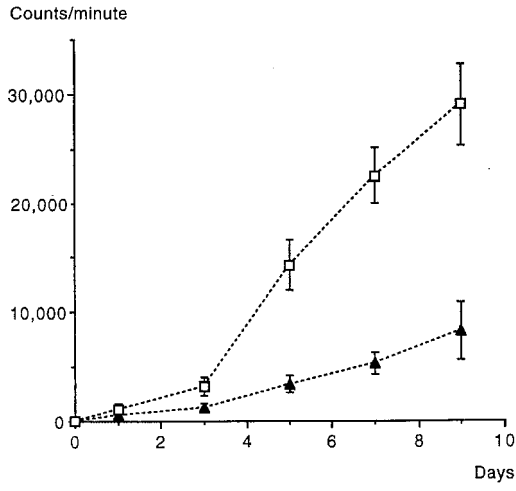


Figure 1 A. Graph comparing daily thymidine incorporation of human osteoblasts cultured in AHS and FBS, combined data of three patients, male 77 year, male 69 year and female 81 year.

B. Cumulative thymidine incorporation in the 3 patients.

mean 11) in FBS and 2.6–12 ng/mg protein (n 3, mean 6.8) in AHS. In general, less cellular osteocalcin was detected in cells treated with AHS than in those treated with FBS. There was no trend apparent in the amount of cellular osteocalcin produced by osteoblasts treated with either serum.

The media from the cells of 2 patients, analysed for secretory proteins by 2D-PAGE, were similar. The secretory activity in the AHS group was greater when the same patient's cells were cultured in FBS. More specifically, there was an increase in the production of osteonectin and collagen I.

The numerical system used for protein comparison is that previously described by Hankey et al. (1993) and identifies group 43 as osteonectin and 41 as the C-telopeptide of collagen type I. Groups 6, 7, 10 and the spot arrowed have been included as comparative reference points (Figure 2).

Discussion

We found that human osteoblasts are capable of proliferation and differentiation in AHS. In each patient studied, human serum was significantly more effective at stimulating osteoblast growth than heat-treated FBS. The findings of the morphological and the phenotyping tests showed that in addition to stimulating osteoblast growth, the autologous serum supplement resulted in increased levels of cellular alkaline phosphatase and an elevation in the secretory activity of the cells, as revealed by 2D-PAGE of conditioned medium.

Ideally, in vitro models should mimic the in vivo conditions as closely as possible, but such conditions are often difficult to recreate because of the physiological fluctuations of growth factors and hormones that occur in vivo. The results presented here suggest that not only does AHS supplementation of osteoblast cultures result in increased cell proliferation, they also show that this supplement maintains the differentiated cellular phenotype. Although it is argued that FBS contains high levels of hormones and growth promoters, the autologous serum may contain factors more specific for the patient's cells and at more appropriate physiological concentrations.

It was, perhaps, not surprising, however, that heat-treated autologous serum was more effective than untreated autologous serum and produced the highest growth response (134% heat-treated compared to 116% for untreated AHS). Serum contains proteins, polypeptides, hormones, metabolites, nutrients and minerals. In addition, serum may also contain substances inhibiting cell proliferation (Harrington and Godman 1980) and, for this reason, serum is heat-treated, a process that removes complement from the serum and reduces

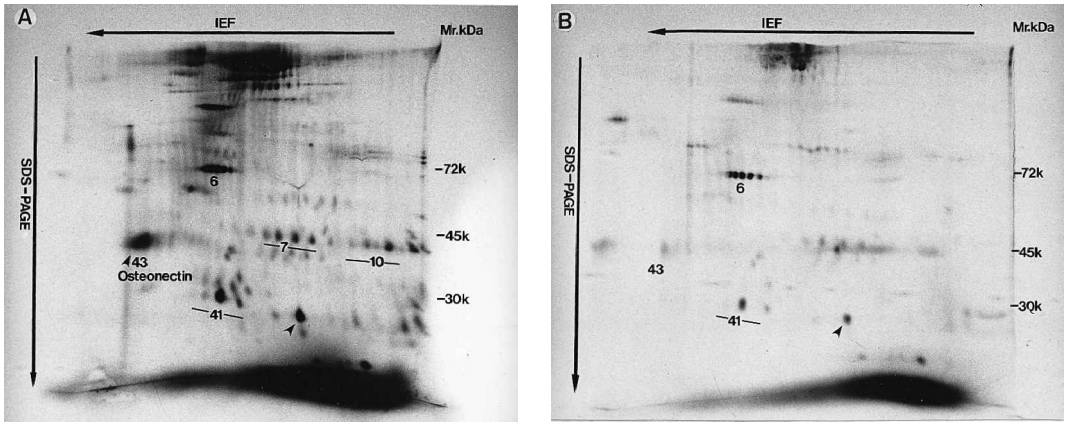


Figure 2. 2D-PAGE analysis of extracellular proteins revealed an elevation of secretory activity in the AHS group (A), as compared with the FBS group (B). The directions of isoelectric focusing (IEF) and electrophoresis (SDS-PAGE) are arrowed and molecular weight standards (Mr. kDa) have been included. The numerical system used for protein comparison is that previously described (Hankey et al. 1993) and identifies group 43 as osteonectin and 41 as the C-telopeptide of collagen type I. Groups 6, 7, 10 and the spot arrowed have been included as comparative reference points. Patient male 68 year. These changes were replicated on sample from female 72 year (data not shown).

the cytotoxic action of immunoglobulins without damaging polypeptide growth factors. This may explain why heat-treated AHS produced a greater growth response than untreated AHS. Serum allowed to clot naturally, such as that used in this study, stimulates cell proliferation more than serum from which the cells have been removed physically (Freshney 1987), and is thought to be due to the release of growth factors from the platelets during clotting. Piche and Graves (1989) noted that cultures of bone-derived cells were maximally stimulated by addition of a combination of three growth factors (EGF, TGF-β and PDGF) rather than by these factors given singly. A combination of these factors is released by platelets.

Supplementation of media with Ultroser G in the recommended concentration resulted in a lower increase in cell number, approximately 60% that of FBS. This result differs from those obtained by Myara et al. (1986) who, after supplementing cultured fibroblasts with 2% Ultroser G, noted that the cell growth was greater than in cells supplemented with 10% FBS. Interestingly, these authors advised against the use of Ultroser G for metabolism experiments on human fibroblasts because Ultroser G altered the metabolism of glucose and lactate. It should be noted that fibroblasts exhibit a different growth response to growth factors than osteoblasts (Schmidt and Kulbe 1993), so these results may not be strictly comparable to the current study.

Given these findings, it would appear that Ultroser G is unsuitable for human osteoblast cultures. Cells grown in Ultroser G by Schmidt and Kulbe (1993) were characterised as osteoblasts by the expression of a high alkaline phosphatase activity and the secretion of osteocalcin stimulated by 1,25 $(OH)_2D_3$. Although they noted that after long-term culture there was less dedifferentiation of the osteoblasts in Ultroser G supplemented media, it may be argued that a lower growth rate in the synthetic medium accounted for the maintenance of differentiated phenotype.

A study of the parameters associated with osteoblast phenotype, although only performed on a small sample, showed increased levels of cellular alkaline phosphatase, increased secretion of osteonectin and collagen and increased DNA synthesis, which corresponded to an increase both in cell number and total cellular protein for cells cultured in AHS compared to cells cultured in FBS.

Surprisingly, no osteocalcin was detected in the medium from cells cultured in either AHS or FBS and the level of cellular osteocalcin was, in general, lower in cells cultured with AHS. The synthesis of osteocalcin is stimulated in vivo and in culture by 1,25 $(OH)_2D_3$ (Price and Baukol 1980, 1981, Marie et al. 1989). The level of cellular osteocalcin in cells treated with FBS (mean 11;

s.e.m. 3.1) is similar to the level of osteocalcin detected in the medium from osteoblast cells by Marie et al. (1989) (mean 18; s.e.m. 7.5). The ages of the patients from whom the osteoblasts were cultured may be an important factor: it has been reported that the amount of osteocalcin secreted from cells in vitro from osteoblast cultures derived from older people is low (Schmidt and Kulbe 1993). The sex of the donor also plays a role, since it has been reported that unstimulated osteoblasts from male donors of the same age secreted nearly twice as much osteocalcin as osteoblasts obtained from female donors (Schmidt and Kulbe 1993).

One of the more definitive osteoblast phenotyping tests is the identification of the proteins secreted into the matrix by 2D-PAGE. The results from 2 patients tested in this study were similar, with the secretory activity in the AHS group being greater than in the FBS group. More specifically, there appeared to be an increase in the production of osteonectin and collagen Type I (Fisher et al. 1987, Hankey et al. 1993). Osteonectin is one of the structural proteins synthesised by the osteoblast which interacts with both collagen and hydroxyapatite (Romberg et al. 1986). Although not bone-specific, this protein is accumulated in significant quantities in bone. It can, therefore be concluded that the cells treated with AHS exhibit an osteoblast phenotype.

In terms of cellular proliferation, the results showed that after an initial high [^{3}H] thymidine incorporation into DNA, the rate of incorporation started to decline on day 5, reaching a plateau between 7–9 days of culture. This is in accordance with the results obtained by Marie et al. (1989) who argued that this decline occurs when the cells become contact-inhibited. There was a consistently higher level of uptake by AHS supplemented cells than by FBS cells, suggesting a higher proliferation rate, or perhaps less contact inhibition.

Similarly, the results from the crystal violet assay indicated that an increase in cell numbers in both FBS and AHS cultures, but supplementation with AHS resulted in a greater increase in cell number.

In conclusion, this study has demonstrated that human osteoblasts grow more efficiently in autologous serum than in FBS, and a hierarchy of response was established: AHS, heat-treated > Pooled human serum, heat-treated and pooled human serum, untreated > AHS, untreated > FBS, untreated and FBS, heat-treated > Ultroser G. This has important implications, not just for osteoblast culture, but for any human cells grown for re-implantation.

Abbreviations used in this paper: AHS, autologous human serum; ALP, alkaline phosphatase; ht, heat-treated; 1,25 $(OH)_2D_3$, 1,25-dihydroxy vitamin D_3; 2D-PAGE, two dimensional polyacrylamide gel electrophoresis.

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