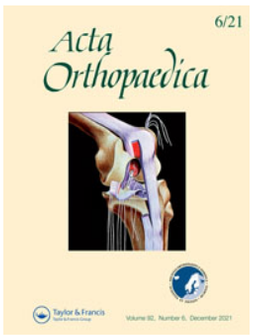

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In vitro osteoblast-like cell metabolism in spondylodesis—a tool that may predict fusion capacity

A prospective study in 50 patients with a 1-year follow-up

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ABSTRACT In vitro cultures of human primary osteoblast-like cells provide a model for studying cellular mechanisms associated with human bone biology. We investigated in vitro osteoblast-like cell metabolism as a method for predicting the occurrence of spinal fusion in the individual patient.

A bone biopsy was taken from the iliac crest of 50 patients, median age 49 (23–77) years, who were undergoing lumbar spine fusion. First-passage osteoblast-like cells were established by the bone-tissue-explant method. We then estimated ^{3}H-thymidine incorporation, alkaline phosphatase activity and procollagen I production. Fusion rates were evaluated at the 1-year follow-up.

Primary human osteoblast-like cell cultures showed an age-dependent decline in their capacity for cellular outgrowth and expression of alkaline phosphatase, which suggested a useful biological response pattern of the osteoblast culture. However, such cultures were unsatisfactory as an in vitro tool for predicting fusion capacity.

Despite progress in spinal instrumentation technologies, which have greatly improved the benefits of mechanical stabilization, the reported nonunion rates (0–40% (Gibson et al. 1999)) indicate that physiological, biological, and molecular events in the individual are essential to ensure graft-incorporation. Recently, Gould et al. (2000) provided considerable evidence that graft-derived cells from fresh bone grafts contributed to all stages in the fusion mass. An understanding of these factors may help to detect patients with poor fusion capacity and even modify the selection of patients who will undergo spinal fusion surgery. During the last two decades, the culture of osteoblast-like cells using primary cultures of untransformed cells derived from cancellous bone explants has become an important experimental model (Christensen et al. 1999, Kudelska-Mazur et al. 1999). These cultured osteoblasts provide a tissue-culture model for studying cellular and molecular mechanisms associated with bone biology (Termine et al. 1990, Fedarko et al. 1992, Pfeilschifter et al. 1993, Sutherland et al. 1995, Kassem et al. 1997, D'Ippolito et al. 1999, Katzburg et al. 1999). We hypothesized that primary human osteoblast-like cell metabolism in cultures could provide an in vitro biological model for predicting the fusion capacity in the individual patient. Therefore we assessed the predictive value of in vitro osteoblast-like cell cultures as regards the fusion capacity in patients undergoing lumbar or lumbosacral spine fusion.

Patients and methods

From January 1999 to February 2000, we obtained bone specimens from the iliac crest of 50 patients, 24 women, with a mean age 48 (23–77) years and

26 men, with a mean age 49 (26–71) years undergoing spinal fusion surgery. They all had severe chronic low back pain due to localized lumbar or lumbosacral instability caused by spondylolisthesis grades I–II (10 patients) or degeneration (40 patients). Posterolateral instrumented fusion or 360° instrumented fusion was performed in 36 and 14 patients, respectively.

Osteoblast-like cell culture

Cancellous iliac crest bone was obtained perioperatively, immediately stored in Earle's Minimal Essential Medium (MEM) at 4 ºC, and osteoblast-like cell cultures were started within 20–24 hours of harvest by the explant technique, as described elsewhere (Robey and Termine 1985). Briefly, trabecular bone fragments were cleaned of adherent tissue, cut into 1–2 mm pieces, and thoroughly washed in phosphate-buffered saline (PBS). The bone sample was digested with crude bacterial collagenase type IV (Sigma, USA) for 2 hours at 37 °C in an agitated water bath. After collagenase treatment, bone chips were washed in PBS, Earle's MEM w/o phenol red supplemented with Glutamax (later referred to as medium) (Life Technologies, DK) and then in medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biotech Line). Bone explants were cultured in 80 cm^2 culture flasks, in medium supplemented with 10% FCS, 0.1mg/mL streptomycin, and 100 U/mL penicillin G. The medium was changed to antibiotic-free 10% FCS after 24 hours and thereafter twice weekly. Cells from the individual bone-donor were grown in an incubator for 4 weeks at 37 °C in 5% CO_2 and 95% humidity. The cell cultures were examined twice a week under an inverted microscope. Cells were released from each culture by 120 sec. trypsinization (0.01% trypsin and 5 mM EDTA in PBS) (Amersham Pharmacia Biotech and Sigma) and agitation.

The cells from each culture were characterized by proliferation/mitogenic response for osteoblastic phenotype by procollagen type C-terminal propeptide (PICP) and osteoblast-like cell alkaline phosphatase (AP).

The cells used in the experiments were from the first passage. Assays were performed in triplicate as regards AP activity and ^{3}H-thymidine incorporation. The PICP assay was done in duplicate. Culture medium from cell cultures suspected of being infected and cultures without cell growth were all examined as regards bacterial contamination.

Cell count

The concentration of cells in all the cell suspensions after culture was determined with a hemocytometer.

DNA synthesis assay

Osteoblast-like cell proliferation and the mitogenic effect were studied by the incorporation of ^{3}H-thymidine into DNA. Cells were plated at a density of 1×10^4 in 96 well plates in 200 μL Earle's MEM containing 10% FCS and permitted to adhere for 24 hours. The medium was then changed to one with 1% or 10% FCS for an additional 48 hours to determine the mitogenic response of the cultures. (This response was estimated by the proliferation ratio in wells supplemented with 10% FCS and 1% FCS (cpm (10%)/cpm (1%)).

^{3}H-thymidine (25 μCi/mL) was added in the last 16 hours of the incubation. The incorporation of ^{3}H-thymidine into trichloroacetic acid-precipitable DNA was measured by liquid scintigraphy (ß-counter, Wallac). Intra-assay CV: 9.4%.

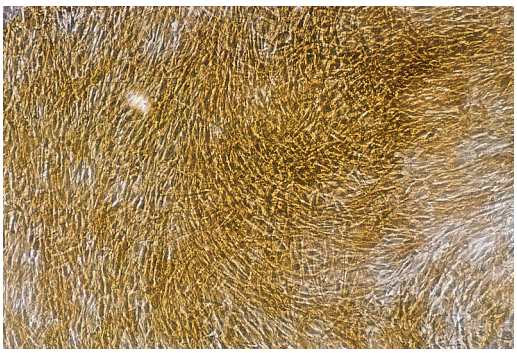
Alkaline phosphatase activity

To assess AP, osteoblast-like cells were plated in 96 well plates with 4×10^4 cells/well in 200 μL medium containing 10% FCS, and permitted to adhere for 24 hours. The culture medium was then changed to one of 1% and 10% for an additional 48 hours. AP activity was measured in the cell layer after 30 min. incubation with p-nitro phenylphosphate as the substrate at 37 °C (Sigma). Absorbance of p-nitrophenol was determined with a microspectrophotometer at 405 nm. Intra-assay CV: 4.6%.

Histochemical staining for AP was done randomly in the cultures (Figure). The cells were fixed and incubated with a substrate containing variamine blue B salt (Fluka) and sodium naphthyl-phosphate (Merck) at 4 °C for 5 min, and then counterstained with Mayer's hematoxylin

Procollagen type I synthesis

Procollagen type 1 C-terminal (PICP) propeptide was measured in the conditioned medium from 96



Histochemical staining for AP in an explant culture (×10). (Female donor, age 23 years).

wells (4×10^4 cells/well, 200 μL/well) after incubation for 48 hours in medium containing 1% and 10% FCS. PICP was quantitated in the conditioned medium, using a commercial radio-immune assay, with an antibody that recognizes procollagen C-terminal propeptide. (PICP (^{125}I) (Orion Diagnostica, Finland). The ^{125}I radioactivity was counted in a gamma counter (Wallac, Turku, Finland). Intra-assay CV: 9%.

Surgical technique

The PLF procedure (36 patients) included a posterior midline subperiosteal dissection, followed by posterior decompression—i.e., hemilaminectomy for neural decompression—as indicated, preserving as much of the stabilizing posterior structures as possible. Under C-arm image intensifier control, a supplementary rigid pedicle screw fixation was done, using the Cotrel-Dubousset (CD) instrumentation system. Autologous bone graft was harvested from the iliac wing shortly before the grafting procedure. Decortication and preparation of the posterolateral fusion bed was done before positioning the graft. In patients in whom a 360° fusion (14) was performed, the PLF procedure was followed by an additional anterior retroperitoneal approach to expose the lumbar segments to be operated on. The disc space was carefully emptied of all disc tissue, and a carbon-fiber (Brantigan) cage was filled with autogenous bone graft (ALIF). Patients were mobilized immediately after surgery and given a regimen consisting of 40 mg s.c. Enoxaparin daily until full mobilization to prevent thromboembolism. NSAIDs were not allowed during hospitalisation and patients were advised to avoid these preparations during the first 6 postoperative months.

RadŠlogy

At the 1-year radiographic follow-up, fusion rates and implant status were evaluated on plain anteroposterior and lateral radiographs by two independent observers (bone radiologist and orthopedic surgeon), using our classification system (Christensen et al. 2001). Each level and each side were judged separately, and continuous intertransverse bony bridges at one of the two sides or a continuous intervertebral bony bridge indicated the presence of fusion at that level. Doubtful fusion was indicated by a fusion mass hidden behind the instrumentation device or suboptimal quality of the fusion mass, and nonunion indicated no fusion at the intended fusion level. In patients with more than one segment subjected to intervention, "fusion" indicated fusion at all intended levels, "doubtful fusion" indicated doubtful fusion at one or more levels and "nonunion" meant definitely no fusion at one or more of the intended levels.

Each of the two observers completed a form using the classification. All identification labels on the radiographs were concealed and were given a random case number.

Smoking assessment

When the study was concluded, the patients received a questionnaire in the mail, asking them about their smoking habits during the first 6 postoperative months. They were asked to choose 1 of 5 answers: 1) none, 2) 5 cigarettes or less daily, 3) 6–10 cigarettes daily, 4) 11–20 cigarettes daily, 5) more than 20 cigarettes daily. 34 of the patients did not smoke, 12 smoked, and 4 patients gave no data concerning their smoking habits.

Statistics

Descriptive statistics are given as mean (SD) for normally distributed data and median (range) for material, which did not comply with the assumptions of normally distributed data. The Mann-Whitney U-test was used to compare the age-distribution in cultures ± cells and osteoblast-like cell metabolism within genders, smokers/nonsmokers and union/nonunion. The odds ratio (OR) was estimated within culture ± cells and ± infection for

Table 1. Distribution of fusion rates in cultures with (+) or without (–) cellular growth and infection

	Union n = 40	Nonunion n = 4	Doubtful n = 6
+ Cells	26	4	4
– Cells	7	0	1
Infection	7	0	1

No predictive value of (+)/(–) cell outgrowth in relation to union/nonunion rates

Table 2. Analysis of distribution of age, gender, tobacco consumption and fusion rates in cultures with (+) or without (–) cellular growth and infection

	+ Cells n = 34	– Cells n = 8	Infection n = 8
Donor age			
median	48	52	49
(range)	(23–77)	(43–71)	(26–65)
Gender (n)			
Female	16	3	5
OR		0.7	0.7
(95%CI)		(0.14–3.2)	(0.14–3.2)
Male	18	5	3
OR		2.0	0.5
(95%CI)		(0.4–8.8)	(0.1–2.3)
Tobacco (n)			
0–5 cigarettes	24	6	4
OR		0.75	0.3
(95%CI)		(0.1–4.7)	(0.05–1.3)
> 5 cigarettes	6	2	4
OR		1.3	3.8
(95%CI)		(0.2–8.3)	(0.7–18)
missing data	4	–	–
Fusion rates (n)			
union	26	7	7
nonunion	4	0	0
doubtful	4	1	1

OR – Odds ratio.
(95%CI) – 95% confidence interval.

the dichotomous variables of gender, smokers/nonsmokers and union/nonunion.

Osteoblast-like cell assays. Values used in the statistical tests were based on single weight data for each parameter (mean of triplicate (AP and ^{3}H-thymidine) or duplicate (PICP) wells) in each individual. The Wilcoxon test was used to compare the mitogenic effect of the FCS concentration, 10% vs. 1% and Kendall's tau-b rank correlation test to assessassociations between osteoblast-like cell metabolism (10% FCS) and age. The data were significant when $p < 0.05$ (two-tailed). The statistical analyses were done using SPSS/PS 6.1.3

Ethics

The trial was approved by the Medical Ethics Committee of Aarhus County, J. no. 1997-3872 and was done in accordance with the recommendations of Helsinki Declaration II. Before inclusion, patients gave their written informed consent.

Results

Explant cultures from all 50 patients were started. Osteoblast-like cell growth occurred in 34 cultures, 8 cultures few, if any, cells and 8 cultures became infected. The cell cultures, examined twice a week with an inverted microscope, showed that the cells growing from the explants initially were thin and spindle-shaped, but became larger at near-confluence. In some of the cultures containing only a few cells from elderly donors, the cells were large and irregularly shaped.

According to our radiographic classification (Christensen et al. 2001), "successful radiographic fusion" was seen in 40 (36 PLF) (4 ALIF+PLF), "doubtful fusion" in 6 (4 PLF), (2 ALIF+PLF) and "nonunion" in 4 (2 PLF), (2 ALIF+PLF) of the patients.

Bone explant cultures by means of cell growth were of no value as regards the prediction of fusion capacity in patients undergoing spinal fusion surgery. 7 of the cultures from the 40 patients with "union" contained few, if any, cells. In contrast, all explant cultures from patients with "nonunion" showed cell growth (Table 1). The distribution of patients concerning age, gender, smoking and fusion, in cultures ± cells revealed no disparities, except age-distribution. In cultures without cell growth, the median age and the lower limit in the age range of the explant donors tended to be higher than in cultures + cell outgrowth ($p = 0.08$). In explant cultures from patients who smoked, 4 of 12 cultures were infected (OR = 3.8), as compared to 4 of 34 cultures in nonsmokers (Table 2).

In explant cultures, in which the number of cells was sufficient for assays, a significant mitogenic effect was found in the proliferation rate, AP activity and PICP production, as regards the concentration of FCS, 10% and 1%, respectively

Table 3. Osteoblast–like cell metabolism in 34 explant donors. Values are median (total range)

	^{3}H–thymidine [a] cpm	AP activity [a] nmol/10^4/min	PICP [a] μg/L	Cell count (×10^5) Primary culture
1% FCS	237 (898)	2.3 (14)	175 (340)	–
10 % FCS	820 (5771)	2.8 (15)	330 (463)	8.26 (36)
Age-correlation (r)	–0.15	–0.29	–0.05	–0.35
p-value	0.3	0.02	0.8	0.01
Mitogenic response [b]	2.9 (13)	–	–	–

[a] First passage
[b] Mitogenic response: cpm (10% FCS/1% FCS)

Table 4. Osteoblast-like cell metabolism in 34 donor cultures according to gender, tobacco consumption and fusion rates. Values are median (total range)

	n	Age	Cell count (×10^5)	Proliferation cpm [b]	Mitogenic [a] response	AP activity [b] nmol/10^4 s/min	PICP [b] μg/L
Gender							
Female	16	47 (54)	9.2 (29)	818 (5771)	3.1 (12.8)	3.4 (14)	331 (463)
Male	18	48 (35)	12 (36)	820 (1080)	2.7 (4.9)	2.8 (14)	330 (400)
Tobacco							
0–5 cig.	24	49 (54)	5.7 (36)	831 (5740)	3.0 (12.5)	2.5 (6.1)	301 (463)
>5 cig.	6	47 (24)	12 (21)	818 (1290)	2.3 (6.5)	2.2 (14)	384 (279)
missing	4						
Fusion							
union	26	49 (46)	9.7 (36)	818 (1270)	2.6 (12.7)	2.5 (15)	302 (415)
nonunion	4	47 (26)	19 (19)	818 (1570)	2.2 (2.9)	9.3 (10)	361 (431)
doubtful	4	47 (24)	4.4 (3.7)	2588 (4988)	4.0 (3.0)	3.0 (1)	597 (444)

[a] Mitogenic response: cpm (10 % FCS / 1 % FCS)
[b] (10 % FCS)

($p < 0.0001$). We found that the osteoblast-like cell count and the baseline alkaline phosphatase activity were inversely correlated to an increase in the age of the patients. The proliferation rate, mitogenic response and PICP production showed a negative correlation with patient's age, which was not significant (Table 3).

Descriptive data concerning osteoblast-like cell metabolism in relation to gender, smokers/non-smokers and fusion rates are shown in Table 4. In these groups, no statistical difference was seen as regards age-distribution and osteoblast-like cell metabolism.

Discussion

To find predictors, which can help to detect patients who are less able to incorporate a graft in autografted fusions, an assessment of the osteogenic capacity in bone cells of an individual may be of value. Osteoblast-like cell cultures have been investigated extensively and their osteoblast-like characteristics described—i.e., osteocalcin, alkaline phosphatase, and collagen type I production and their response to osteotropic growth factors and hormones (Bellows et al. 1990, Fedarko et al. 1990, Lian and Stein 1995, Kassem et al. 1997, Rattner et al. 1997, Siggelkow et al. 1999a, b).

In this study, we established in vitro osteoblast-like cell cultures by the tissue-explant method, and were therefore able to obtain cultures from limited bone resources and with phenotype characteristics of osteoblasts—i.e., mitogenic-inducible alkaline phosphatase activity and collagen type 1 production. The risk of confounding by various experimental factors was reduced so far as possible by the peroperative handling, and control-

ling the temperature, medium and the durtion of storage. Moreover, the cultures were maintained for 4 weeks, thereby obviating the bias, which would otherwise have been introduced by a subjective determination of confluence. In addition, deviations as regards the criteria for radiological outcome were reduced to a minimum, as the fusion rates were evaluated on anteroposterior and lateral radiographs by two independent observers in accordance with our standardized classification system (Christensen et al. 2001).

Although only a few patients developed "nonunion", this in vitro model failed to predict fusion capacity in the individual, using the current study design—i.e., cellular growth did not occur in 7 explant cultures from patients with "union". On the other hand, all explant cultures from patients with "nonunion" showed cellular growth. Furthermore, the culture of primary human osteoblast-like cells had experimental limitations as regards the scope of the current study, because of the slow rate of cellular growth, which in addition to being time-consuming, increased the vulnerability of the cultures to infection. This vulnerability, however, was most marked in cultures from the group of smokers, with an odds ratio of 3.8. A hypothetical assumption would be an increased risk of infection due to tobacco-related changes in explant cultures, since tobacco consumption has been reported to be a significant risk factor as regards the development of postoperative infections (Thalgott et al. 1991, Capen et al. 1996). However, 2 of the cultures were infected simultaneously, and in this case, the risk of bacterial contamination in cultures was obvious. Secondly, none of the patients developed clinical signs of infection.

Cultures, in which a sufficient number of cells for assays were produced, showed that osteoblastic cells derived from iliac crest trabecular bone showed a significant age-dependent decline in cellular growth and alkaline phosphatase activity. However, collagen type 1 production, proliferation capacity and mitogenic response did not decline significantly with age. Although osteoblast-like cell metabolism within genders, smokers/nonsmokers and patients with union/nonunion was not significant, a type 2 error can not be excluded.

The negative correlation between cellular growth and an increase in age was shown in a study by Evans et al. (1990). This finding, however, was not reflected by the proliferation capacity, as would have been expected, and was at variance with the results of studies by Christensen et al. (1999). An explanation may be the small size of the sample due to the limitations of experiment. Differences caused by age in the expression of AP activity were apparent, since AP declined with the age of the bone donors, an observation which accords with that of other studies (Pfeilschifter et al. 1993, Sutherland et al. 1995). Sutherland et al. suggested a defect in the synthesis of AP mRNA in older donors, because they found an age-dependent decline in AP mRNA levels. Collagen type 1 production did not decrease with age in our study, which corresponds to the results of Fedarko et al. (1992). They found that collagen type 1 reached a maximum in cells obtained in puberty and declined to 1/3 of the maximum levels after the age of 20 (Fedarko et al. 1992). However, Koshihara and Honda (1994) found that procollagen type 1 mRNA levels increased up to the age of 65 years and fell thereafter.

The literature contains conflicting reports about the effect of age on osteoblastic function. While age-related reductions in metabolic activity (Chavassieux et al. 1990, Fedarko et al. 1992, Pfeilschifter et al. 1993, Sutherland et al. 1995, Christensen et al. 1999) and proliferative ability (Termine 1990, Fedarko et al. 1992, Pfeilschifter et al. 1993, D'Ippolito et al. 1999) have been reported, other studies report no age-related effect (Chavassieux et al. 1990, Evans et al. 1990, Katzburg et al. 1999). Uncontrolled basic experimental conditions, such as the number of passages, confluence stages, various sources of bone tissue and small sampling sizes in some studies may have contributed to these discrepancies (Chavassieux et al. 1990, Matsuyama et al. 1990, Pfeilschifter et al. 1993, Schmidt and Kulbe 1993, Kassem et al. 1997, Rattner et al. 1997, Siggelkow et al. 1999b).

In our study, in vitro osteoblast-like cell cultures showed an age-dependent decline in osteoblast-like cell outgrowth, rather than the fusion capacity in the individual. This finding may indicate that the failure to incorporate a graft is mainly affected by other factors. However, on the basis of our previous study in which the fusion capacity depended significantly on the amount of the bone graft, it

is possible that an age-dependent reduction in osteogenic capacity can be compensated by using a large enough graft (Laursen et al. 2002).

No competing interests declared.

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