The influence of human intervertebral disc tissue on the metabolism of osteoblast-like cells

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ABSTRACT – Extensive studies have been performed to evaluate different factors that may affect on spinal interbody fusion, but the role of intervertebral disc tissue in the fusion process remains unclear.

To study the influence of intervertebral disc tissue on osteoblast metabolism, we harvested disc tissue from patients who had undergone spinal surgery. The nucleus pulposus and annulus fibrosus were separately co-cultured with osteoblast-like cells SaOS-2 by means of culture inserts or organ culture. We assayed alkaline phosphoatase activity, ^{3}H-thymidine incorporation into the DNA, and production of collagen type I, IL-1β, IL-6, IL-10, and TNF-α.

Exposure of the nucleus pulposus (NP) to osteoblast-like cells revealed stimulation of alkaline phosphatase production, ^{3}H-thymidine incorporation and collagen type I production. Exposure of the annulus fibrosus (AF) stimulated ^{3}H-thymidine incorporation and collagen type I production, but did not affect ALP activity. IL-6 was detected after application of NP and AF. Interleukin IL-10, IL-1β and TNF-α were all below detection levels after application of disc tissue.

Our findings show that frozen disc tissue stimulates the metabolism of osteoblast-like cells in vitro.

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The NP and AF cells represent two kinds of cells present in intervertebral disc tissue. The NP is surrounded by gelatinous proteoglycan matrix, and the AF is rich in concentric collagen fibers. They function as a unit to protect the vertebrae from compressive, rotational, and tensile stresses (Humzah and Soames 1988). Both NP and AF cells secrete the aggrecan matrix and collagens. They show a phenotype close to that of chondrocytes (Olmarker et al. 1993, 1997).

The intervertebral disc has attracted attention due to its direct cause of lower back pain and nerve root injury in pathological conditions (Nachemson 1981, Yasuma et al. 1990). Research has focused on its neurological effects. NP can reduce the nerve conduction velocity of the adjacent nerve roots, when applied epidurally; furthermore, application of vital or dead NP cells have the same effect on nerve roots (Olmarker et al. 1993, Kayama et al. 1998). Little is known about its effect on spinal fusion rates. Interbody spinal fusion has been performed extensively in clinical practice, but the fusion rate varies from 52% to 96%, regardless of the method used (Roye and Farcy 1987).

A discectomy is a conventional procedure prior to intervertebral fusion. With the development of minimal invasive operations, such as laparoscopic anterior spinal fusion with threaded cages, disc tissue may not be removed completely. Disc tissue, especially NP, has been reported to possess inflammatory properties (Olmarker et al. 1995, Gertzbein et al. 1977) and to secrete cytokines (Rand et al. 1997), which may affect the metabolism of osteoblasts (Zheng et al. 1992). Furthermore, exposure of NP to the fusion environment may cause immune reactions (Gertzbein et al. 1977, Pennington et al. 1988, Kanerva et al. 1997). Therefore, we sought a possible effect of human disc tissue on osteoblast-like cell proliferation and differentiation in vitro.

Material and methods

Preparation of nucleus pulposus

We obtained nucleus pulposus from 16 patients who had undergone spinal surgery due to degenerative disease or scoliosis (Table 1). The patients had no systemic immune disease or metabolic bone disease, as determined by their medical history, serum biochemistry and radiographs of the spine. Immediately after removal of the disc tissue, it was transferred to the laboratory and frozen at -80 °C until the experiments were started.

Table 1. Patient data

Case	Age	Sex	Diagnosis	Operation level
1	34	M	Lumbar vertebral fracture	L3–L5
2	15	F	Scoliosis	T5–T10
3	41	M	Juvenile scoliosis	T10–L3
4	19	M	Neuromuscular scoliosis	T6–T10
5	41	M	Osteochondrosis	L1–L5
6	12	F	Congenital scoliosis	Thoracolumbar
7	40	F	Idiopathic scoliosis	T10–L4
8	15	F	Idiopathic scoliosis	T10–L2
9	52	F	Spondylolisthesis	L5–S1
10	45	F	Intervertebral disc degen.	L4–S1
11	26	F	Spinal deformity	T6–L1
12	65	M	Spinal stenosis	L3–L4
13	18	M	Kyphosis	T9–T12
14	16	F	Kyphosis	Thoracolumbar
15	20	F	Scoliosis	T7–T10
16	31	F	Lumbago	L5–S1

Osteoblast-like cell culture

We used the SaOS-2 osteosarcoma cell line in this experiment. Cells were cultured in Nunc culture flasks (Nunclon, Nunc A/S, Roskilde, Denmark) with McCoy's 5A medium (Gibco BRL, Grand Island, NY) supplemented with penicillin and streptomycin (100 μ/mL, 100 μ/mL, respectively) and 15% fetal calf serum. The cells were incubated at 37 °C in a humidified (95%) atmosphere with 5% CO_2. Culture medium was changed twice weekly. After the cells had become confluent, they were trypsinized and counted with a hemocytometer.

Application of disc tissue to the culture of osteoblast-like cells

SaOS-2 cells were seeded in 24-well plates at a density of 5000 cells/well in 1 mL McCoy's medium supplemented with 15% FCS and allowed attachment for 24 hours. Then the medium was changed to McCoy's 5A without serum before adding the disc tissue. This tissue was rinsed twice with PBS, and NP and AF were visually identified and separated. NP and AF were cut into small pieces in a Petri dish and weighed. It was applied in two ways: 1. Directly put into the medium. If there was an adequate amount of NP tissue, it was divided into two parts: NP1 (0.2 g) and NP2 (0.3 g). AF was always applied directly to the medium. 2: in a 10 mm tissue culture insert (Anopore, Nunc A/S, Denmark) NP_{in} (0.2 g). The culture insert separated the disc tissue from the SaOS-2 cells by a porous membrane. The porous membrane we used had a pore size of 0.2 μm, which permits the exchange of proteins and molecules but not cells. Since our main interest was NP tissue, only NP was put into the inserts for the test. The co-culture was maintained for 48 hours, then the ALP activity and the cell proliferation assays were performed in the cell layer. Medium was collected and stored at –80 °C, pending assays for collagen type I and interleukins.

Alkaline phosphatase activity

After a 48-hour co-culture of osteoblast like cells with disc tissue, we determined ALP activity in the cell layer by incubating with p-nitrophenyl phosphate substrate for 20 minutes. The p-nitrophenolate produced was assessed spectrophotometrically at 405 nm, using an automated microtiter plate ELISA reader. ALP concentrations were calculated from the standard curve of substrate, and expressed in μmol/mL, which means μmol of p-nitrophenol per milliliter (or per well) after 20 min of incubation at 37 °C. Culture wells were in triplicates. Wells without the disc tissue were used as controls.

Cell proliferation

We determined the cell proliferation by ^{3}H-thymidine incorporation. 16 hours before stopping the co-culture, 25 μCi/mL ^{3}H-thymidine (Lifescience, Amersham) were added to the plate with 125 μL per well. The ^{3}H-thymidine incorporation in DNA was measured in the cell layer after being dissolved with 0.5N NaOH. An aliquot from each well was added with 3 mL of scintillation fluid

Table 2. Results

	Control	NP_{in}	NP_1	NP_2	AF
ALP (μmol/mL)	0.04 (0.02) n 15	0.07 (0.04) n 9	0.06 (0.03) n 13	0.06 (0.02) [a] n 9	0.07 (0.05) n 9
^{3}H-T incorporation (cpm)	4867 (3821) n 10	8765 (6640) [a] n 10; p = 0.002	10543 (6783) [a] n 14; p = 0.0007	8880 (5215) [a] n 8; p = 0.04	9647 (7328) [b] n 8; p = 0.008
Collagen I (μg/L)	2.1 (1.4) n 10	15 (11) [a] n 11, p = 0.03	25 (27) [c] n 9, p = 0.001	85 (77) [c] n 7, p = 0.001	132 (105) [c] n 5, p = 0.002
IL-6 (Pg/L)	< detectable level n 16	37 (58) n 3	60 (115) n 8	51 (85) n 4	1.3 (1.6) n 2

ALP is expressed as μmol/mL of p-nitrophenolate per mL (or per well) after 20 min of incubation at 37 °C
[a] Significant difference was found with the paired t-test (test of normality and equal of variance passed)
[b] Wilcoxon signed rank test was used when test of normality passed but the equal of variance failed.
[c] Mann-Whitney rank sum test was used when test of normality failed.
Difference in numbers between ALP and ^{3}H-T groups is due to lack of sufficient amount of disc tissue from each patient. The numbers shown in collagen I and IL-6 groups are positive detectable numbers.

and counted by a liquid scintillation counter. Culture wells were in triplicates, and the averages were used for the statistical analysis.

Collagen synthesis

Collagen synthesis was determined by the production of collagen type I carboxyterminal peptide (PICP) released in the medium. The PICP levels were measured with a radioimmunoassay kit (Orion Diagnostica, Espoo, Finland) and the ^{125}I radioactivity was counted by gamma counter (Wallac, Turku, Finland).

Cytokines assay

IL-6, IL-10, IL-1β and TNF-α were analyzed in a culture medium using ELISA kits (Quantikine, R&D Systems Europe).

Statistics

Values are given as mean (SD). Comparisons of different conditions were made using the paired t-test when test of normality and equal variance passed, otherwise, the Mann-Whitney rank sum test or Wilcoxon signed rank test was used. Results were considered significant when the p-value was less than 0.05. Every culture was in triplicate wells, so each value evaluated by statistical testing is a mean (SD) of the triplicate.

Results (Table 2)

Alkaline phosphatase activity

The ALP activity of osteoblasts showed no significant change in the presence of NPin (weight 0.2 g) by means of culture inserts (n 9, p = 0.515).

9 patients had enough disc tissue that could be separated into two groups: NP1 (0.2 g) and NP2 (0.3 g). No difference was found between them in their action on ALP activity of osteoblast-like cells (p = 0.5), but NP2 stimulated ALP activity, compared to the control group (p = 0.007) while NP1 did not (p = 0.1).

AF (wt 0.2 g, n 9) did not affect ALP activity when compared to the control (p = 0.9).

Proliferation

In the culture inserts group, NPin (0.2 g) enhanced SaOS-2 proliferation by increasing the 3H-thymidine incorporation (n 10, p = 0.002). For direct organ co-culture, NP1 (0.2 g) showed enhancement of ^{3}H-thymidine incorporation, compared to the control (p = 0.0007). When a higher amount was added, NP2 did not differ from NP1 in its action on the osteoblast (n 8, p = 0.8), but it also showed a stimulating effect, compared to the control (p = 0.04). AF (n 8) also increased cell proliferation (p = 0.008).

When age was taken into consideration, samples from the older (above 30 years, n 6) and younger (below 30 years, n 7) patients all showed

enhancement either by means of inserts (p = 0.04 and 0.009, respectively) or by organ culture (p = 0.02 and 0.007, respectively).

Collagen type I production

The collagen type I level increased after the application of NP in the inserts (p = 0.03, n 7). The same significant results were found in the NP1, NP2 and AF groups (Table 2).

IL-1ß,IL-6,IL-10 and TNF-α production

IL-6 was detected in medium samples from 10 patients. 9 of these were from co-culture medium with NP (NP_{in}, NP_1, and NP_2) tissue, 1 from AF, and none from the controls. The highest level of IL-6 was seen from patient number 10 who had a diagnosis of lumbar intervertebral disc degeneration. IL-10, IL-1β and TNF-α were not detected in the medium of both controls and co-cultures.

Discussion

We found that when disc tissue was applied to the osteoblast culture, the osteoblast growth was stimulated, as shown by increased ALP activity, collagen type I production, and ^{3}H-thymidine incorporation. We found IL-6 but not IL-10, IL-1β and TNF-α in the co-culture medium. NP tissue put into the culture inserts showed the same results on SaOS-2 cells as that of direct contact in small amount (NP_1).

Although the disc tissue we used was frozen, the NP and AF cells may already have been lysed after thawing. The bioactive mediators like interleukins, enzymes and chemical components should still be preserved (Olmarker et al. 1997). These mediators or protein molecules may be responsible for the overall effect of increased ALP activity and osteoblast proliferation. This is supported by our culture insert method, in which the communication between disc tissue and osteoblasts is effected through a 0.2 μm pore-size membrane.

Both osteoblast and disc cells can secrete cytokines. We detected IL-6 in the co-culture medium, but the levels of IL-10, IL-1β, TNF-α were below detection. Either too little disc tissue was placed in each well or the frozen disc tissues did not have secretion potential, which could account for the undetected interleukins. It is difficult to say which cell type plays a major role in this IL-6 secretion. Nucleus pulposus tissue could have contained IL-6 that entered the medium during the co-culture. SaOS-2 cells could also secrete IL-6, and could be enhanced by some unknown stimulators in the disc tissue. Indeed, the highest concentration of IL-6 was found in the co-cultured medium of the disc that was taken from a patient with disc degeneration (patient number 10). The interaction between IL-6 and osteoblasts is very complicated (Fang and Hahn 1991, Littlewood et al. 1991), and we found no correlation between specific interleukin levels and osteoblast proliferation in this experiment.

The collagen type I level in the culture medium increased after the application of disc tissue. Although most of the collagen that NP and AF cells produce is collagen type II, there may still be some collagen type I present in the matrix (Chiba et al. 1997 ,Wang et al. 1997). Thus it is not certain that this increased type I collagen level is contributed by SaOS-2 or the disc matrix.

^{3}H-thymidine incorporation increased greatly in the disc tissue groups (NP_{in}, NP_1 and NP_2), so there may be a difference in the cell numbers from the controls. Thus the increase in ALP and interleukins could also be due to increased cell numbers. We used human osteosarcoma cell line SaOS-2 in our study. This cell line possesses the main features of an osteoblast (Rodan et al. 1987), and it has been used in many in vitro studies(Farley et al. 1989, Farley et al. 1994, Holbein et al. 1995). Owing to difficulties due to general availability and time-schedule coordination in obtaining fresh human intervertebral disc tissue and cancellous bone chips, we used frozen disc tissue and an always-ready cell line. More studies should, of course, be done on primary human osteoblasts.

The patients' ages ranged from 12 to 65 years. They were of different ages because we wanted to observe the different effects of normal and degenerated disc tissue on osteoblasts. A comparison of the disc tissue from the two patient groups above and below 30 years of age showed the same effect on the osteoblasts, as judged by the ALP activity and ^{3}H-incorporation.

It is still too early to say what the in vivo effect of intervertebral disc tissue on bone healing may be. Given the influence of an immune system, in vivo effects of disc tissue may be more complex, and the results may differ from those of an in vitro study. Our preliminary results of an in vivo study on pigs showed that the spinal fusion was not affected when the autogenous bone graft was implanted together with NP (unpublished data). The precise relation of the disc to bone interaction requires further studies on the inflammatory mechanism of disc cells.

In conclusion, our data suggest that NP and AF tissue can mediate a stimulating effect on osteoblast-like cells in vitro.

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