

Cytokine profiles in conditioned media from cultured human intervertebral disc tissue

Implications of their effect on bone marrow stem cell metabolism

Haisheng Li, Xuenong Zou, Anette Baatrup, Martin Lind and Cody Büngrer

Orthopaedic Research Laboratory, Orthopaedic Department E, Clinical Institute, Aarhus University Hospital, Nørrebrogade 44, DK-8000 Aarhus C, Denmark

Correspondence HL: haisheng.li@ki.au.dk

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Background Cytokines released from intervertebral discs cultured in vitro have not been profiled, and the effect of these cytokines on human bone marrow stem cells is yet to be studied.

Materials and methods Intervertebral discs from 14 patients who had undergone spinal fusion surgery were cultured separately in vitro. Conditioned media were collected after 48 and 96 h of culture in serum-free Minimum Essential Medium (MEM). Profiling of the cytokines was conducted using pooled media. Conditioned medium from each patient was also tested in human bone marrow stem cell culture, and incorporation of alkaline phosphatase and ^3H -thymidine incorporation was evaluated.

Results Of the 18 cytokines screened, 12 were found to be positive, but only eotaxin, IP-10, Rantes IL-6 and IL-8 seemed to be present at high levels. There was a close correlation between IL-6 and IL-8 levels in the medium ($R = 0.90$, $p < 0.001$). When the conditioned media were added to human bone marrow stem cell cultures, cellular proliferation was stimulated ($p = 0.02$), but alkaline phosphatase activity remained unchanged. Cellular proliferation correlated negatively with IL-6 levels ($R = -0.44$, $p = 0.04$).

Interpretation Intervertebral discs secrete certain cytokines into the medium when cultured in vitro, and conditioned media from cultured intervertebral discs stimulate proliferation of bone marrow stem cells.

Total resection of intervertebral disc tissue is an important procedure in spinal interbody fusion, but remnants of disc tissue may still remain in the disc space, especially when a minimally invasive approach has been employed (Riley et al. 1997).

Herniated or in vitro cultured intervertebral disc tissue, especially nucleus pulposus (NP), has been shown to produce proinflammatory cytokines (Kang et al. 1997, Rand et al. 1997, Brisby et al. 2002, Burke et al. 2002a). These proinflammatory cytokines have been shown to be involved in the pathophysiology of NP-induced nerve root injury and the spontaneous resorption of herniated disc (Kawakami et al. 1998, Olmarker and Larsson. 1998, Burke et al. 2002b). Cytokine and chemokine networks are implicated in stem cell proliferation and differentiation (Moore 2002). Using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique, Kawaguchi et al. (2002) detected MCP-3, MCP-4, RANTES, and IP-10 in disc material from idiopathic scoliosis, and MCP-3, MCP-4, RANTES, IP-10, MIG and MGSA- α were detected in herniated human intervertebral discs. However, the cytokines released from cultured human intervertebral discs have not yet been characterized.

We screened 18 kinds of cytokines in conditioned media from cultured human intervertebral discs in order to profile these cytokines and to find possible differences between the profiles of younger and older patients. The conditioned media were further tested in human bone marrow stem cell cultures in

an attempt to verify their involvement in affecting the metabolism of bone cells.

Materials and methods

Intervertebral disc tissue

We obtained intervertebral disc tissue from 14 patients (7 males) who underwent spinal fusion surgery as a result of spinal deformity or degenerative disorders. The average age was 28 (14–48) years. Patients were classified into 2 groups: (a) younger patients with normal intervertebral discs, the diagnoses being idiopathic scoliosis (3), juvenile scoliosis (3) and secondary kyphosis (1), and (b) older patients with intervertebral disc degeneration, including discogenic pain (5) and spondylolisthesis with disc degeneration (1). The presence of disc degeneration was confirmed by MRI. Upon removal of the disc tissue, the nucleus pulposus (NP) and annulus fibrosus (AF) were separated by the surgeon and immediately transported to the laboratory in sterile tubes containing 25 mL phosphate-buffered saline (PBS) containing antibiotics (penicillin 100U/mL and streptomycin 100 µg/mL).

Preparation of the conditioned medium

Each of the intervertebral disc tissues, together with 25 mL PBS, were weighed and the results recorded. (The tubes with the 25 mL PBS had been pre-weighed). Each was cut into small pieces (about 3 mm³) with a pair of scissors, placed in a Petri dish and subsequently washed 3 times with PBS (containing penicillin 100 u/mL and streptomycin 100 µg/mL) in order to eliminate possible interference from serum and growth factors in the blood. The washed tissue pieces were then cultured for 48 and 96 h at 37°C (95% humidity) in serum-free Minimum Essential Medium (MEM) in 75cm² culture flasks with a density of about 1.0g tissue/5 mL medium.

The resulting conditioned medium was collected after 48 h and centrifuged at 1 200 rpm for 10 min. The supernatant was next put into sterile tubes in aliquots (1.5–2 mL) and stored at –80°C until use. The precipitated disc tissue was cultured for another 48 h in fresh serum-free MEM as before, and the conditioned medium was again collected

(as supernate) after 96 h.

Human bone marrow stem cells

The human bone marrow stem cells used (hMSC-TERTS; kindly provided by Prof. Kassem, Department of Endocrinology, University Hospital of Odense, Denmark) were bone marrow stromal cells (hMSCs) from a 33-year-old male and had been transduced by a retroviral vector containing the gene for human telomerase (hTERT). The gene modification inhibits cellular changes due to ageing. To date, the cells have undergone more than 260 population doublings and have continued to maintain the production of osteoblastic markers and differentiation potentials during continuous subculture (Simonsen et al. 2002). In contrast to cell lines such as SaOS-2 which came from tumor cells, hMSC-TERT cells have a normal karyotype.

Cytokine array assay in pooled medium

In order to obtain an overall profile of cytokines from different patients, samples of media from each patient were pooled into 2 groups: those of younger patients (mean age 17.4 years, SD 4.3, n = 5) and those of older patients (mean age 38.4 years, SD 8.4, n = 5). In both groups, only media with clear coloration during the culture process were pooled. Conditioned media from NP and AF were pooled separately. Cytokines were screened using the TransSignal human cytokine antibody array (Panomics Inc., Redwood City, CA, USA). Based on the sandwich ELISA method, 18 kinds of cytokines were screened simultaneously. Each array membrane containing immobilized capture antibodies for the cytokines was incubated with 2 ml of conditioned medium. Thereafter, the biotin-conjugated secondary antibody was introduced, which created a “sandwich” around specific cytokines. Finally, by means of a biotin-streptavidin interaction, enzyme-conjugated streptavidin complexed to the the antibody-protein complex was detected by a chemiluminescent image system and quantified using LabWorks software ((UVP Inc., Upland, CA, USA, version 4.0) and the UVP BioImaging System. According to the manufacturer, the array consistency between the same spots on 2 of the same type of membrane was between 0 and 10%.

Cytokine assay with ELISA

Following the cytokine screening, IL-6 and IL-8 were selected because differences in levels of the 2 cytokines had been found in NP from younger and older patients. The 2 cytokines were individually determined by ELISA testing of the conditioned media from NP and AF after 96 h of cultivation (Quantikine ELISA kits, R&D System, Abingdon, UK). The procedures used conformed to the manufacturer's guidelines. All samples and standardized solutions were analyzed in duplicate. According to the manufacturer, the intra-assay variation is less than 3.4%, and the sensitivity is 0.2 pg/mL.

Application of conditioned medium to hMSC-TERT cell cultures

Conditioned media from each of the 14 patients were tested individually with the hTERT cells. Only media from 96 h of culture were tested. Human MSC-TERT cells were seeded at a concentration of 2 000 cells/well in 96-well plates with 200 μ L MEM containing 10% FCS. The cells were allowed to adhere for 24 h, after which the medium were replaced with fresh medium containing 1% FCS. 100 μ L conditioned medium and 200 μ L MEM was added to each of the test wells, while 300 μ L MEM was added to the control wells. Cultures were terminated after 2 days for cell proliferation test, and after 5 days for alkaline phosphatase activity assay.

Evaluation parameters

Alkaline phosphatase (ALP) activity. ALP activity in the cell layer was determined by incubation with p-nitrophenylphosphate as a substrate for 30 min at 37°C. The p-nitrophenolate produced was assessed spectrophotometrically at 405nm using an ELISA reader for microtiter plates. Values for cell number were adjusted by counting the cells in similar wells.

Cell proliferation. Cell proliferation was determined by ^3H -thymidine incorporation. 20 h before terminating the culture, 25 μ L of 25 $\mu\text{Ci/mL}$ ^3H -thymidine (Amersham Pharmacia Biotech UK limited, Buckinghamshire, England) was added to each well in the plate. The incorporation of ^3H -thymidine into DNA was measured in the cell layer after treatment of the cells with 0.5N NaOH. An aliquot from each well was added to 3 mL scintil-

lation fluid and the radioactivity was counted in a liquid scintillation counter (Wallac, Finland).

Statistics

Data are presented as mean and SD. Student's t-test was employed when data were normally distributed with an equal variance. Mann-Whitney test was used for data that were either not normally distributed or showed unequal variance. Spearman rank correlation test was used for correlation analysis. Significance was assumed at $p < 0.05$.

Results

Cytokine array assay

Of the 18 kinds of cytokines and chemokines being tested for in the array, 12 were found to be positive (Figure 1). High levels of Eotaxin, IP-10, and RANTES were detected in the pooled conditioned media from both NP and AF. MIP-5, IL-12, leptin, IL-4, TNF- α , GM-CSF, and MIP-1 α were detected at low levels from both NP and AF.

There was a difference in IL-6 and IL-8 levels in the pooled media from NP between younger and older patient groups, while in the media from AF there were equally high levels of both at 48 h. The difference in IL-6 and IL-8 levels between the younger and older patient groups was still evident at 96 h (Figure 2). However, the levels of cytokines released from the NP had decreased in the pooled media at 96 h (Figure 3).

ELISA assay of IL-6 and IL-8

When IL-6 and IL-8 were measured in the media from NP and AF taken at 96 h for each patient, the difference between the younger and older patients, which had been found in the pooled media from NP using array membranes, was not statistically significant (IL-6, $p = 0.9$; IL-8, $p = 0.3$). There were large variations in the levels of IL-6 (mean 23, SD 35) and IL-8 (mean 583, SD 1252) in the media from NP. The media from AF also showed no difference in terms of IL-6 and IL-8 levels between younger and older patients (IL-6, $p = 0.9$; IL-8, $p = 0.5$).

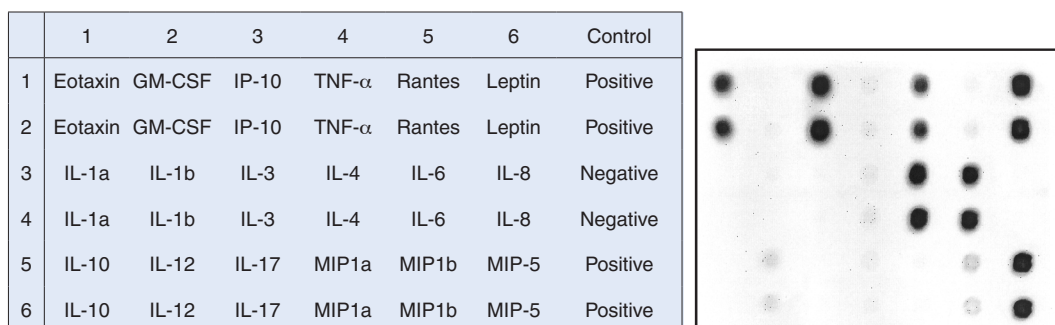


Figure 1. Left frame: configuration of the cytokine array. Right frame: result (chemiluminescence image) from pooled media from NP-derived medium taken at 48 h. The NP were from the older patient group. Eotaxin, IP-10, Rantes IL-6 and IL-8 appear as dense dots.

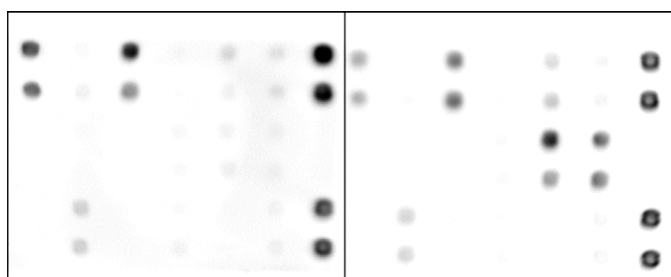


Figure 2. Array images showing cytokine levels at 96 h in the pooled media from NP taken from younger (left) and older (right) patient groups. The differences in IL-6 and IL-8 levels between groups, which had been noted in the pooled media harvested at 48 h, can still be seen.

Relative optical density

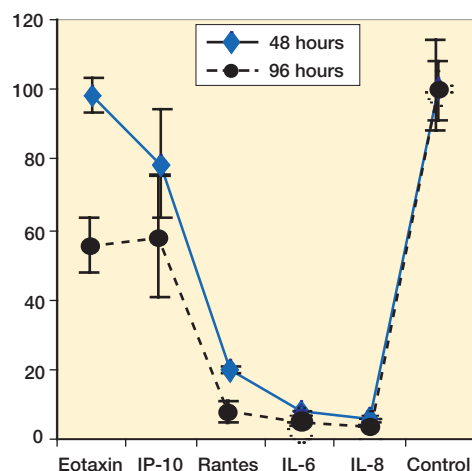


Figure 3. Cytokine levels in the pooled media of NP from the same patients ($n = 5$) after 48 and 96 h of culture. Decline in cytokine levels could be observed.

Alkaline phosphatase (nmol/min/1000 cells)

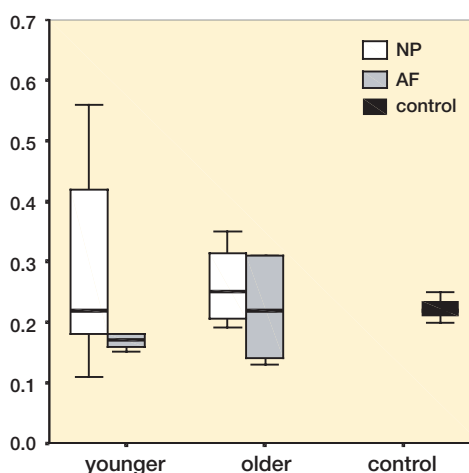


Figure 4. Box plot showing alkaline phosphatase activity of the hTERT cells 5 days after adding the conditioned media. No statistical difference was found ($p = 0.4$)

Alkaline phosphatase activity and cellular proliferation

Because of the individual variations seen from the IL-6 and IL-8 assay, media from each patient were tested separately. The conditioned media from both

NP and AF were individually applied to the hTERT cell cultures, but alkaline phosphatase activity remained unchanged ($p = 0.4$). The conditioned media from younger and older patients did not differ from each other in their effect ($p = 0.5$) (Figure 4).

Cellular proliferation (CPM)

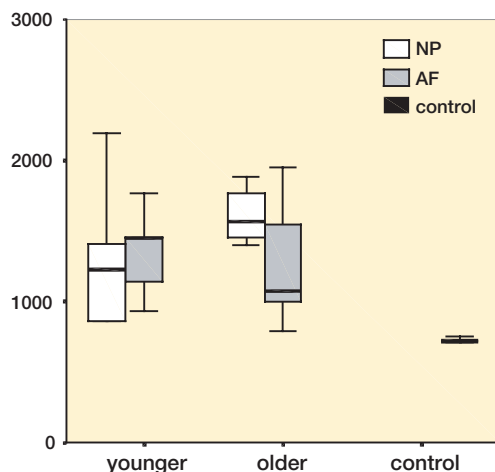


Figure 5. Cellular proliferation 2 days after adding the conditioned media. Significant differences were found between the control and NP or AF ($p = 0.02$ and 0.01 , respectively).

IL-6 (pg/mL)

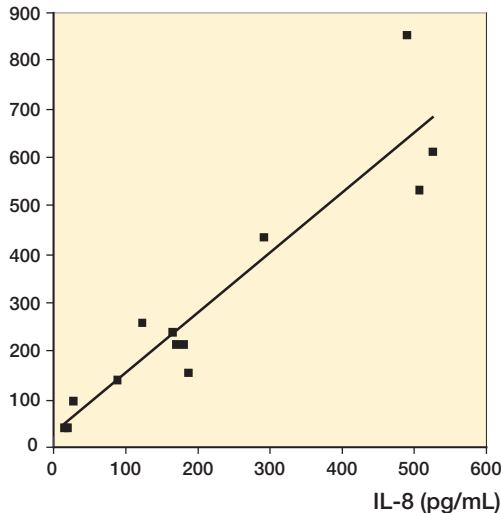


Figure 6. IL-6 and IL-8 levels show a close correlation ($R = 0.90$, $p < 0.001$, Spearman's rank correlation test). Two extreme values for both IL-6 and IL-8 have not been shown on the plot.

Cellular proliferation was slightly increased when conditioned media from either NP or AF were added to the cell culture ($p = 0.02$ and $p = 0.01$, respectively) (Figure 5). No difference in stimulatory effect was found, however, between NP and AF ($p = 0.6$) or younger and older patients ($p = 0.5$). There was a close correlation between the IL-6 and IL-8 levels ($R = 0.90$, $p < 0.001$, Figure 6) when data from NP and AF were analyzed together. There was also a significant correlation between IL-6 and cellular proliferation ($R = -0.44$, $p = 0.04$), but not between IL-8 and cellular proliferation ($R = -0.38$, $p = 0.08$).

Discussion

Cytokines released by the disc tissue may possibly influence the process of bone graft healing. In one of our previous studies, we reported that mixing autograft with NP could result in delayed or inferior healing following anterior spinal interbody fusion in pigs (Li et al. 2002). In the present study we have screened, for the first time, 18 kinds of cytokines in conditioned medium from cultured human intervertebral discs. High levels of Eotaxin, IP-10, Rantes, IL-6 and IL-8 were found in pooled

conditioned media, and 7 other cytokines were also detectable. Our test did not, however, cover all the cytokines that may be released by the discs.

Macrophages, T- and B-lymphocytes and neutrophils have been found in the human intervertebral disc (Kanerva et al. 1997, Gronblad et al. 2000). This accumulation of inflammatory cells can be explained by the finding of chemokine production by the disc tissue. Eotaxin and Rantes have been suggested to be chemotactic factors for monocytes/macrophages and Th1 cells. IP-10 is a ligand for CXCR3 receptors on Th1 cells, and IL-8 is known to be important for chemotaxis of neutrophils and angiogenesis (Loetscher et al. 1996, Mackay 1996, Ponath et al. 1996, Schrum et al. 1996). The overall action of these chemokines will probably result in leukocyte, macrophage/monocyte recruitment when NP is herniated or exposed. It is reasonable to speculate that when NP tissue is exposed to a spine fusion environment or is mixed with bone graft, inflammatory cells may be recruited. By inducing monocyte recruitment, chemokines could indirectly affect bone resorption or formation. Burke et al. (2002a) reported that intervertebral discs that cause low back pain secrete high levels of IL-6 and IL-8. Due to the limited number of patients, we were not able to come to a firm conclusion as to

whether or not this difference existed in the present study. IL-6 and IL-8 levels in the conditioned media were found to be closely correlated, possibly because they were produced both by the NP and AF. Although there was a negative correlation between IL-6 and cellular proliferation, IL-6 may not be the only factor involved (Frost et al. 1997); cellular proliferation may be the result of multiple factors.

In our previous study, we found that the influence of NP tissue on SaOS₂ cells was the result of mediators being released from the disc tissue (Li et al. 2000). We confirmed this in the present study by using conditioned media from cultured NP and AF material. When conditioned media were added to hTERT cell cultures, cellular proliferation was increased significantly. The reason why alkaline phosphatase activity remained unchanged, which is contrary to the results from our previous study, may be that different cells were used. We used hTERT cells in this study because these cells have a normal karyotype and more closely resemble the primary human osteoblast. Furthermore, they possess differentiation potential. The main aim of our experiments was to provide some evidence for clinical practice concerning the importance of complete removal of intervertebral disc. Due to the complexity of cytokine interactions, it is as yet impossible for us to state the exact mechanism underlying the influence of intervertebral discs on osteoblast cells. Moreover, bone graft modeling and remodeling proceed under the mutual interaction of osteoblasts and osteoclasts. Depiction of isolated effects on osteoblasts cannot be the whole story. This is probably the reason for the apparent discrepancy between the present results and our previous studies.

From the design of the present study, we are not able to say whether the disc cells secreted these cytokines. Other possibilities might be that these cytokines had diffused into the disc from the circulation or surrounding tissue because there was a reduction in cytokine levels from 48 to 96 h. However, studies with murine nucleus pulposus-derived cells have demonstrated that cytokine levels increased up to 150-fold with LPS stimulation in vitro (Rand et al. 1997). Thus, the reduction in cytokine levels from 48 to 96 h could also result from loss of proper stimuli in vitro, which

may include nutritional, biomechanical or inflammatory factors. To avoid systemic influences that could remain in the fresh disc tissue, the media from 96 h were chosen for the cell test. On the other hand, media from 48 h may be more reflective of what was happening in the in vivo situation. Further study is warranted to address this question in detail.

In conclusion, we found that intervertebral disc material can secrete various chemokines such as Eotaxin, IP-10, Rantes, IL-6 and IL-8 into culture media. Cytokines MIP-5, IL-12, leptin, IL-4, TNF- α , GM-CSF, and MIP-1 α were present at detectable levels. Furthermore, conditioned media from both NP and AF can stimulate cellular proliferation of human hTERT cells, which are bone marrow stem cells.

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No competing interests declared.

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