Effects of synovial fluid from aseptic prosthesis loosening on collagen production in osteoblasts

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Abstract Synovial fluid from a loose prosthesis may act as a vehicle for factors that regulate bone turnover. The effect of such synovial fluid on osteoblasts has been studied. Synovial fluid obtained from patients who underwent revision hip arthroplasty because of aseptic prosthesis loosening was studied regarding the effect on protein synthesis, procollagen I mRNA expression, the secretion of procollagen I carboxyterminal propeptide (PICP) and osteocalcin in MG63 osteoblasts. Protein synthesis was increased and procollagen I mRNA expression was decreased by synovial fluid from patients with prosthesis loosening. Synovial fluid stimulated the total PICP in cell medium, but there was no change after correction for cell

protein content in the cells. Synovial fluid in patients with prosthesis loosening has a general stimulatory effect on collagen formation and osteoblast proliferation because of a stimulatory effect on cell growth. Aseptic prosthesis loosening may be associated with an increase in bone formation.

Résumé Le liquide synovial provenant de prothèses descellées peut être considéré comme le support de facteurs de la régulation du turn over osseux. Pour cela ont été étudiés les effets de ce liquide sur les ostéoblastes. Le liquide synovial obtenu de patients ayant nécessité une révision de prothèse de hanche descellée pour descellement aseptique a été utilisé afin d'évaluer les effets de ce liquide sur la synthèse des protéines, pro collagène, I mRNA expression sur la sécrétion du pro collagène I carboxyterminal propeptide et sur l'ostéocalcine dans les ostéoblastes MG63. Cette synthèse protéinique est augmentée et l'expression du pro collagène I mRNA diminue lorsque le liquide synovial de ces patients est adjoint. Le liquide synovial stimule le PICP mais il n'y a pas de modification des protéines cellulaires contenues dans les ostéoblastes. Conclusion, le fluide synovial chez ces patients présentant un descellement prothétique a un effet stimulant sur la formation du collagène et la prolifération des ostéoblastes. Le descellement aseptique des prothèses peut être associé avec une augmentation de la formation osseuse.

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Introduction

The pathogenesis of aseptic prosthesis loosening is multifactorial [3]. Several studies have suggested a role of wear debris from the prosthetic components, which are phagocytosed by macrophages that subsequently release cytokines and other factors that change the balance between the

resorption and formation of bone [4, 6, 8, 15]. Under such conditions, macrophages may even differentiate into bone-resorbing osteoclasts [14]. Given that the formation and resorption of bone are coupled to each other [5], a change in bone formation could also be important for bone loss in aseptic loosening. Bone morphological studies on periprosthetic bone around loose prostheses have shown that there is an accelerated turnover, with a high osteoclastic surface and also signs of increased bone formation [17]. Increased turnover may lead to a fragility of the bone structure. The synovial fluid around a loose prosthesis can be regarded as a part of an inflammatory foreign body reaction, which may induce the formation of brittle bone around a loose prosthesis. Such synovial fluid contains different levels of cytokines and chemokines that regulate bone turnover as compared to synovial fluid from patients with osteoarthritis [7, 11, 12, 16, 18]. This implies that the synovial fluid can act as a vehicle for molecules which regulate bone metabolism. We have previously shown that such synovial fluid induces bone resorption and markers of osteoblast differentiation in whole-bone cultures [2]. The object of our study was to measure the effects of synovial fluid from patients with aseptic loosening on procollagen I mRNA expression, general protein synthesis and the secretion of collagen and osteocalcin in cultured osteoblasts.

Methods

Synovial fluid and serum samples

Synovial fluid samples were obtained from patients with aseptic prosthesis loosening following primary surgery for osteoarthritis who then underwent revision total hip arthroplasty. Synovial fluid was obtained from six patients (age 71 ± 2.7 years) for protein synthesis, seven patients (age 74 ± 2.6 years) for the procollagen I expression, five patients (71 ± 5.3 years) for procollagen I carboxyterminal propeptide (PICP) and six patients for osteocalcin (68 ± 6.2 years). Three patients were included in studies of both protein synthesis and procollagen I mRNA expression. Different patients and concentrations of synovial fluid were used because variable amounts of synovial fluid were available and because the experiments were done at different points in time. The synovial fluid samples were aspirated intraoperatively and before incision of the joint capsule. All samples were centrifuged and aliquoted before storage at -70°C . The Ethical Committee of Karolinska University Hospital approved this study.

Cell culture

MG63 human osteosarcoma cells were grown in α -MEM containing penicillin (50 U/l), streptomycin (50 $\mu\text{g/l}$), L-

glutamine (2 mM) and 10% foetal bovine serum (all from Gibco, Grand Island, NY). The cells were incubated at 37°C in 5% CO_2 saturated humidity. After reaching near-confluency, the cells were detached with trypsin-EDTA (Gibco) and plated at a density of 12,000 cells/well for procollagen (PICP) and osteocalcin measurement, 20,000 cells/well for protein synthesis and 50,000 cells/well for procollagen I mRNA expression in α -MEM medium. After 3 days, the medium was replaced to serum-free α -MEM supplemented with 0.1% bovine serum albumin, together with synovial fluid.

Osteoblast protein synthesis

MG63 osteoblasts were grown for 24 h in the presence of 1 $\mu\text{Ci/well}$ of [^3H]proline (Perkin Elmer Life Sciences, Boston, MA) and 25 $\mu\text{g/ml}$ of ascorbic acid, together with synovial fluid or 5% FBS (positive control). After two washes with ice-cold 0.9% NaCl, the cells were incubated with 5% ice-cold trichloroacetic acid for 15 min. The cells were then dissolved in 0.1 M NaOH and supplemented with scintillation liquid (Ultima Gold, Groningen, Holland) before measurement for 120 s in a scintillation counter. Each value represents an average of three culture wells.

Expression of procollagen I mRNA

MG63 osteoblasts' total RNA was isolated with the RNeasy Mini Kit (Qiagen Inc.) after incubation with 10% synovial fluid. One microgram of total RNA per sample was used as a template for the synthesis of cDNA using a commercial kit (First Strand cDNA Synthesis Kit for RT-PCR (AMV), Roche Diagnostics Scandinavia AB). Real-time semiquantitative polymerase chain reaction (PCR) was performed on a LightCycler (Roche) using commercially available fluorescent probes from Applied Biosystems (Foster City, CA). Collagen, type I, alpha 1 (public reference sequence NM_000088, item number Hs00164004_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, public reference sequence NM_002046, item number Hs99999905_m1) probes were used. PCR reagents were obtained from Roche (LightCycler FastStart DNA Master Hybridization Probes Kit). Crossing point (Cp) values were calculated by the LightCycler software using the second derivative maximum method.

The calculation of relative changes in the gene expression is as follows. Three cDNA samples (arbitrarily selected) for each gene product to be analysed were diluted 10 times prior to each round of amplification in the LightCycler. The Cp values from these samples were subtracted from corresponding undiluted samples (ΔCp). PCR efficiency (E) was then calculated by the formula

$E=10^{(1/\Delta C_p)}$. The relative levels of gene expression (RL) were then calculated using the formula $RL=E^{(C_{p\text{ ref}}-C_{p\text{ test}})}$, where $C_{p\text{ ref}}$ is the mean C_p value of the three undiluted cDNA samples used for calculation of E and $C_{p\text{ test}}$ is the C_p value of each cDNA sample. A ratio between procollagen I RL and GAPDH RL was calculated for each sample. The RNA from unstimulated cells were used as controls. The number of experiments in the respective groups and time points varied because some samples had to be excluded due to RNA degradation.

Procollagen I carboxyterminal propeptide analysis

Procollagen I carboxyterminal propeptide (PICP) in conditioned medium was measured with a radioimmunoassay (RIA) according to the instructions from the manufacturer (Orion Diagnostica, Espoo, Finland). Medium with synovial fluid that had not been in contact with the osteoblasts was assayed separately and the values were subtracted from the respective samples from osteoblast-conditioned medium in order to determine the amount of PICP secreted from the osteoblasts. Each value represents an average of three cell culture wells.

Osteocalcin

Osteocalcin was analysed with the ELSA-Osteo kit (CIS Biointernational, Gif-sur-Yvette Cedex, France), detection limit 0.4 ng/ml, inter-assay variation <5%. Medium with synovial fluid that had not been in contact with the

Fig. 1 [^3H]proline incorporation in MG63 osteoblasts cultured for 24 h in the presence of 0.2, 0.6, 2, 6 and 10% synovial fluid from patients with aseptic loosening undergoing revision surgery ($n=5$), expressed as a relative amount compared to 0% synovial fluid. $**=p<0.01$ compared to 0% (RM ANOVA)

Fig. 2 Procollagen I mRNA/GAPDH expression in MG63 osteoblasts incubated with 10% synovial fluid from patients with aseptic loosening undergoing revision surgery ($n=7$), expressed as a relative amount compared to 0% synovial fluid (=1). $**=p<0.01$ compared to 0% (t -test)

osteoblasts was assayed separately and the values were subtracted from the respective samples from osteoblast-conditioned medium in order to determine the amount of osteocalcin secreted from the osteoblasts. Each value represents an average of three cell culture wells. Ten nM of vitamin D_3 (Abbot Scandinavia AB, Solna, Sweden) was used as a positive control.

Total cell protein

The total protein in the cell culture wells was determined as described to correct for differences in the cell number. After washing with PBS, dye reagent (Bio-Rad Laboratories, Hercules, CA) was added to NaOH-digested cells and the absorbance at 595 nm was determined by spectrophotometry. Known amounts of transferrin (Collaborative Research Inc., Waltham, MA) were used as the standard. Each value represents an average of three culture wells.

Fig. 3 Procollagen I carboxyterminal propeptide (PICP) in conditioned medium from MG63 osteoblasts stimulated for 24 h with 0.2, 0.6, 2, 6 or 20% synovial fluid from patients undergoing revision surgery ($n=5$), expressed as a relative amount compared to 0% and PICP/total cell protein from the same experiments. $*=p<0.05$ compared to 0% (RM ANOVA)

Statistics

The data is presented as median±standard error of the mean (SEM). One-way repeated measurement analysis of variance (RM ANOVA) for paired data or ANOVA for unpaired data with the Student-Newman-Keuls post hoc test or Student's *t*-test was used to determine the statistical differences. *p*-values less than 0.05 were considered to be significant. Sigma Stat software was used for all calculations (Jandel Corp., Sausalito, CA).

Results

Protein synthesis

Osteoblast protein synthesis, measured as [^3H]proline incorporation, increased dose-dependently after exposure

to synovial fluid from patients with aseptic loosening undergoing revision surgery (Fig. 1).

Procollagen I mRNA expression

After 24 h of incubation with 10% synovial fluid, the levels of procollagen I mRNA were significantly lower compared to the control (Fig. 2b). There was no change after 6 h of incubation (Fig. 2a).

PICP

Synovial fluid from patients with aseptic loosening undergoing revision surgery induced a dose-dependent increase of PICP in conditioned medium from MG63 osteoblasts cultivated for 24 h (Fig. 3a). When the PICP level was normalised for the total cell protein content in the culture well from where the conditioned medium came, there was no change (Fig. 3b).

Osteocalcin

Synovial fluid from patients with aseptic loosening undergoing revision surgery had no effect on osteocalcin secretion in MG63 osteoblasts after 96 h of incubation (Fig. 4). As a positive control, 10 nM of vitamin D₃ induced a robust increase.

Discussion

We have used the cultivation of osteoblasts together with synovial fluid from patients with aseptic prosthesis loosening who underwent revision surgery as a model for

Fig. 4 Osteocalcin in conditioned medium from MG63 osteoblasts stimulated for 96 h with 10% synovial fluid from patients undergoing revision surgery ($n=6$) or 10 nM vitamin D₃ expressed as a relative amount compared 0% (=1) $***=p<0.001$ compared to 0% (ANOVA)

studying bone metabolism in this condition. Synovial fluid from patients with a loose prosthesis increased the general osteoblast protein synthesis dose-dependently, as well as the total secretion of PICP, a marker for collagen synthesis [13]. However, there was no increase when PICP was corrected for cell protein. Collagen synthesis studied at the pre-translational level showed a downregulation of procollagen I mRNA expression in response to the synovial fluid. Taken together, these data suggest that there may be an increase in collagen I formation by osteoblasts in aseptic loosening because of a general stimulatory effect on cell growth, even if there is a relative decrease in the gene expression of procollagen I, possibly because of a decreased osteoblast differentiation. However, the secretion of osteocalcin, which is another marker of osteoblast differentiation, was not influenced by synovial fluid.

There are several pathways that may lead to aseptic prosthesis loosening. Mechanical insufficiency due to either inadequate initial fixation or the mechanical loss of fixation over time can lead to loosening. Much progress has also recently been made in the research regarding biological loss of fixation due to implant interface wear, which leads to the resorption of periprosthetic bone [1]. Wear debris leads to the expression of bone resorbing factors in periprosthetic tissue, i.e. RANK-L, that may induce the resorption of bone [4]. We previously showed that the synovial fluid from patients with aseptic loosening induces mineral release and gene expression related to the activation of both osteoclasts and osteoblasts [2]. The synovial fluid around a loose prosthesis may, thus, be a vehicle for molecules produced in the periprosthetic tissue that regulate bone turnover. Since the status of bone tissue is dependent on both the resorption and formation of bone, we studied the effect of synovial fluid on osteoblasts. Our data indicate that the synovial fluid may have a stimulatory effect on osteoblasts in terms of cell growth and the total production of collagen I, even if there was a relative decrease of procollagen I mRNA. Studies on periprosthetic bone morphology and biochemical bone markers have also shown signs of increased bone formation in aseptic prosthesis loosening [9, 10, 17]. Since the resorption and formation of bone are intimately coupled to each other [5], it is important to consider both of these aspects when attempting to develop strategies to decrease bone loss in patients who have undergone total hip replacement.

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