Periprosthetic strain magnitude-dependent upregulation of type I collagen synthesis in human osteoblasts through an ERK1/2 pathway

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Abstract Human osteoblasts sense mechanical stimulation and synthesise type I collagen in periprosthetic osseointegration following total hip arthroplasty. However, the regulation of type I collagen synthesis by periprosthetic strain is unclear because the cellular-level strain magnitude remains unknown to date. Fortunately, the tissue-level strain in implanted femurs is measurable. According to the mechanism of strain amplification, the tissue-level strain was amplified 20 times to stretch human osteoblasts in this study. Elongation of 0.8–3.2% enhanced the mRNA level of type I collagen, whereas the release of procollagen type I C propeptide only increased at 2.4% and 3.2% elongation. Type I collagen expression increased with the activation of ERK1/2 phosphorylation in a strain-magnitude-dependent manner, whereas JNK and P38 were unaffected. The responses were completely inhibited by blocking the ERK1/2 pathway with U0126. The results indicate that type I collagen synthesis in human osteoblasts depends on the level of periprosthetic strain and ERK1/2 activation.

Résumé Les ostéoblastes humains peuvent, dans les suites d'une intervention de prothèse totale de hanche être stimulés et synthétiser du collagène de grade I notamment lors des mécanismes d'ostéo intégration. Cependant, la régulation de cette synthèse secondaire aux contraintes péri prothétiques et leur mise en jeu n'est pas claire. Fort heureusement, le niveau de contrainte tissulaire dans les fémurs peut être mesuré. Selon un mécanisme d'amplication des forces, pour cette étude ces contraintes tissulaires peuvent être multipliées par 20 de façon à étirer les otéoblastes. 0,8C3 2% d'élongation permet d'élever le niveau de l'ARN messenger du collagène de type I alors que le relargage du prototype du procollagène de type IC augmente seulement de 2,4 et 3,2%. L'expression du collagène de type I augmente avec l'activation de la phosphorylation ERK1/2, cependant que JNK et P38 ne sont pas affectés. Les réponses sont complètement inhibées par le blocage de ERK1/2 par l'U0126. Ces résultats nous démontrent que la synthèse de collagène de type I par les otéoblastes humains dépend du niveau des tensions péri prothétiques et du niveau d'activation de ERK1/2.

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Introduction

Periprosthetic osseointegration after total hip arthroplasty (THA) plays a key role in the stability of cementless stems. By producing a mineralised matrix, osteoblasts play a leading role in postoperative osseointegration of the stem. Type I collagen accounts for 90% of the bone matrix proteins [9]. Most investigations indicated that tensile strain regulated the expression of type I collagen in cells [14, 17, 22], and some of these responses may be related to the strain magnitude. The carboxyterminal propeptide of type I procollagen (PICP) is cleaved from the biochemical

precursor of type I collagen and used as a new marker of the bone turnover after THA. The quantification of PICP in the culture medium is an accurate method for the assessment of type I collagen synthesis [11]. However, there is limited research on the stretch-induced release of PICP; furthermore, the regulation of type I collagen synthesis by periprosthetic strain after THA is unclear because of the absence of measuring methods for cellular-level strain magnitude. Fortunately, the tissue-level strain in implanted femurs is measurable. Most of the data reported in the literature varied from 400 $\mu\epsilon$ to 1,600 $\mu\epsilon$ [3, 26]. According to the mechanism of strain amplification at the cellular level [2, 32], which is the basis of this study, the strain magnitude at the tissue level can be converted to the corresponding magnitude at the cellular level.

The signalling pathways by which bone cells sense mechanical stimulation and induce collagen gene expression are poorly characterised. The mitogen-activated protein kinase (MAPK) has been implicated in the response of the cells to the mechanical stimulation. However, the reported activation of the MAPK family members including extracellular signal-regulated kinase (ERK1/2), c-jun N-terminal kinase (JNK), and p38MAPK (p38) differed widely from each other. They were all activated in some studies [15, 16, 21], while only a certain member was activated in others [10, 23, 33]. Aside from the cell type and the character of mechanical stimulation, the various activation of MAPK may be determined by additional regulatory factors. We postulate that these various responses are also at least partially attributed to the different strain magnitudes. To investigate the role of postoperative periprosthetic strain in the regulation of type I collagen synthesis, as well as the effect of different magnitudes of tensile strain upon the selective activation of MAPK members, 8000–32,000 $\mu\epsilon$ of tensile strain was applied to stimulate human osteoblasts *in vitro*. The strain range was chosen based on the tissue-level strain in implanted femurs and the theory of strain amplification at the cellular level.

Materials and methods

Cell culture

SV40 human osteoblast (SV 40hOB, ATCC) were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Science, China. The mechanosensitivity of SV 40hOB cells was verified in previous studies [4–6]. Cells were seeded to six-well BioFlex culture plates (Flexcell International, Hillsborough, NC, USA) with flexible membranes at a density of 3.0×10^4 cells/cm². They were cultured in 2 ml DMEM/F12 medium supplemented with 0.3 mg/ml G418, 50 mg/l ascorbic acid,

10 mM b-glycerophosphate (Sigma, St. Louis, MO, USA) and 10% foetal bovine serum (HyClone, USA). Finally, they were incubated under 5% CO₂ atmosphere at 37°C for four days to reach 90% confluence. The medium was changed every two days.

Application of mechanical strain

Radial and circumferential strains were provided by an FX-4000T Flexcell BioFlex Tension Plus Unit (Flexcell International, USA). BioFlex culture plates were placed on a 25 mm-diameter loading station. When vacuum pressure was applied to the plates through a vacuum pump, the membrane was deformed to create a regulated equibiaxial strain. After reaching 90% confluence, the cells were respectively subjected to tensile strains of 0.8% (8,000 $\mu\epsilon$), 1.6% (16,000 $\mu\epsilon$), 2.4% (24,000 $\mu\epsilon$), and 3.2% (32,000 $\mu\epsilon$) for 48 h. The static controls were not stretched. Half of the sine wave and a work frequency of 1 Hz were selected in this experiment. For inhibition of ERK1/2, 2 h prior to the stretch, the MEK1/2 inhibitor U0126 (Sigma) was added to the medium at a final concentration of 10 μ M.

Semiquantitative RT-PCR

After being stretched, total RNA was extracted from the cells by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Then 1 μ g RNA was reverse-transcribed for first strand cDNA synthesis (RevertAidTM M-MuLV, Fermentas, USA). The PCR products were obtained in the linear range of amplification. For this reason, the cycle number was determined by serial PCR with a decreased cycle number to obtain a faint reproducible signal. Twenty-two amplification cycles for type I collagen, and 21 cycles for GAPDH were run. Each cycle consisted of 45 s denaturation at 94°C, 45 s of annealing at 52°C, and 45 s of extension at 72°C. Final extension was allowed to run ten minutes at 72°C. The following sequences of the primers were used: GAPDH, sense: 5'-GTTCCAATATGATTCCACCC-3', antisense: 5'-AGGGATGATGTTCTGGAGAG-3' and type I collagen, sense: 5'-acagccgcttaccctacagc-3', antisense: 5'-tgcacttttggttttgggtcat-3'. RT-PCR products were electrophoresed on 1% agarose gel with 0.5 mg/ml ethidium bromide. Bands were detected by UV illumination of ethidium bromide-stained gels. Band intensities were quantitatively analysed by Quantity One software (Bio-Rad Laboratories) for each gene and were normalised to the corresponding GAPDH values.

Quantification of PICP

After being stretched, the supernatant in each well was collected and frozen at -80° C until it was processed for

PICP quantification. The concentration of PICP was determined using a specific radioimmunoassay from Orion Diagnostica (Espoo, Finland), according to the manufacturer's instructions. The results were displayed as the percentage of concentration as compared to the control group.

Western blot analysis of MAPK

After being stretched, the cells were rinsed with PBS and lysed in 0.2 ml of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue). The samples were kept on ice and then boiled for five minutes. Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Millipore, USA). After being blocked with 5% skim milk for two hours at room temperature, the membranes were probed overnight at 4°C with anti-ERK, anti-JNK, anti-p38, anti-phospho-p38, anti-phospho-ERK, and anti-phospho-JNK (Cell Signaling, USA). After extensive washing, the membranes were incubated for one hour at room temperature with an anti-rabbit secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were detected by an enhanced chemiluminescence system (Pierce) followed by exposure to X-OMAT Kodak films. The density of the bands was analysed, and the results were normalized to total ERK1/2, JNK, and p38 MAPK.

Statistical analysis

All assays were repeated in two independent experiments with a minimum of $n=3$ for each data point. Statistical analysis among groups was performed by ANOVA and SNK test using the SAS6.12 software package (SAS Institute, NC, USA). The statistically significant values were defined as $P<0.05$.

Results and discussion

Tissue-level strains in intact human bone are usually less than $1,000 \mu\epsilon$ [7]. After the insertion of an untapered femoral prosthesis, the interface bone strain increased along the stem axis. A peak strain of $1,868 \mu\epsilon$ was reported near the tip [27]. Owing to the improved prosthetic material and shape design, the tissue-level periprosthetic strain magnitudes reported in the literature now mostly varied from $400 \mu\epsilon$ to $1,600 \mu\epsilon$ [3, 26]. We focussed on the regulation of type I collagen synthesis by periprosthetic stress in human osteoblasts, which was important for the postoperative stability of cementless stems. However, the strain loaded on cells within the bone is different from the tissue-

level strain and remains unknown to date. In previous studies, few cell responses were induced by foregoing tissue-level strains, whereas efficient mechanical strain in vitro seemed to be unrelated to periprosthetic strain because the two kinds of magnitude greatly differed from each other. Recently, a quantitative cellular-level strain model and the strain amplification mechanism stated that strains loaded on the whole bone might be amplified about 20 times at the membrane of osteoblasts [2, 13, 32]. Based on this information, a strain range from $8,000 \mu\epsilon$ (0.8%) to $32,000 \mu\epsilon$ (3.2%) was chosen as the magnitude of tissue-level periprosthetic strain in this study.

The skeletal system is an extremely dynamic environment which is subjected to constant remodelling [1]. Most investigations demonstrated that tensile force increased the mRNA level of type I collagen in bone marrow stromal cells [17], anterior cruciate ligament cells [14] and human osteoblast-like cells [22]. Unfortunately, the corresponding increase of type I collagen protein synthesis was not indicated in these studies. Hypergravity induced the increased type I collagen synthesis in human osteoblast-like cells, which was indirectly indicated by increased total collagen protein [25]. As a new bone metabolic marker for the assessment of type I collagen synthesis, the release of PICP has been investigated in many studies [18, 19, 24]. In this study, the stretch-induced expression of type I collagen was enhanced at both mRNA and protein levels. Compared to static control, the mRNA level of type I collagen increased in a strain-magnitude-dependent manner

Fig. 1 Different magnitudes of tensile strain increased the mRNA level of type I collagen in human osteoblasts. **a** Agarose gel electrophoresis of PCR products. **b** Semiquantitative results of type I collagen gene normalised for GAPDH expression. The mRNA level of type I collagen increased in a strain-magnitude-dependent manner. $P<0.01$; * compared to static control; ++ compared to the group with 1.6% elongation

Fig. 2 Higher magnitudes of tensile strain induced the release of procollagen type I C propeptide (PICP) in human osteoblasts. After being stretched, the release of PICP increased at 2.4% and 3.2% elongation. Pretreated with the MEK1/2 inhibitor U0126, no significant increase of PICP was induced by tensile force. The release of PICP is given as the percentage of concentration compared to the control group. * $P < 0.01$

(Fig. 1), whereas the release of PICP only increased at 2.4% and 3.2% elongation (Fig. 2). To our knowledge, this is the first report on stretch-induced release of PICP in human osteoblasts. This result suggests that mechanically induced type I collagen synthesis of human osteoblasts is dependent on the magnitude of periprosthetic strain. Higher magnitudes of strain are more beneficial than lower magnitudes for the gene expression. Similarly, this conclusion was justified at the protein level in the study. Lower magnitudes of strain lost the ability to induce the release of PICP in human osteoblasts. It indicates the presence of a mechano-transduction threshold in the stretch-induced release of

Fig. 3 The selective activation of MAPK phosphorylation induced by different magnitudes of tensile strain. Western blot of MAPK was shown as p-ERK and ERK (a), p-p38 and p38 (b), and p-JNK and JNK (c). **d** Quantification graph for the protein levels of the phosphorylated kinases. The ERK1/2 was phosphorylated in a load-dose-dependent manner, whereas JNK and P38 were unaffected. Pretreated with the MEK1/2 inhibitor U0126, no significant increase of ERK1/2 phosphorylation was induced by tensile force. The results were normalised to total kinase levels and expressed as a ratio to the corresponding static control. $P < 0.01$; * as compared to the static control; ++ as compared to the group with 1.6% elongation

Fig. 4 Pretreated with the MEK1/2 inhibitor U0126, no significant increase of type I collagen mRNA level was induced by the tensile strain. **a** Agarose gel electrophoresis of PCR products. **b** Semiquantitative results of type I collagen gene normalized for GAPDH expression

PICP. The different induction of PICP from the mRNA level could be attributed to the post-transcriptional modifications and protein–protein interaction, since the collagen synthesis was regulated by glucocorticoids and various growth factors [8, 11, 14]. As the initial step in bone tissue formation, the synthesis of type I collagen provides the organic scaffold for the subsequent deposition of mineral. New bone mass can only be acquired by increased matrix synthesis [25]. In terms of this viewpoint, 2.4% and 3.2%

of tensile strain would enhance periprosthetic bone formation by initiating the increase of type I collagen synthesis in human osteoblasts. This finding was consistent with a postoperative histological phenomenon that hyperostosis was observed near the tip of stems where postoperative stress distribution markedly increased. Meanwhile, bone loss took place around the proximal part of the femoral prosthesis where stress shielding occurred [20]. The presence of the mechanotransduction threshold in the stretch-induced release of PICP may inversely justify a starting point in postoperative bone loss induced by stress shielding.

The selective activation of MAPK family members has been involved in the mechanically induced cell responses. In bone marrow stromal cells subjected to a shear stress of 2.3 dyn/cm², the ERK and p38 pathways were immediately activated, while the JNK kinase pathway remained unaffected [29]. In contrast, 9% of tensile force applied in human osteoblasts activated JNK, whereas ERK and p38 were unaffected [28]. Figure 3 shows that ERK1/2 was phosphorylated in a load-dose-dependent manner, whereas JNK and P38 were unaffected. All these evidences indicate that the selective activation of MAPK family members is related to the strain magnitude. It appears that the strain magnitudes used in this study were excluded from the magnitude spectrum for the activation of JNK and p38 phosphorylation. Within 3.2%, the increasing cellular-level tensile strain gradually upregulates the activation of ERK1/2 phosphorylation. Interestingly, the activation of ERK1/2 phosphorylation was highly consistent with the stretch-induced expression of type I collagen gene (Figs. 1b, and 3d). ERK1/2 is activated by phosphorylation of p42/44 MAPK kinase (MEK1/2). In our study, the MEK1/2 inhibitor U0126 was applied to determine whether the strain magnitude-dependent induction of type I collagen synthesis was regulated through the activation of ERK1/2 phosphorylation. Figures 2 and 4 show that the stretch-induced increases of PICP and mRNA levels of type I collagen were both completely inhibited by blocking the ERK1/2 pathway with U0126. This is different from previous reports that the mechanical responses of cells were partially inhibited by a certain MAPK inhibitor. The results suggest that the ERK1/2 signalling pathway plays a vital role in the induction of type I collagen gene transduction by periprosthetic strain. The association between type I collagen synthesis and ERK1/2 activation would be further justified by the upregulation of transcription factor AP-1, since AP-1 complex was implicated in type I collagen expression [25, 31] and correlated with the activation of the ERK1/2 pathway [12, 30].

In summary, the results indicate that type I collagen synthesis of human osteoblasts is dependent on the level of periprosthetic strain and ERK1/2 activation. Higher magnitudes

of periprosthetic strain enhanced both the mRNA level of type I collagen and the release of PICP, while lower magnitudes lost the ability to induce the protein synthesis of type I collagen. Associated with the analysis of strain distribution in various implanted femurs, this finding may provide clues for speculating the status of periprosthetic osseointegration. Two promising strategies for improving periprosthetic osseointegration should be emphasised as follows: (1) regulate periprosthetic strain by improving prosthetic material and shape design, and (2) increase the level of ERK1/2 phosphorylation in human osteoblasts.

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