Note

Antisense Suppression of Collagen VI Synthesis Results in Reduced Expression of Collagen I in Normal Human Osteoblast-like Cells

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A transient increase in collagen VI expression precedes the accumulation of collagen I associated with interleukin-4 (IL-4)-induced mineralization in human osteoblast-like cells. Transfection with an antisense oligonucleotide specific for α1(VI) collagen mRNA was shown to attenuate mRNA levels of collagens VI and I. Incubating IL-4 treated cells with anti-collagen VI antisem decreased expression of α1(I) mRNA. The results suggest that collagen VI may regulate collagen I expression in the early phase of IL-4-induced mineralization.

Key words: osteoblast; collagen VI; collagen I; human; antisense oligonucleotide

Previous studies have indicated that osteoblasts initiate synthesis of extracellular matrix proteins, particularly collagen I, during the early stages of differentiation.1,2) It has been shown that collagen I plays important roles not only as a guide for calcification in bone tissue but also as a regulator of osteoblast differentiation.3,4) We have previously reported that recombinant human interleukin-4 (rhIL-4) increased collagen I and osteocalcin accumulation and caused mineralization in normal human periosteal osteoblast-like cells, named SaM-1 cells.5) The rhIL-4 also transiently elevated the mRNA levels for α1(VI) and α2(VI) collagens and the accumulation of collagen VI in the cell layer.6,7) Collagen VI expression peaked at day 5, and the α(I) procollagen mRNA level was greatest at day 10 after treatment with rhIL-4 for 72 h, suggesting that deposition of collagen VI into the extracellular matrix may be a prerequisite for the increase in collagen I expression.7)

Immunological and biochemical studies have established that collagen VI is a ubiquitous component of most connective tissues, where it usually exists as a heterotrimer of three genetically distinct α chains termed α1(VI) and α2(VI) (both of 140 kDa) and α3(VI) (250 kDa).8,9) Collagen VI appears to be involved in the structural stabilization of the extracellular matrix via a direct interaction with other macromolecules, including collagen I.10) Collagen VI has been reported to be a significant constituent of cartilaginous matrices11) and fetal bone.12) Although the amount of collagen VI has been found to be low in mature bone,13) decreased levels have been reported in some cases of osteoporosis.14) Another study has detected the continuous presence of α2(VI) collagen mRNA in callus tissue, suggesting that this collagen is produced throughout the fracture-healing process.15) Interestingly, the transient expression of collagen VI mRNAs has also been reported during the differentiation in vitro of a range of cell types including chondrocytes,16) muscle cells,17) and adipose cells.18)

The aim of this study was to clarify the role of collagen VI in the in vitro mineralization induced by rhIL-4 in SaM-1 cell cultures. We have used an antisense oligonucleotide (ASODN) to α1(VI) collagen mRNA to suppress the transient up-regulation of collagen VI expression by rhIL-4 and identify the effect on the expression of a range of bone-related genes using a semiquantitative reverse transcriptase polymerase chain reaction (SQ RT-PCR) and immunoblot analysis. Furthermore, we have added specific anti-collagen VI antibodies to the cultures to disrupt the extracellular function of the molecule.

SaM-1 cells, demonstrating functional and morphological characteristics typical of osteoblasts, were cultured as previously reported.5,19) More than 90% of the collagen they produced was type I.19) SaM-1 cells

Abbreviations: ALP, alkaline phosphatase; ASODN, antisense oligonucleotide; SODN, sense oligonucleotide; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OC, osteocalcin; ON, osteonectin; PBS, phosphate-buffered saline; PDL, population doubling levels; rhIL-4, recombinant human interleukin-4; SQ RT-PCR, semiquantitative reverse transcriptase polymerase chain reaction.
were cultured with or without 3.0 ng/ml rhIL-4 (kindly supplied by Ono Pharmaceutical, Japan). Transfection with an antisense oligonucleotide (ASODN) for the \( \alpha1(VI) \) collagen chain was done as follows. An ASODN (5'-AGAGCAGGGGCCTTG-ACT-3') complementary to a sequence directly downstream from the initiation codon of \( \alpha1(VI) \) collagen was selected, as it was particularly effective in blocking collagen VI synthesis in preliminary experiments (not shown). The corresponding control sense oligonucleotide (SODN) was 5'-ATGAGCCGGTCGGTCTT-3'. Both were synthesized as phosphorothionate oligonucleotides and subsequently purified, using high pressure liquid chromatography, by Toa Gosei Ltd. Japan.21 For transfection, 5 \( \mu \)M of each oligonucleotide was mixed with 3 \( \mu \)l of FuGENE 6 (Roche Diagnostics) in medium (400 \( \mu \)l) containing 1.2 ng of rhIL-4 and 10% FBS, and added to subconfluent SaM-1 cells in 24-well multiwell plates (Becton Dickinson) according to the manufacturer's instructions. The cells were then incubated for a further 72 h and total RNA was isolated as described below. Transfection with either oligonucleotide did not affect cell number, cell growth, or viability (data not shown). Total RNA was prepared from SaM-1 cells by the acid guanidine-phenol-chloroform method.22 First-strand cDNA libraries were made from total RNA by using random primers (Takara, Japan) and Moloney Murine Leukaemia Virus reverse transcriptase (Toyobo, Japan) as described by the manufacturer's instructions. PCR was done in a 50-\( \mu \)l reaction containing 1 \( \times \) PCR reaction buffer, 200 \( \mu \)M dNTPs, 2 mM MgCl\(_2\), 1 \( \mu \)l of single strand cDNA solution, 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.6 \( \mu \)M of sense and antisense primers (Table 1). Amplifications were done by incubation in a GeneAmp 9700 thermal cycler (Applied Biosystems) using step-cycle programs under the linear amplification conditions. The PCR program was as follows: 94°C for 45 s, 55°C for 40 s, and 72°C for 1 min for \( \alpha1(I) \) collagen, \( \alpha1(VI) \) collagen, alkaline phosphatase (ALP), CBFA1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 94°C for 45 s, 70°C for 40 s, and 72°C for 30 s for \( \alpha1(III) \) collagen and osteonectin (ON); 94°C for 45 s, 62°C for 30 s, and 72°C for 30 s for osteocalcin (OC). The PCR products were electrophoresed on a 2% agarose gel and made visible by ethidium bromide staining with ultraviolet light illumination. The internal control for RT-PCR was GAPDH mRNA. The authenticity of all PCR products was confirmed by sequencing.

First, SQ RT-PCR analysis was used to confirm that the ASODN specifically attenuated the level of \( \alpha1(VI) \) collagen mRNA in the rhIL-4 treated SaM-1 cells (Fig. 1A). Clearly transfection of the cells with the ASODN resulted in markedly less mRNA levels for \( \alpha1(VI) \) collagen than those transfected with the SODN. In contrast, no differences were observed in the amounts of GAPDH amplicon between the two treatments, demonstrating that the two oligonucleotides had no discriminating effects on this control gene and that the RNA samples had equal efficiency of cDNA synthesis. The effect of the antisense attenuation of the \( \alpha1(VI) \) collagen mRNA level on the expression of various genes associated with osteoblastic differentiation was also investigated. Using SQ RT-PCR, relative mRNA levels for \( \alpha1(I) \) collagen, \( \alpha1(III) \) collagen, ALP, CBFA1, OC, and ON were measured (Fig. 1B). CBFA1 expression was examined, as a key role of this runt-containing transcription factor has recently been identified in the regulation of bone cell differentiation.23,24 Collagen III has been already identified in SaM-1 cells.25 Messenger RNAs for each of these mRNAs were detected in the SaM-1 cells by SQ RT-PCR. No differences were detected in mRNA levels for ALP, CBFA1, OC, and GAPDH between the cells treated with the SODN and the ASODN of \( \alpha1(VI) \) collagen. However, there was a significant reduction in the level of \( \alpha1(I) \) collagen mRNA, and a slight reduction in the level of \( \alpha1(III) \) collagen mRNA, in the cells.

### Table 1. Oligonucleotide Primers Used in the PCR

<table>
<thead>
<tr>
<th>Target cDNA</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>( \alpha1(I) ) collagen</td>
<td>S: 5'-GTTCGACTTCAGCTTCGTCG-3'</td>
<td>813</td>
</tr>
<tr>
<td>( \alpha1(III) ) collagen</td>
<td>A: 5'-GTTCGACTTCAGCTTCGTCG-3'</td>
<td>485</td>
</tr>
<tr>
<td>( \alpha1(VI) ) collagen</td>
<td>A: 5'-GTTCGACTTCAGCTTCGTCG-3'</td>
<td>500</td>
</tr>
<tr>
<td>ALP</td>
<td>A: 5'-ATCGCCTACCAGCTCATGCAT-3'</td>
<td>291</td>
</tr>
<tr>
<td>CBFA1</td>
<td>A: 5'-CCGCCCCACGACAACCGCACCAT-3'</td>
<td>365</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>A: 5'-GCTCCTGTGATAGGTAGCTAC-3'</td>
<td>292</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>A: 5'-CGGGAGAGCCGCTCTGCCTGCCC-3'</td>
<td>546</td>
</tr>
<tr>
<td>GAPDH</td>
<td>A: 5'-ACCACAGTCCATGCCATCAC-3'</td>
<td>452</td>
</tr>
<tr>
<td>( \alpha1(I) ) collagen</td>
<td>A: 5'-TCCACACACCCTGTTGCTGTA-3'</td>
<td>813</td>
</tr>
</tbody>
</table>

S = Sense primer  A = Antisense primer
transfected with the ASODN of α1(VI) collagen relative to cells treated with the control SODN. We confirmed that the ASODN of α1(VI) collagen did not directly block α1(I) collagen mRNA expression in the cultured SaM-1 cells without rhIL-4 (data not shown). Thus the suppression of rhIL-4-induced α1(VI) collagen mRNA up-regulation by antisense treatment also appeared to have the relatively specific effect of attenuating the rhIL-4-induced up-regulation of α1(I) collagen mRNA expression.

To find if the antisense treatment of α1(VI) collagen also attenuated the accumulation of collagens VI and I by IL-4 treated SaM-1 cells, cell layer extracts were immunoblotted for these collagens and the intensities of the antibody-staining bands were measured. Preparation of the cell lysate and immunoblot analyses of collagens VI, I and GAPDH in the cell lysates were done as described previously. Antibodies were obtained from Chemicon (anti-human collagen I antibody) and Trevigen (anti-human GAPDH antibody). The rabbit polyclonal antiserum to collagen VI was described previously. Relative amounts of collagens VI, I and GAPDH on western blots were then measured using a CS-9000 dual-wavelength flying-spot scanner (Shimadzu, Japan). The values of 46 and 37 were obtained from cultures treated with the control SODN (Table 2). Interestingly, the ASODN treatment also reduced the relative protein levels of α1(I) and α2(I) collagens to about 46 and 37, respectively, relative to the values obtained from cultures treated with the control SODN. Thus transfection of the ASODN of α1(VI) collagen attenuated protein levels of collagen I as well as the mRNA levels. Further study is necessary to examine whether the attenuation of collagen I expression could be improved by exogenous collagen VI.

Table 2. Effects of the Antisense Oligonucleotide against the Protein Levels of Collagens VI and I

<table>
<thead>
<tr>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen VI</td>
<td></td>
</tr>
<tr>
<td>α1</td>
<td>104.8 ± 2.324</td>
</tr>
<tr>
<td>α2</td>
<td>158.0 ± 1.550</td>
</tr>
<tr>
<td>α3</td>
<td>56.5 ± 3.06</td>
</tr>
<tr>
<td>Collagen I</td>
<td></td>
</tr>
<tr>
<td>α1</td>
<td>164 ± 2.02</td>
</tr>
<tr>
<td>α2</td>
<td>174 ± 17.4</td>
</tr>
<tr>
<td>GAPDH</td>
<td>21.6 ± 1.00</td>
</tr>
</tbody>
</table>

* Protein levels assayed in arbitrary unit by densitometry of western blots. Values are mean ± S.E. (n = 3).

A) SQ RT-PCR analysis of mRNA levels for α1(VI) collagen and GAPDH. SaM-1 cells were cultured for 72 h with the α1(VI) collagen SODN (S) or ASODN (AS) in the medium also containing rhIL-4 (3.0 ng/ml). The mRNA encoding α1(VI) collagen (α1(VI)) was amplified by SQ RT-PCR after normalization with GAPDH expression as described in the text. (B) SQ RT-PCR analysis of osteoblast marker mRNA levels. The mRNAs encoding collagen VI, I, and GAPDH on western blots were made visible on ethidium bromide-stained agarose gels.

To investigate further the influence of collagen VI protein on collagen I mRNA expression in the early stage of rhIL-4 induced mineralization, we added anti-collagen VI antisera to cultures to perturb the normal function of extracellular collagen VI. The cells were treated with rhIL-4 (3.0 ng/ml) for 72 h, together with 1/400 dilution of rabbit anti-collagen VI antisera (Ab). Control cultures contained no antiserum (Con) or a 1/400 dilution of preimmune rabbit serum (Pre). SQ RT-PCR analysis showed that the cultures treated with anti-collagen VI antisera showed no effect on the expression of the mRNAs for α1(VI) collagen and GAPDH relative to theuntreat-
ed and preimmune treated control cultures (Fig. 2). However the antiserum treatment significantly decreased expression of α(I) collagen mRNA relative to the controls. A slight decrease in expression of α(III) collagen mRNA was also noted. The results suggest that the observed modulation of collagen I expression in the IL-4 treated SaM-1 cells is specifically mediated by the levels of extracellular collagen VI.

In this study, these findings suggest that up-regulation of collagen I mRNA in rhIL-4 treated SaM-1 cells may be modulated by their interaction with collagen VI accumulated in the surrounding extracellular matrix. Thus, collagen VI may serve as a matrix-derived inducer of integrin-mediated collagen I expression in the early stages of osteoblastic differentiation. In osteoblastic cell lines, except SaM-1 cells, the existence and function of collagen VI described here need to be examined. Moreover, it will be important to investigate the modulation of collagen I expression in osteoblast cultures isolated from collagen VI-deficient and IL-4-deficient bones.

A candidate cell surface receptor for collagen VI, α2β1 integrin, was identified on the SaM-1 cells by immunoblot analysis (data not shown). Forced expression of α2β1 integrin has recently been shown to up-regulate α(I) procollagen mRNA transcription by osteosarcoma cells in response to contact with three-dimensional collagen. The signalling has been found to be mediated by p38 mitogen-activated protein kinase. Moreover α2β1 integrin is known to bind collagen VI and a signal transduction pathway for collagen VI involving the α1 integrin has been partly described for fibroblasts. However, it should be noted that collagen VI also associates with several other transmembrane receptors such as α2β1 integrin and the transmembrane proteoglycan NG2. A number of extracellular modulators of collagen I expression have been identified. It has been shown, using rat calvaria RNA in a reticulocyte cell-free translation system, that amino-terminal peptides of α(I) and α(III) procollagen chains inhibit the production of α(I) and α(II) procollagen chains. Recently, SPARC (secreted protein acidic and rich in cysteine), a matricellular glycoprotein (also known as BM-40, osteonectin, or 43-kDa protein) has been shown to regulate the expression of collagen I via activation of the TGF-β signalling pathway in mouse mesangial cells. Further studies will focus on the identification of the signaling pathway in SaM-1 cells activated by collagen VI and finding if its up-regulation of collagen I gene expression involves direct or indirect signalling.

References


10) Bonaldo, P., Russo, V., Bucciotti, F., Doliana, R.,


20) Chu, M. L., Pan, T. C., Conway, D., Kuo, H. J., Glanzville, R. W., Timpl, R., Mann, K., and Deutzman, R., Sequence analysis of alpha I(VI) and alpha 2(VI) chains of human type VI collagen reveals internal triplication of globular domains similar to the A domains of von Willebrand factor and two alpha 2(VI) chain variants that differ in the carboxy ter-


31) Chu, M. L., Pan, T. C., Conway, D., Kuo, H. J., Glanzville, R. W., Timpl, R., Mann, K., and Deutzmann, R., Sequence analysis of alpha I(VI) and alpha 2(VI) chains of human type VI collagen reveals internal triplication of globular domains similar to the A domains of von Willebrand factor and two alpha 2(VI) chain variants that differ in the carboxy ter-


