Collagen metabolism in cultured osteoblasts from osteogenesis imperfecta patients

Martin MÖRIKE,* Rolf E. BRENNER, Gregor B. BUSHART, Walter M. TELLER and Ulrich VETTER†
Connective Tissue Laboratory, Department of Pediatrics I, University of Ulm, Parkstraße 11, W-7900 Ulm, Germany

Collagen produced *in vitro* by bone cells isolated from 19 patients with different forms of osteogenesis imperfecta (OI) was analysed. Clinically, four patients were classified as OI type I, 10 patients as OI type III and five patients as OI type IV. Bone cells of 12 of the 19 OI patients produced structurally abnormal type I collagen. Electrophoretically uniformly slower migrating collagen type I α -chains were found in one case of OI type I, in seven cases of OI type III and in one case of OI type IV; two cultures of OI type III produced two different populations of collagen type I α -chains, and one culture of OI type IV showed reduction-sensitive dimer formation of $\alpha 1(I)$ chains, resulting from the inadequate incorporation of a cysteine residue into the triple helical domain of $\alpha 1(I)$. Quantitative analysis of collagen metabolism led to the distinction of two groups of cultured OI osteoblasts. In osteoblasts of OI type I, mainly production of collagen was decreased, whereas secretion, processing and pericellular accumulation of (pro)collagen type I was similar to that in control osteoblasts. In contrast, in osteoblasts of OI types III and IV, production as well as secretion, processing and pericellular accumulation of (pro)collagen type I were significantly decreased. Low levels of type I collagen were found irrespective of the presence or absence of structural abnormalities of collagen type I in all OI types.

INTRODUCTION

Osteogenesis imperfecta (OI) is a rare congenital disease of connective tissue. Bone fragility and skeletal deformation are the most prominent clinical features, although other tissues such as teeth, sclerae, tendon, heart, kidney and skin can also be affected. The Sillence classification defines four subtypes of this clinically variable disease [1]. OI type II is the most severe perinatal lethal form, whereas type III patients are the most severely affected surviving patients. Type I and IV patients suffer from a mild or intermediate form of the disease. Biochemical studies with skin fibroblasts of OI patients revealed a variety of abnormalities of collagen type I metabolism. In many OI patients, mutations of the genes coding for procollagen type I chains have been identified [2–5]. However, in a small number of other patients, a linkage of OI to the collagen genes could be excluded [6,7].

In spite of progress in understanding the molecular and genetic basis of OI, little is known about collagen metabolism in cells isolated from bone of OI patients [8,9]. We therefore studied quantitative and qualitative aspects of collagen metabolism in osteoblasts isolated from 19 patients with different clinical types of OI.

EXPERIMENTAL

Patients

Bone specimens were obtained during orthopaedic surgery from 19 OI patients who were clinically classified according to Sillence *et al.* [1]. Clinical data of the patients are summarized in Table 1. Bone biopsies of six normal individuals (four males and two females) aged 12–20 years were obtained from surgery or iliac crest biopsies.

Osteoblast cultures

Osteoblast cultures were established as previously described [10]. Briefly, biopsies of cortical bone were cleaned carefully,

minced and incubated for 4 h at 37 °C with 400 units of collagenase type IV $\cdot$ ml⁻¹ (Sigma). The collagenase-treated bone particles were placed into Ca²⁺-free Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), ascorbate (50 μ g $\cdot$ ml⁻¹), penicillin (400 units $\cdot$ ml⁻¹), nethilmycin (50 μ g $\cdot$ ml⁻¹) and glutamine (2 mM). Osteoblasts growing out of the bone particles were subcultured by trypsin

Table 1. Clinical data of OI patients from whom osteoblast cultures were established

The fracture rate is given as the mean per year in the first decade: +, ≤ 2 ; ++, 3 or 4; +++, > 4 . The height below normal is the number of s.d.s by which the individual's height is below the mean of the population for that age. n.k., not known.

Patient number	OI type	Sex	Age (years)	Fracture rate	Height below normal (s.d.s)
1	I	m	10	++	-0.8
2	I	m	12	+++	-2.4
3	I	m	19	n.k.	n.k.
4	I	m	19	n.k.	n.k.
5	III	f	4	+	-7.3
6	III	f	3	++	-7.8
7	III	m	7	+++	-6.5
8	III	m	7	n.k.	n.k.
9	III	m	8	n.k.	n.k.
10	III	f	8	++	-5.5
11	III	m	9	++	-9.5
12	III	m	9	+++	-11.1
13	III	f	15	+++	-11.5
14	III	m	18	+	-8.6
15	IV	m	2	+	-3.1
16	IV	m	2	++	-7.3
17	IV	f	3	+	-3.4
18	IV	f	4	++	-7.7
19	IV	m	18	+	-2.9

Abbreviations used: OI, osteogenesis imperfecta; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

* To whom correspondence should be addressed.

† Present address: Department of Pediatrics, Johann Wolfgang Goethe University, 70 Theodor Stern Kai 7, W-6000 Frankfurt, Germany.

treatment (0.2% trypsin, 0.5 mM-EDTA) in DMEM/10% FCS after reaching confluency. Cells were fed every 3 days. Biochemical analysis was carried out on cells in the third or fourth passage.

Labelling of osteoblast collagen with [³H]proline

Confluent osteoblasts seeded at a density of 10^4 cells·cm⁻² were cultivated for 2 days under conditions as described above before preincubation in fresh medium without antibiotics for 24 h. The cells were labelled with 10 µCi of L-[5-³H]proline·ml⁻¹ (Amersham; specific radioactivity 35 Ci·mmol⁻¹) in DMEM without serum and glutamine, but supplemented with fresh ascorbate and β-aminopropionitrile (100 µg·ml⁻¹). A parallel Petri dish was used for cell counting.

Qualitative analysis of labelled collagens

Cell layer and medium were harvested separately and dialysed for 48 h (with three changes) in 250 ml of 0.5% acetic acid at 4 °C. Cell-layer lysates and medium were incubated for 6 h with 0.1 mg of pepsin·ml⁻¹ (Boehringer) at 16 °C.

SDS/PAGE and fluorography

Pepsin-treated samples were separated electrophoretically [11] without or with prior reduction using 0.1 M-2-mercaptoethanol. Procollagen samples were reduced prior to SDS/PAGE. After electrophoresis, gels were impregnated with 2,5-diphenyloxazole, dried and exposed to preflashed films (Hyperfilm; Amersham) for various times (8 h to 14 days). Fluorograms were scanned with a laser densitometer (Ultrosan XL; Pharmacia) and evaluated using a calibration curve to determine the relative intensities of single bands.

Quantification of collagen

Cell layer and medium were harvested separately and dialysed as described above. Non-dialysable radioactivity was determined in triplicate by using a β-scintillation counter. Samples of cells and medium were hydrolysed (6 M-HCl, 110 °C, 18 h). The concentrations of [³H]proline and [³H]hydroxyproline were determined in triplicate after amino acid separation by h.p.l.c. (Na High performance column; Beckman) and scintillation counting of the fractions. The collagen content as a percentage of total protein synthesized was determined using a modified formula described by Wiestner *et al.* [12]:

$$\text{Collagen (\%)} = \frac{2.17 \times (\text{Hyp c.p.m.}) \times 100}{2.17 \times (\text{Hyp c.p.m.}) + 5.4 \times [(\text{Pro c.p.m.}) - 1.17 \times (\text{Hyp c.p.m.})]}$$

assuming that (a) hydroxyproline (Hyp) is found only in collagen, (b) collagen type I contains hydroxyproline and proline (Pro) at a ratio of 0.855 [13], and (c) collagen type I contains about 5.4 times as many imino acids as do non-collagenous proteins [14]. The same assumptions were underlying the determination of total radiolabel incorporation into collagen (collagen c.p.m.):

$$\text{Collagen (c.p.m.)} = \frac{\text{Total non-dialysable c.p.m.} \times \text{collagen (\%)} \times 5.4}{\text{Collagen (\%)} \times 5.4 + 100 - \text{collagen (\%)}}$$

Quantification of secreted, processed and pericellular collagen

Radiolabel incorporated into collagen in the cell layer and in the medium was distributed between procollagen type I chains (proα1, proα2), partially processed procollagen type I chains (pCα1/α2, pNα1/α2) and completely processed α-chains of collagen type I (α1, α2) based on the relative intensities of their bands on fluorograms.

Only unprocessed chains of procollagen type I from the cell layer were considered to be intracellular collagen. Partially and

completely processed chains of collagen type I from the cell layer were termed pericellular collagen. Pericellular collagen and all (pro)collagen in the medium was considered to be extracellular collagen. Collagen type I chains lacking both the N- and C-terminal propeptides were termed 'processed collagen'.

Amounts of intra-, extra- and peri-cellular (pro)collagen were expressed as ratio relative to total (pro)collagen. Amounts of processed collagen were expressed relative to extracellular (pro)collagen.

Statistics

Statistical analysis was performed using the SAS statistical package (SAS, Cary, NC, U.S.A.). The data were to be distributed in a non-parametric fashion. Differences between all groups were determined by analysis of variance using the Kruskal-Wallis test. Chi-squared significance was calculated, corrected for ties, and considered significant when *P* was less than 0.05. When the variance among the groups was distributed so that there was a probability less than 0.05 of random distribution, the null hypothesis was rejected. Consequently, differences between single OI types and controls were determined using the Wilcoxon test together with the Holms adjustment.

RESULTS

Pattern and qualitative analysis of interstitial collagens

Because of the connection between OI and abnormalities of collagen structure and pattern, collagen produced *in vitro* by osteoblasts derived from 19 patients with different types of OI (see Table 1) was analysed. SDS/PAGE after limited pepsin digestion revealed that collagen produced by control and OI osteoblasts consisted mainly of type I collagen (Fig. 1). Densitometric analysis of the fluorograms showed that osteoblasts of all OI types synthesized only slightly more type III (0–6.8%) and type V (0–4.3%) collagen than did controls (type III, 0.6–3.8%; type V, 0–3.0%). Abnormally migrating collagen type I chains were found in osteoblast cultures of each OI subtype (Table 2). Fig. 1 shows the different types of abnormally migrating collagen type I chains found with OI osteoblasts. Osteoblasts from two OI type III and one OI type IV patients produced both normally and abnormally migrating α1(I)/α2(I) chains (Fig. 1, lanes 2 and 4).

The OI type IV culture synthesizing normal and slightly retarded migrating α1(I) chains additionally showed a very slowly migrating pepsin-resistant band (Fig. 1a, lane 4, arrowhead) which did not co-migrate with collagen type III and disappeared upon prior reduction (Fig. 1b, lane 4). Direct protein sequencing revealed a substitution of glycine-610 by cysteine in α1(I)

(D. Eyre, personal communication). Osteoblasts from one OI type I, seven OI type III and one OI type IV patient synthesized only abnormally migrating collagen type I α-chains, as shown in Fig. 1, lane 6. Only normally migrating collagen type I α-chains were found in three OI type I, one OI type III and three OI type IV osteoblast cultures.

Production, processing and localization of collagen

Collagen production was significantly decreased in cultured

Fig. 1. SDS/PAGE of pepsin-treated collagens synthesized by control (C) and OI osteoblasts *in vitro*

Osteoblast cultures were labelled for 24 h with [3 H]proline. The medium was incubated with pepsin. Collagen was electrophoresed on SDS/6%-polyacrylamide gels without (a) or after (b) reduction with 0.1 M-2-mercaptoethanol. Lanes 1, 3, 5 and 7, controls; lane 2, OI osteoblast producing normally and abnormally migrating collagen type I α -chains; lane 4, OI osteoblasts producing additionally disulphide-linked α 1(I) dimers (arrowhead in a, lane 4); lane 6, OI osteoblasts producing only abnormally migrating collagen type I α -chains.

OI osteoblasts in terms of a decreased ratio of collagen to total protein and in terms of labelled collagen per cell (Table 2). Total protein synthesis did not differ significantly between controls and OI osteoblasts (results not shown).

To determine the relative amounts of intra- and extra-cellular processed and pericellular (pro)collagen, the single bands in fluorograms of electrophoretically separated procollagen and collagen chains isolated from the cell layer (Fig. 2a) and the medium (Fig. 2b) were quantified by densitometry. Consequently the total amount of radiolabel incorporated into collagen found in the cell layer or in the medium was attributed to the single bands according to their respective intensities in the fluorograms.

Assuming that procollagen processing takes place only outside the cell, processed collagen chains isolated from the cell layer were attributed to the pericellular compartment and therefore considered, together with all (pro)collagen in the medium, to be located extracellularly. Only unprocessed chains from the cell layer were considered to be located intracellularly.

The proportion of intracellular procollagen was similar in control and OI type I osteoblasts, and significantly higher in cultures of OI types III and IV osteoblasts (Table 2).

The proportion of extracellular (pro)collagen which was completely processed was similar in controls and OI type I osteoblasts, but markedly decreased in OI type III and type IV osteoblasts (Table 2). Radiolabel incorporation into processed collagen expressed on a per cell basis was highest in controls, moderately but significantly decreased in OI type I and IV osteoblasts, and very low in OI type III osteoblasts.

Pericellular collagen, which constituted 9–18% of total collagen in control and OI type I osteoblasts, was very low or

Table 2. Quantitative analysis of collagen metabolism and structural defects in collagen type I of OI osteoblasts

Experimental details are given in the text. Sample numbers are identical to patient numbers in Table 1. Structural defects were determined by SDS/PAGE of pepsin-treated samples. Significance of differences from controls was calculated using the Kruskal-Wallis/Wilcoxon test: * $P < 0.05$; ** $P < 0.01$.

Sample	Structural defect in collagen I	10 ⁻³ × Total collagen (c.p.m./10 ⁵ cells)	Collagen (% of total protein)	Intracellular collagen (% of total collagen)	Processed collagen (% of extracellular)	Pericellular collagen	
						(% of total)	(10 ⁻³ × c.p.m. /10 ⁵ cells)
Controls (<i>n</i> = 6)							
Median		82.5	17.2	6.1	24.4	15.3	12.9
Minimum value		59.2	12.3	4.5	15.1	9.8	5.8
Maximum value		97.1	23.3	9.5	28.9	18.3	17.3
OI type I							
1	None	45.9	12.1	5.5	17.1	9.2	4.2
2	α1 + α2 retarded	54.2	9.3	6.4	17.6	9.5	5.2
3	None	20.1	10.6	10.4	24.4	15.7	3.2
4	None	42.5	11.1	5.8	20.9	10.0	4.3
OI type III							
5	α1 + α2 retarded	37.6	6.1	8.3	3.5	2.8	1.1
6	α1 + α2 retarded	59.5	13.4	13.9	10.7	6.8	4.0
7	α1 + α2 retarded	43.7	15.4	16.2	9.0	4.5	2.0
8	α1 + α2 retarded	67.9	10.9	10.9	9.3	4.2	2.9
9	α1 + α2 double band	22.7	15.5	13.6	1.5	0.0	0.0
10	α1 + α2 double band	56.6	9.0	18.6	8.0	4.7	2.7
11	α1 + α2 retarded	51.7	11.2	8.3	7.4	3.1	1.6
12	None	31.0	11.5	11.2	7.6	0.3	0.1
13	α1 + α2 retarded	79.5	11.9	13.6	5.2	2.6	2.1
14	α1 + α2 retarded	40.3	8.6	27.7	5.2	0.0	0.0
OI type IV							
15	None	53.3	7.2	11.0	9.4	7.6	4.1
16	None	49.4	13.0	13.7	13.3	2.6	1.3
17	α1 + α2 retarded	40.8	11.5	18.2	9.0	0.0	0.0
18	α1 S-S dimer	76.7	13.2	11.6	10.5	3.1	2.4
19	None	51.8	11.5	12.0	7.0	2.4	1.3

Fig. 2. SDS/PAGE of procollagens from (a) the cell layer and (b) the medium, synthesized by cultured osteoblasts of controls (C) and patients with different types of OI (I, III and IV)

Osteoblast cultures were labelled for 24 h with [3 H]proline. Cell layer and medium were harvested separately and separated by SDS/PAGE after reduction with 2-mercaptoethanol. The positions of procollagen type I chains (pro α 1, pro α 2), partially processed type I chains (pC α 1/ α 2, pN α 1/ α 2) and completely processed collagen type I chains (α 1, α 2) are shown.

even absent from osteoblast cultures of OI types III and IV. Osteoblasts from all OI types incorporated decreased levels of radiolabel into pericellular collagen (Table 2).

Comparison of OI osteoblasts and OI fibroblasts *in vitro*

For comparison of certain aspects of collagen metabolism in cultured OI skin fibroblasts and osteoblasts, we compared our results with the data published by Brenner *et al.* [15] (Table 3). The ratio of collagen to total protein was similar in control and OI fibroblasts, whereas OI osteoblasts had lower values than control osteoblasts. OI fibroblasts synthesized increased amounts of collagen types III and V in comparison to control fibroblasts. Distribution of collagen types I, III and V was similar in control and OI osteoblasts.

DISCUSSION

We report for the first time on collagen metabolism in cultured osteoblasts from patients with different types of OI. Cultured osteoblasts of all OI types produced significantly less collagen than control osteoblasts. In contrast to OI skin fibroblasts, osteoblasts derived from OI patients produced collagen types I, III and V in similar proportions as in controls, but the ratio of collagen to total protein was significantly decreased in osteoblasts of all OI types compared with controls [15,16].

Quantification of intra- and extra-cellular collagen and procollagen was based on the assumption that only completely unprocessed procollagen isolated from the cell layer is located in the cell, since activity of procollagen-cleaving enzymes is re-

Table 3. Comparison of cultured fibroblasts and osteoblasts derived from controls and OI patients

Data concerning osteoblasts were derived from the cells described in the present paper. Data concerning fibroblasts were derived from cells described by Brenner *et al.* [15]. Collagen as a percentage of total protein was determined by the hydroxyproline/proline ratio (for details, see the Experimental section). Amounts of collagen types I, III and V were determined by densitometric evaluation of fluorograms of pepsin-treated samples. OI data are mean values of all cell cultures studied, which were derived from patients with OI types I, III and IV.

	Collagen (% of total protein)	Collagen types (% of total collagen)		
		I	III	V
<hr/>				
Osteoblasts				
Controls (n = 6)	17.2	96.7	2.2	1.1
OI (n = 19)	11.2	96.0	2.7	1.3
Fibroblasts				
Controls (n = 10)	12.4	94.2	7.6	1.2
OI (n = 27)	12.4	88.8	9.4	2.3

stricted to the extra- and peri-cellular compartments [17–19]. Processed collagen chains found in association with the cell layer were considered to be located pericellularly, as reported in studies with fibroblasts [17,20,21].

The distribution of type I procollagen and collagen in the intra-, extra- and peri-cellular compartments was similar in controls and OI type I osteoblasts, but markedly different in OI type III and IV osteoblasts. The elevated proportion of intracellular procollagen in OI type III and IV osteoblasts indicates impaired secretion and intracellular accumulation of procollagen type I. Similar findings have been reported from OI skin fibroblasts synthesizing structurally abnormal collagen type I [22,23]. In OI type III and IV osteoblasts, intracellular retention of collagen was found irrespective of the production of electrophoretically abnormal collagen.

The ratio of processed to extracellular (pro)collagen was similar in control and OI type I osteoblasts, decreased in OI type IV and lowest in OI type III. In cultures of control osteoblasts, about 25% of the extracellular collagen was processed. This indicates that, because of the cell culture conditions (i.e. dilution of procollagen in the culture medium), procollagen processing is low compared with the situation *in vivo*. This cell culture effect influences all cultured cells in a similar way. Therefore the low levels of processed collagen found with OI type III and IV osteoblasts have to be regarded in relation to controls; they result in part from the cell culture conditions, but most likely also from a slower processing rate, suggesting that procollagen from OI type III and IV osteoblasts is a suboptimal substrate for the procollagen-cleaving enzymes. Alternatively the activities of these enzymes might be decreased in OI types III and IV.

Osteoblasts of all OI subtypes were found to have decreased levels of processed collagen in association with the cell layer, i.e. pericellular collagen. Keeping the restrictions of the cell culture in mind, it is nevertheless tempting to hypothesize that decreased accumulation of collagen in the pericellular compartment of cultured OI osteoblasts corresponds to the low collagen content found in OI bone [24]. In OI type III and IV osteoblasts, the low level of pericellular collagen is probably due in part to decreased secretion and processing of procollagen. Failure to aggregate and to interact properly with other constituents of the extracellular matrix may also contribute to low pericellular accumulation. Similar proportions of pericellular collagen in OI type I

and control osteoblasts indicate that the low level of pericellular collagen in OI type I is mainly due to the low collagen synthesis, but not to impaired aggregation or interaction with other matrix constituents.

Abnormally slowly migrating collagen type I α -chains were found among all OI types. Osteoblasts from one OI type IV patient produced disulphide-linked $\alpha 1(I)$ chains similar to those found in some OI skin fibroblasts [25,26]. Further studies revealed a glycine to cysteine conversion at position 610 in $\alpha 1(I)$ (D. Eyre, personal communication). With the exception of two OI type III osteoblast cultures producing normal and abnormal collagen type I α -chains, nine osteoblast cultures from OI type I, III and IV patients showed only abnormally slowly migrating α -chains of collagen type I, whereas normally migrating chains were absent. Studies with OI skin fibroblasts revealed various uniallelic mutations in one of the genes coding for collagen I [2-5]. Uniallelic mutations are not compatible with the absence of normally migrating α -chains. Therefore, in addition to mutations, dysregulation of collagen type I hydroxylation and glycosylation may play an important role in the pathogenesis of OI, as reported previously [27].

Some osteoblast cultures of all OI types studied produced collagen type I α -chains indistinguishable from those of controls. OI type I can be caused by one 'null' allele of the collagen-coding genes [2], explaining the absence of structurally abnormal chains. In the cases where we found normal collagen type I in OI type III and IV osteoblasts, minor structural or conformational alterations in the triple helix or propeptides may have been undetected. However, in these cases, as in all other OI type III and IV osteoblasts, the synthesis, secretion, processing and pericellular accumulation of collagen were significantly decreased.

In summary, two distinct groups of OI osteoblasts could be distinguished *in vitro* according to the quantitative analysis of collagen metabolism. In OI type I osteoblasts, the major effect was a decrease in collagen synthesis, whereas secretion, processing and pericellular accumulation of collagen appeared similar to controls. In contrast, in OI type III and IV osteoblasts, the pools of secreted, processed and pericellular collagen were greatly decreased. Qualitative abnormalities of collagen type I were found among all OI types. We conclude that cultured OI osteoblasts represent an appropriate *in vitro* system reflecting the severe deficiency of collagen type I in OI bone.

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