Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 217-222

DIRECT VISUALIZATION OF AFFECTED COLLAGEN MOLECULES SYNTHESIZED BY CULTURED FIBROBLASTS FROM AN OSTEOGENESIS IMPERFECTA PATIENT

Kunihiko KOBAYASHI[1], Ryu-ichiro HATA*, Shin-ya NAGAI**, Jun-ichi NIWA, and Takeshi HOSHINO

Department of Anatomy, Nagoya University School of Medicine, Nagoya 466, Japan

*Department of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101, Japan

**Department of Pediatrics, Matsuyama Red-Cross Hospital, Matsuyama 790, Japan

Received August 30, 1990

Human skin fibroblasts obtained from normal controls and a patient with osteogenesis imperfecta were cultured in the presence of ascorbic acid 2-phosphate, a long-acting vitamin C derivative. Crude collagen samples extracted from the cell layer were made to form lateral aggregates of collagen molecules, segment-long-spacing crystallites. Under the electron microscope, normal and abnormal crystallites of type I collagen were identified with the patient's collagen. While the carboxyl-terminal half of the abnormal crystallite was tightly packed, the amino-terminal half was loose and spreading, indicating the site of abnormality in the amino-terminal half of one of type I collagen alpha chains. The method is simple and useful to detect abnormal collagen and to predict the site of mutation.

The defects of the collagen molecules cause deformity of connective tissues in various parts of the body with various severity. Recent studies have also revealed the genetic heterogeneity of the congenital connective tissue diseases (for reviews, see Ref. 1-3), including single point mutations within triple-helical region of collagen for as many as 20 cases of osteogenesis imperfecta (OI). The substitution of glycine into any other amino acid results in the delay of triple-helix formation, over-modification of the N-terminal side of the α chains, and the reduced secretion of the molecules. However,

[1] To whom correspondence should be addressed.

Abbreviations used: SLS, segment-long-spacing crystallite; OI, osteogenesis imperfecta.

0006-291X/90 $1.50

the consequence of the biochemical defects into morphogenic abnormalities has not been fully understood. Molecular shape of the affected collagen is only reported for in a case of OI patient (4), in which, substitution of Cys for Gly 748 in α 1(I) chain results in a sharp kink at the site of mutation.

Fiber-forming collagen molecules assemble in uni-directional, in-register, lateral aggregation form, segment-long-spacing crystallites (SLS), in the presence of ATP in acetic acid (see Ref. 5 for review). While the cross-striations of positively stained SLS correspond to the location of the polar amino acids along the collagen molecules (5), those of the negatively stained SLS correlate with the distribution of hydrophobic amino acids (6, 7). From the banding patterns, we can deduce the type(s) of constituent collagen(s) and distinguish abnormal molecules, if any, in a given crude preparation, and predict the site of irregularity within the molecule.

The present paper describes the ultrastructure of abnormal collagen molecules synthesized by fibroblasts from an OI type II patient.

MATERIALS AND METHODS

Proband

A proband (OI-4) with a variant (type II) of osteogenesis imperfecta (OI) was a new-born girl from a 28-year-old woman and a 34 year-old man, both were unaffected and unrelated each other. Ultrasound study prior to delivery showed the short limb, suggesting the impaired osteogenesis. Following Caesarian delivery, the infant had severe respiratory distress. The infant had short arms and legs, and suffered from multiple bone fractures; the sclerae were blue.

Fibroblast Culture and Collagen Preparation

Fibroblasts from the patient (OI-4) and normal controls were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for one month. The medium was also supplemented with L-ascorbic acid 2-phosphate in order to stimulate collagen production and to accumulate fully hydroxylated collagen molecules (8). Collagen was extracted from the cell layer either by pepsin (100 μ g/ml 0.5 M acetic acid) treatment or with 0.5 M acetic acid (fraction SUP0). Pepsin-solubilized collagen was precipitated with acidic 2M-NaCl, washed with 4.4 M NaCl in 0.05 M Tris-HCl at pH 7.4, and extracted with neutral 2.6 M NaCl (fraction SUP4).

SLS Formation and Electron Microscopy

SLS was formed by dialyzing collagen solution (0.05-0.3 mg/ml) against 0.2% ATP·2Na/0.1M acetic acid (final pH 2.8-2.9) at 4 ℃ for one day or more. Aggregates were taken on collodion-coated grids, negatively stained with 2% ammonium molybdate, and examined in a Hitachi H-800 electron microscope (9).

Primary Structure and Computation

Data for the primary structures (triple helical portion, 1014 residues) of human type I collagen α chains were: Ramirez

and de Wet (10) for α 1(I), and de Wet et al. (11) for α 2(I). Distribution profile of large hydrophobic amino acids was computed as described (7) by a program COLLAGEN for the computation of heterotrimers, originally made by Dr. T. Ito, Kyoto Institute of Technology.

RESULTS AND DISCUSSION

Collagen, synthesized by and extracted from cultures of OI-4 fibroblasts, was dialysed against acidic ATP solution to aggregate into SLS crystallite form. Under low-power electron microscopy, two types of SLS were identified; normal-shaped SLS and abnormal one (Fig. 1). The normal SLS is assigned to be that of type I collagen by comparing with the standard pattern (3) or with the SLS of normal fibroblast collagen (data not shown) and by aligning the negative staining banding pattern with the distribution of hydrophobic amino acids along the molecule (Fig. 2B). The abnormal SLS did not form rectangular outlines. This was tightly packed only in one side of the bundles, and loose in the other side, spreading the end like a sea-anemone. Comparison of the high-power electron micrographs (Figs. 2A and 2B) indicates that the tight end is the carboxyl terminal (downstream of translation), and the loose end is the amino terminal (downstream of helix formation). In the amino-terminal half of the bundles, individual filaments lost straightness and rigidity and were gradually bending and folding. In our case, any kink at a specific site was not observed, as reported by Vogel et al. (4).

The presence of the two distinct SLS indicates that the mutation is heterozygous for a certain gene. The presence of sea-anemone-like SLS also indicates that one species of the collagen molecules synthesized by the OI fibroblasts has irregularity in amino-terminal half of the molecule which prevent the molecule from packing into regular SLS in this side. Starting points of the spreading were localized to light band numbers 22-34, corresponding to residue numbers around 460-710 of the triple-helical region. If a single mutational event is assumed, the most probable prediction is that a point mutation converting Gly to the other amino acid within this region of either α 1(I) or α 2(I) chain results in the delay of triple-helix formation leading to the over-modification of N-terminal side of the chains, the accumulation of which, in turn, distort the triple-helical structure completely at the end. Alternatively, regular bundle formation may be disturbed by the steric hindrance of a globular portion inserted into the

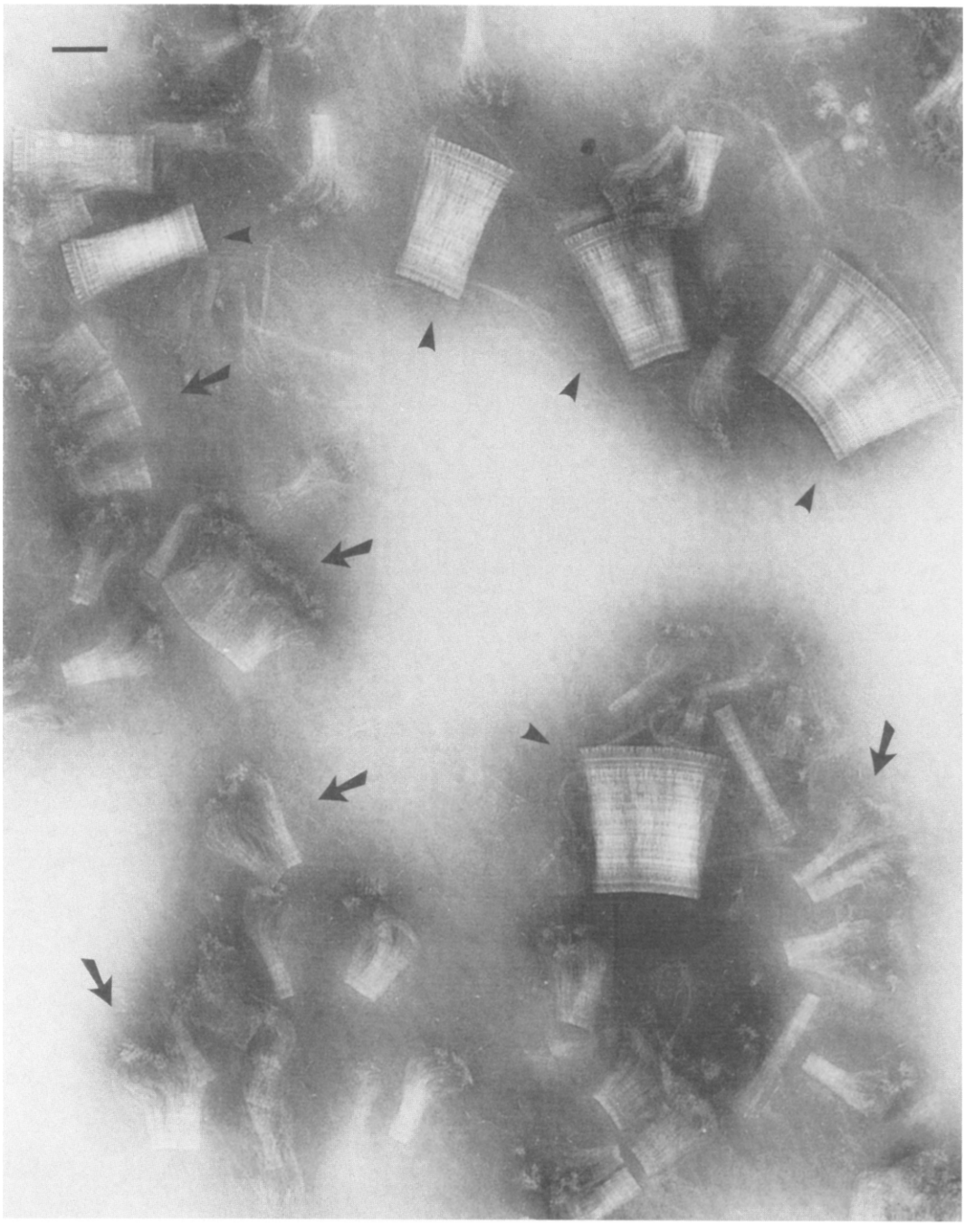

Fig. 1. Electron micrograph of SLS of collagen obtained from OI-4 fibroblast culture by pepsin extraction, negatively stained with ammonium molybdate. Normal rectangular SLS (arrowheads) and irregular, sea-anemone-like SLS (arrows) co-exist in a single field. Bar indicates 100 nm. Magnification, x 75,000.

N-terminal side of the helix. Further studies including nucleotide and amino acid sequencing are awaited for to explain the molecular structure. Nevertheless, the method introduced

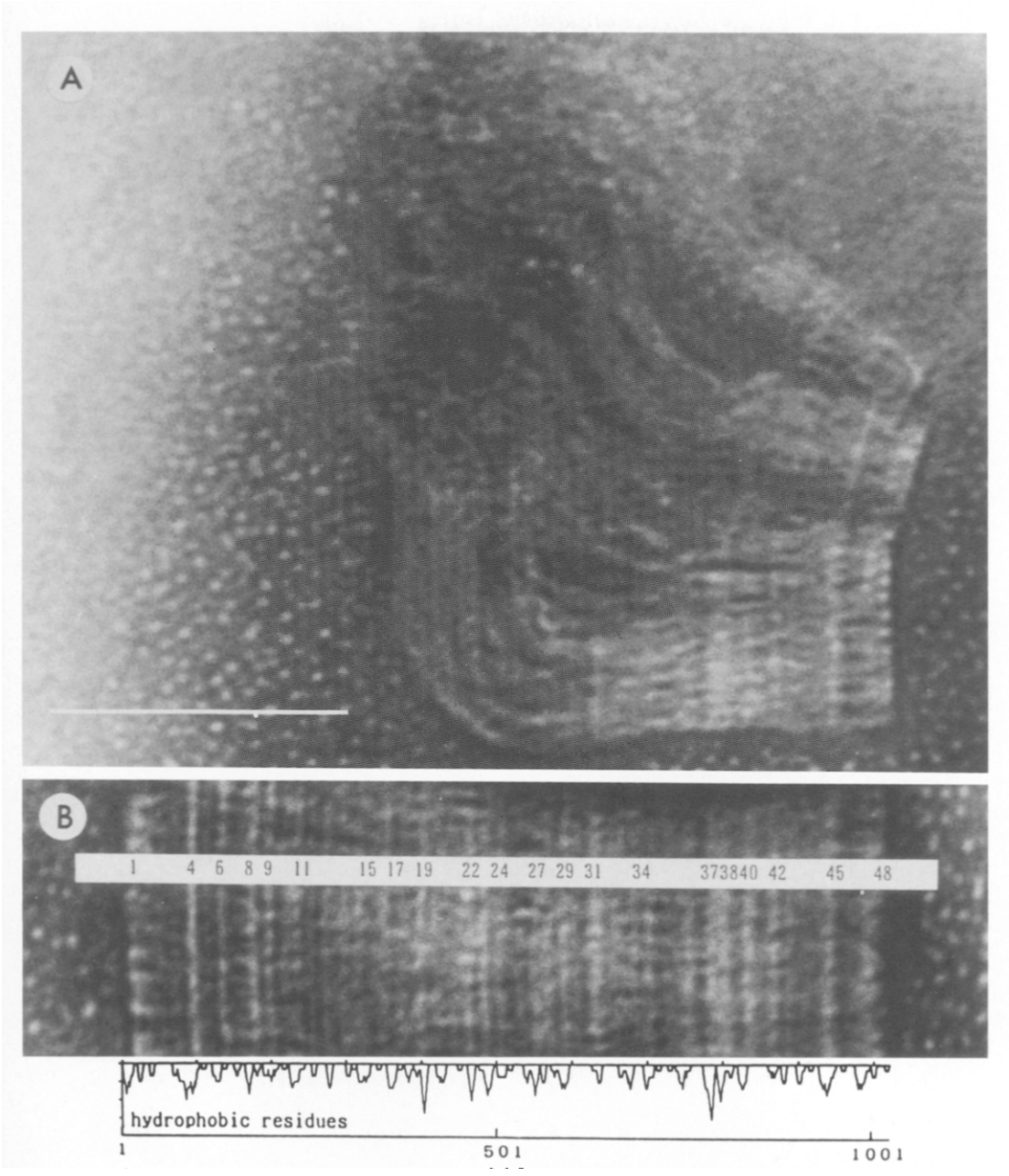

Fig. 2. Higher magnification of two types of SLS made from the OI collagen. Banding pattern of the tight end of the sea-anemone-like SLS (A) matches with the C-terminal half of the normal-shaped SLS (B). Major light bands of human type I collagen SLS are numbered consecutively from the N-terminal. Location of hydrophobic amino acids along type I collagen are shown as a histogram with residue numbers from the N-terminal (left)(C). Bar, 100 nm. Magnification, x 425,000.

here has several advantages and may be used as a tool for screening abnormal collagen in pathological samples. Collagen used was unlabeled, prepared from cultured cells, and not

necessarily fully purified: SUP0 fraction also formed similar abnormal SLS as well as normal SLS. The amount required was minute (1 μ g). Understanding of the ultrastructure of the collagen molecule is a step toward the understanding of the higher order of molecular aggregation, i.e., fibrillogenesis and morphogenesis.

ACKNOWLEDGMENT

This study was supported by a Grant-in-Aid for Scientific Research (01570008) from Japanese Ministry of Education, Science and Culture to K.K.

REFERENCES

1. Cheah, K.S.E. (1985) Biochem. J. 229:287-303.
2. Hollister, D.W. (1987) Curr. Probl. Derm. 17:76-94.
3. Byers, P.H. (1989) Am. J. Med. Genet. 34:72-80.
4. Vogel, B.E., Doelz, R., Kadler, K.E., Hojima, Y., Engel,J., and Prockop, D.J. (1988) J. Biol. Chem. 263:19249-19255.
5. Kühn, K. (1982) Collagen Rel. Res. 2:61-80.
6. Kobayashi, K., Ito, T., and Hoshino, T. (1986) J. Electr. Microsc. 35:272-275.
7. Kobayashi, K., Hashimoto, Y., Hayakawa, T., and Hoshino, T. (1988) J. Ultrastruct. Mol. Struct. Res. 100:255-262.
8. Hata, R. and Senoo, H. (1989) J. Cell Physiol. 138:8-16.
9. Kobayashi, K., Ito, T., and Hoshino, T. (1985) Collagen Rel. Res. 5:253-260.
10. Ramirez, F. and de Wet, W. (1988) Ann. N. Y. Acad. Sci. 543: 109-116.
11. de Wet, W., Bernard, M., Benson-Chanda, V., Chu, M.-L., Dickson, L., Weil, D., and Ramirez, F. (1987) J. Biol. Chem. 262:16032-16036.