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IMPAIRED SECRETION OF TYPE III PROCOLLAGEN IN EHLERS-DANLOS SYNDROME TYPE IV FIBROBLASTS: CORRECTION OF THE DEFECT BY INCUBATION AT REDUCED TEMPERATURE AND DEMONSTRATION OF SUBTLE ALTERATIONS IN THE TRIPLE-HELICAL REGION OF THE MOLECULE

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SUMMARY The amount of type III procollagen secreted by fibroblasts from *from two patients with type IV Ehlers-Danlos syndrome is reduced to* 25% and 20%, respectively, of that of control cells after incubation at 37°C, but reverts to 70% and 110% when cells are incubated at 32°C. The type III procollagen molecules secreted only at the lower temperature are of normal size but apparently contain different mutations which disrupt the triple-helical region and lower the thermal stability of the molecule. These data suggest that subtle mutations in the pro α1(III)-chains produce Ehlers-Danlos syndrome type IV by disrupting the triple-helical region of the molecule, lowering its thermal stability, and thus impairing its secretion. At the lower temperature, stabilization of the defective molecules result in more efficient secretion. This approach may be useful for the characterization of other unstable collagens.

The Ehlers-Danlos syndrome type IV (EDS IV) is a dominantly inherited disease characterized by severe fragility of the blood vessel walls, the internal organs, and the skin (1,2). A deficiency in type III collagen is considered to be responsible for the fragility of tissues in EDS IV patients (2-11). Severely reduced secretion of type III procollagen is a frequent finding in fibroblasts from EDS IV patients (4-7); however, little is known about the underlying molecular defects. We recently identified a patient with a deletion within one of the two alleles coding for type III collagen (COL3A1)(9,10); in fibroblast culture, secretion of procollagen molecules containing the shortened pro α1(III)-chains was found

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Abbreviations used are: EDS IV, Ehlers-Danlos syndrome type IV; COL3A1, the genetic locus coding for the α-chains of type III collagen; Tm, melting temperature; Mr, relative mobility.

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to be enhanced by a reduced incubation temperature (11). This observation led us to investigate the effect of reduced incubation temperature on procollagen secretion in three other EDS IV cell strains which had previously been shown to secrete reduced amounts of type III procollagen under standard culture conditions.

EXPERIMENTAL PROCEDURES

Fibroblast cultures - Three of the fibroblast strains used in this study were originally obtained from the American Type Culture Collection (CRL-1243, CRL-1299, and CRL-1384) and maintained in our laboratory. Both CRL-1243 and CRL-1299 were studied in 1975 by Pope et al.(3) and in 1984 by Stolle et al.(7). Autosomal recessive inheritance of EDS IV has been claimed in the patient from which CRL-1243 cells were derived (12). The patient from whom cell strain CRL-1384 was established was originally diagnosed by one of us (B.S.); a second cell strain from this patient (CRL-1397) was studied by Stolle et al.(7). All three cell strains have previously been shown to secrete markedly reduced amounts of type III procollagen (3,7,13,14). In cell strains CRL-1243 and CRL-1384 we found normal-sized type III procollagen mRNA (11). Cells from an EDS IV patient with a 3.3 kb deletion in COL3A1 (EL-007)(9-11), from a baby with dominantly inherited osteogenesis imperfecta (EL-012), and from adult controls were from our laboratory.

Radioactive Labelling - Preincubation of confluent fibroblast cultures with ascorbate, radiolabelling of the cultures with [2,3-^{3}H]proline and [2-^{3}H]glycine (New England Nuclear) in the presence of ascorbate for 16 hours, and harvesting of procollagens from medium and cell layer were performed as described (15). To study the effect of reduced temperature on collagen synthesis, the cultures were grown and preincubated at 37° but radiolabelled at 32°C; further processing was identical.

Purification of Collagens with Pepsin - Because melting temperature determination, and electrophoretic separation and quantitation of type III and type I collagens, are easier and more reproducible than those of procollagens, the harvested procollagens were treated with pepsin (Boehringer) to remove non-triple helical propeptides, as described (15). The samples were then neutralized to inactivate pepsin, and either lyophilized or dialyzed against 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.4, for trypsin digestion.

Melting Profiles - Thermal stability of pepsin-purified collagens was determined as described (16), with minor modifications. 50 ul-aliquots of the pepsin-treated collagens, dissolved in 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.4, were gradually heated in a water bath (ΔT= 12°C/h). Samples were removed at desired temperatures and quickly cooled by immersion in ice-water for a few seconds. A one-tenth volume of a trypsin solution (Worthington; 100 ug/ml in the above buffer) was added, and digestion was allowed to proceed for 2 min at 20°C. The samples were quickly frozen by immersion in isopropanol/solid carbon dioxide, lyophilized, redissolved in electrophoresis sample buffer, heat denatured, and analyzed by electrophoresis.

Electrophoretic Analysis - Samples were analyzed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with the buffer system of Laemmli (17) containing 0.5 M urea (13). Two-dimensional electrophoresis of α-chains and of CNBr peptides was performed as described previously (15). After electrophoresis, gels were processed for quantita-

tive fluorography (18) and exposed to Kodak SO-282 films. The fluorograms were scanned with an Uvikon 810 Gel Scanner (Kontron) equipped with a Hewlett-Packard integrator (Model 3390A). Different exposures of the same gel were scanned in order to determine the range of linear response.

RESULTS

Secretion of Collagens at 37°C and 32°C - In accordance with previous studies, we found that the amount of type III collagen, relative to type I collagen, in the medium of all four EDS IV cell strains was reduced to 10 to 25% of that in the medium of control cells, when labelling was performed at 37°C (3,7,11-14). An increased amount of type III collagen in the cell-layer, suggesting intracellular accumulation, was observed in cell strain CRL-1384, while in EL-007 cells there was retention of the shortened type III collagen (not shown).

Labelling the cultures at 32°C rather than 37°C resulted in an increase in the amount of type III collagen in the medium of three of the four EDS IV cell strains (Fig.1). Type III collagen secreted by cell strain CRL-1299 increased from 25% of the control value at 37°C to 70% of that at 32°C. In cell strain CRL-1384, type III collagen in the medium increased from 20% of the control value at 37°C to 110% of that at 32°C (Fig.1); type III collagen in the cell layer, which was increased at 37°C, was normal at 32°C (not shown). In cell strain EL-007 increased secretion of the type III collagen with a shortened triple-helix was again found (11), whereas secretion of the normal type III collagen was unchanged. No change was observed in the relative proportion of collagens secreted by cell strain CRL-1243.

Melting Profiles of Collagens Secreted at 32°C - The triple-helical conformation of collagens secreted at 32° was tested with trypsin digestion and analysis of reaction products by SDS-PAGE. Type III collagen from control cells resists trypsin digestion up to a temperature of 39°C; because of interchain disulphide bonds located at the COOH-terminus of the triple-helix, it migrates with a Mr of 300 kDa on SDS-PAGE.

At 32°C, all four EDS IV cell strains secreted a population of type III collagen molecules with normal stability; three also secreted a population of unstable type III collagen molecules (Fig.2). In cell strain CRL-1299, 50% of the type III collagen secreted at 32°C was cleaved by trypsin near the NH_2-terminal end of the molecule (as indicated by the fact that the α-chains were still migrating as disulphide-bonded trimers) yielding a doublet at Mrs of about 270 kDa and 260 kDa; cleavage occurred

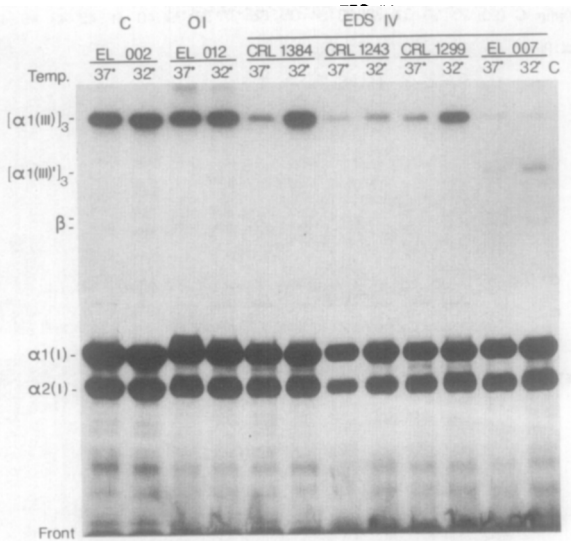

Fig.1 - Collagens Secreted at 37°C and 32°C by Ehlers-Danlos Syndrome type IV (EDS IV), Osteogenesis Imperfecta (OI), and Control Fibroblasts - $[\alpha1(III)]_3$ is type III collagen; $[\alpha1(III)']_3$ is the type III collagen with a shortened triple-helical region produced by cell strain EL-007. The fluorogram was deliberately overexposed to better visualize type III collagen. Note the increase in the amount of type III collagen secreted by cell strains CRL-1384 and CRL-1299, as well as in the amount of shortened type III collagen secreted by cell strain EL-007, after incubation at 32°C. Overmodification of α1(I)-chains from the osteogenesis imperfecta cells is abolished at 32°C.

already at 20°C. The melting temperature of the molecules containing the trypsin-sensitive site was 37°C (Fig.2, top panel). In cell strain CRL-1384, about 80% of the type III collagen secreted at 32°C was degraded by trypsin over a temperature range of 30°C to 36°C; intermediate cleavage products of approximately 285 kDa, 270 kDa, and 235 kDa were observed, suggesting that also in this case, cleavage occurred at the NH_2-terminal end of the molecule, but that the defect was located more towards the middle of the triple-helix. Only about 20% of the type III collagen resisted digestion up to 39°C (Fig.2, middle panel). When collagens secreted at 37° by these two cell strains were analysed, the population of mutant molecules was barely detectable; however, in cell strain CRL-1384 and after incubation at 37°C, the population of unstable type III collagen could be demonstrated in the cell-layer (not shown), suggesting intracellular accumulation because of impaired secretion. The type III collagen with a shortened triple-helix secreted by EL-007 cells at 32°C had a Tm of 36°C (Fig.2, bottom panel).

Two-Dimensional Electrophoretic Analysis of Mutant Type III Collagens

Under non-reducing conditions, the type III collagen secreted at 32°C

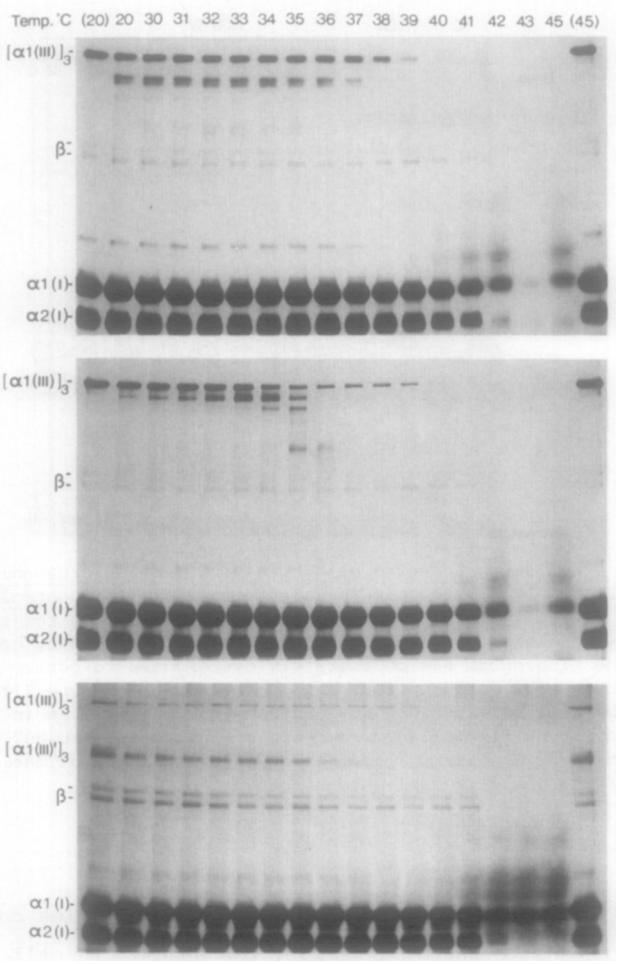

Fig.2 - Melting Profile of Collagens Secreted at 32°C by Three EDS IV Cell Strains - Pepsin-purified collagens were heated to the temperatures indicated and digested with trypsin. Samples in the first and last lanes were processed without trypsin.
Top Panel: Collagens from cell strain CRL-1299. About 50% of the type III collagen is cleaved by trypsin at 20°C to give a doublet (approx. Mr = 270 and 260 kDa); the Tm of the mutant molecules is 37°C. The rest of the type III collagen has normal stability (Tm = 39°C).
Middle Panel: Collagens from cell strain CRL-1384. Approx. 80% of type III collagen is digested by trypsin over a temperature range of 30°-36°C; the intermediate digestion products have a Mr of approx. 285, 270, and 235 kDa. A minor portion of type III collagen has normal stability.
Bottom Panel: Collagens from cell strain EL-007. $[\alpha 1(III)']_3$ is the type III collagen with shortened triple-helical region secreted by this cell strain which carries a deletion in one COL3A1 allele; its Tm is 36°C. The normal-sized type III collagen has normal stability (Tm = 39°C).

by cell strains CRL-1299 and CRL-1384 formed single bands with the same electrophoretic mobility as normal type III collagen (Fig.1). Two-dimensional electrophoresis with reduction of disulphide bonds before the second run confirmed that the monomeric α1(III)-chains were of normal size (not shown). In both cases, the lack of α1(III)-chain overmodification

(4,8) was consistent with a localization of the defects in the NH_2-terminal portion of the triple helix. The CNBr peptide pattern was also similar to that of type III collagen from control cells (not shown).

DISCUSSION

Recently, we identified a patient with a heterozygous deletion in one type III collagen gene (9,10). Secretion of the unstable procollagen molecules synthesized by his fibroblasts was found to be increased when cells were incubated at 34°C rather than at 37°C (11). In the present study, we examined whether three further EDS IV cell strains, which secreted little type III procollagen under standard conditions (37°C), could be induced to secrete more type III procollagen by incubation at a lower temperature. In two cell strains, incubation at 32°C resulted in a marked increase in the amount of type III procollagen secreted.

A triple-helical conformation is required for efficient secretion of procollagens; unhydroxylated procollagens, which cannot form a stable triple-helix at 37°C, or structurally abnormal procollagens with delayed helix formation, are poorly secreted (15,19-21). Accordingly, the intracellular degradation of newly synthesized procollagens is low at temperatures below their denaturation temperature, and high at temperatures above it (22). The finding that secretion of type III collagen in two EDS IV cell strains could be markedly enhanced by lowering the temperature of incubation, and the demonstration of a reduced stability of the collagens secreted only at the lower temperature, strongly suggests that impaired secretion at 37°C is at least in part a consequence of the reduced stability of the mutant molecules. The most likely explanation for our findings is that at 37°C, the molecules containing mutant α1(III)-chains are not secreted because the triple-helical region is partially unfolded, while at 32°C the helix is stabilized and secretion becomes possible. The mechanism by which molecules which are not triple-helical are excluded from secretion is still not known (19).

The type III collagens secreted by fibroblasts at 32°C but not at 37°C were found to be susceptible to trypsin digestion at temperatures at which normal type III collagen is resistant, indicating the presence of structural defects within the triple-helical domain. In both cases, the digestion products suggest that these structural defects are in the NH_2-terminal half of the triple-helix. Since no size alterations in the α1(III)-chains from either cell strain or in the α1(III)-CNBr peptides were observed, the structural alterations probably involve only one or a few amino acids.

This study indicates that subtle mutations with a destabilizing effect on the triple-helix, such as those observed in type I collagen leading to osteogenesis imperfecta (15,20), also occur in type III collagen. These mutations appear to produce the EDS IV phenotype mainly by impairing the secretion of type III procollagen. The finding of some normal type III collagen being secreted in all four EDS IV cell strains is consistent with heterozygosity at the COL3A1 locus (6,7,10) and argues against recessive inheritance (12).

Incubation of fibroblasts at temperatures below 37°C is a simple means by which the production of unstable collagens can be enhanced, and it may be applied to cell strains from patients with both the Ehlers-Danlos syndrome and osteogenesis imperfecta (15) in order to characterize the molecular defect. Also, because the relative amount of type III collagen produced by cultured fibroblasts can vary with culture conditions (13,23), the demonstration of an unstable type III collagen may be a better diagnostic marker for EDS IV than the finding of reduced secretion alone.

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