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Type II Collagen Defects in the Chondrodysplasias. I. Spondyloepiphyseal Dysplasias

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Summary

The spondyloepiphyseal dysplasias (SEDs) and spondyloepimetaphyseal dysplasias (SEMDs) are a heterogenous group of skeletal dysplasias (dwarfing disorders) characterized by abnormal epiphyses, with and without varying degrees of metaphyseal irregularities, flattened vertebral bodies, and myopia. To better define the underlying cause of these disorders, we have analyzed the collagens from costal cartilage from several of these patients, using SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) of intact chains and cyanogen bromide (CNBr) peptides and amino acid analysis. In almost all of the patients in this study group, the type II collagen exhibited a slower electrophoretic mobility when compared with that in normal controls. The mobility of many, but not all, of the CNBr peptides was also retarded. Peptides near the amino terminus were almost always altered, while the mobility of peptides close to the carboxyl terminus were normal in all but the severely affected cases. Analysis of the CNBr peptides on an HPLC sieving column confirmed that the electrophoretically abnormal peptides were of a higher molecular weight than were control peptides. Amino acid analysis indicated that the abnormal collagens have a higher ratio of hydroxylysine to lysine than does control collagen, suggesting that overmodification may be involved in the altered mobility. Our results are consistent with a defect in the collagen helix that results in overmodification of the molecule from that point toward the amino terminus. We propose that some forms of SED and SEMD are associated with abnormalities in type II collagen that results in delayed helix formation and consequent overmodification of the collagen. Cases of SED fit onto a continuous spectrum of clinical severity that correlates positively with both the extent of alteration and the proximity of the defect to the carboxyl terminus.

Introduction

The skeletal dysplasias are a heterogeneous group of disorders, many of which have been well defined by clinical, radiological, and morphological criteria, but the underlying biochemical defect is unknown in most of them (Sillence et al. 1979; Rimoin and Lachman 1983). Within the chondrodysplasias, the spondyloepiphyseal dysplasias (SEDs) and spondyloepimetaphyseal dysplasias (SEMDs) are a heterogeneous group of disorders that are characterized by abnormal epiphyses and flattened vertebral bodies (Lachman et al. 1975; Spranger 1975). In cases of SEMD, the metaphyses are involved as well, with varying degrees of irregularity. A variety of alterations in chondro-osseous morphology have been described in these disorders, including widely dilated cisternae of the rough endoplasmic reticulum of chondrocytes (Sillence et al. 1979; Borochowitz 1985). Most patients exhibit myopia, and vitreous degeneration has also been demonstrated (Spranger and Langer 1970). Type II collagen is present at all of these affected sites, which led us to postulate that type II collagen might be altered in these patients.

Although several distinct syndromes, such as SED congenita and SEMD Strudwick, have been defined within the broader category of the SEDs and SEMDs, the majority of cases still defy classification. To better define the underlying cause of these disorders, we have

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extracted and analyzed the collagens from several patients with SED congenita, several patients with various forms of unclassified SED and SEMD, and one patient with SEMD Strudwick. In a situation that appears analagous to the overmodifications of type I collagen that are found in lethal osteogenesis imperfecta (Byers et al. 1984; Prockop and Kivirikko et al. 1984; Cohn et al. 1986; Traub and Steinmann 1986; Bateman et al. 1987), we have found apparent overmodification of type II collagen in patients with various forms of SED and SEMD (Murray 1985, 1986; Murray and Rimoin 1985, 1988). An analysis by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) of intact type II collagen chains and CNBr peptides from several patients is given in the present report. The altered mobility on SDS-PAGE and HPLC and the increased levels of hydroxylysine suggest that overmodification of the molecule has occurred. This apparent overmodification implies that a structural defect in the type II collagen has resulted in delayed helix formation and in subsequent overmodification. The degree of clinical severity correlates with the extent to which the type II collagen molecule is overmodified; that is, the closer the apparent defect to the carboxyl terminus, the greater the extent of overmodification and the more severely affected the patient. We have found that each patient in our series has a slightly different pattern of overmodification. It appears that some of the phenotypic variability in the SEDs and SEMDs can be accounted for by heterogeneity of the position of the implied structural alteration of the type II collagen molecule.

Methods

Cases

Individual patients are indicated with three-letter designations. All patients were congenital in onset and were sporadic cases in their families. Biopsies of costal cartilage were obtained under general anesthesia. Autopsy material was obtained for case DEL. Diagnoses and clinical severity were assessed by one of us (D.L.R.), on the basis of a combination of radiographic, histologic, and clinical criteria. Height of these patients was compared with a published height-growth curve for patients with SED (Horton et al. 1982).

PET, an 11-year-old female at the time of biopsy, had a mild form of SED. Short limbs were noted at birth. She had rhizomelic shortening of the limbs and an unusual form of brachydactyly in which the third fingers were the shortest and the second fingers were the longest. The great toes were large, and the middle toes were short. She had limitation of motion at the hips and elbows, mild scoliosis, and a waddling gait. There was no evidence of myopia. Her height at age 13½ years was 126 cm. She was seen again at age 26 years, at which time her height had reached 130 cm.

HER, a 7-year-old female at the time of biopsy, had typical SED congenita. Birth weight was 6 lbs 7 oz, and birth length was said to be much shorter than that of her sibs. At age 5½ years she limped and complained of pain in her hip. At age 8½ years, her facies were normal, she had mild hyperopia, her neck was short, and there was mild scoliosis and lordosis. She was a short-trunk dwarf and had generalized laxity of her joints. On X-ray she was found to have odontoid hypoplasia, mild platyspondyly, and flattened femoral heads. At age 8.4 years, her height was 102.9 cm, just slightly less than 1 SD above the mean for SED congenita.

THO, a 13-year-old male at time of biopsy, had an unclassified SED. His birth weight was 6 lbs, and birth length 42.5 cm. At birth he was said to have feet that were twisted and had "knots" on his knees. Short stature was apparently not remarkable until 11 years of age. His height at age 13 years was 132.8 cm, which was close to 2 SD above the mean for SED congenita. His upper/lower segment ratio was 0.79, verifying a short-trunked appearance. He walked with flexion at his hips and knees, genu varum, and kyphoscoliosis. His hands were short, and his fingers were stubby.

JON, a 7-year-old male at time of biopsy, had a form of SED with pectus excavatum, minimal to moderate scoliosis, and valgus of the knees with limited motion of hips and elbows. His birth weight was 7 lbs, and his birth length was 45 cm. At age 14 years, his height was 125 cm, approximately 1 SD above the mean for SED congenita. He had significant myopia.

OHL, an 8-year-old female at time of biopsy, had an unusual form of SED. At birth, she was only 37.5 cm in length, and her legs appeared very short and were hyperflexed at the hips for more than 3 mo. She looked short limbed and short trunked. Her fingers were knobby and short, and her feet were wide and small. She developed bowing of the legs, short limbs, pes planus, and lumbar lordosis. She had myopia and astigmatism which was first noted at age 8 years. There was overgrowth of the first metatarsal and hallux valgus which required surgery.

DUN, a 2-year-old male at time of biopsy, had an unusual form of SEMD. He weighed 6 lbs 13 oz at birth,

and his birth length was 47.5 cm. At age 2 years he had an open anterior fontanelle, somewhat irregular skull shape, and a head shape that appeared proportionally larger than body size. He had unusual facial features, high-arched palate, relatively thick skin, and very short, stubby hands. X-rays showed platyspondyly, as well as changes in the long bones and in both the epiphyses and metaphyses. In contrast to other patients in this series, the metaphyses were much more significantly involved than the epiphyses.

ROB, a female child, had an SED on radiologic examination, but no clinical data were available.

REE, a 5-year-old female at time of biopsy, had a relatively severe SED congenita. Her birth weight was 6 lbs 10 oz, and birth length was 42.5 cm. She had cleft palate, clubfeet, mitral valve prolapse, myopia, and bilateral conductive hearing loss. At age 16 years, she was only 85 cm tall, with a upper/lower segment ratio of 1.33. She appeared to have severe short-trunk dwarfism nevertheless. She was found to have C1/C2 subluxation requiring fusion. She had a flat midface, genu valgum, flat feet, limited motion in her elbows, and relatively normal hands and feet. Her X-rays showed both epiphyseal and metaphyseal changes.

BRO, a 1-year-old male at the time of biopsy, had SED congenita. At birth, he had micrognathia, shortened thorax with a protuberant abdomen, and camptodactyly of the fifth finger. His neck appeared quite short. X-rays showed evidence of SED as well as lead intoxication.

WIS, a 2-year-old male at the time of biopsy, had significant SED, was very short, and had a short-trunked appearance with protruding abdomen and depressed chest. His clinical appearance was closest to that of REE.

IST, an 8-year-old male at time of biopsy, had SEMD Strudwick type. His birth weight was 5 lbs 1 oz, and his birth length was 40 cm. During his infancy, his trunk appeared short and his abdomen protruded. His fingers were long, with bilateral fifth-finger clinodactyly. There was no myopia. X-rays showed both significant SEMD characteristic of the Strudwick type and subluxation of C1/C2, requiring fusion. He had significant pectus carinatum, lumbar lordosis, and hip flexion contractures, as well as valgus deformity of the knees.

DEL, a 22-mo-old male, had severe congenital SED and died at 22 mo following pulmonary and congestive heart failure. At birth he was noted to have bilateral megalocornea, bilateral inguinal hernia, and significant short-limb and short-trunk dwarfism. He had multiple admissions for pneumonia, respiratory distress and developed ventricular hypertrophy. By 21 mo of age he had only reached the average height for a 1-mo-old infant. His early demise was secondary to the significant lung disease and cor pulmonole.

In addition to these patients, specimens were obtained at autopsy for a fetal control, three individual newborn controls, a 5-year-old control, a 9-year-old control, a 16-year-old control, and a 34-year-old control. A biopsy from a diastrophic dwarf was used as a 22-mo-old control.

Extraction of Collagen

Costal cartilage from age-matched controls and from patients with various classified and unclassified types of SED and SEMD was obtained. Sample size ranged between 15 and 100 mg (wet wt) of tissue, after removal of the perichondrium. All operations were carried out at 4°C. Tissue was minced and extracted for 24 h with 4 M guanidine HCl containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM N-ethylmaleimide (NEM), and 25 mM EDTA as protease inhibitors. Tissue was centrifuged to separate the tissue and the guanidine extract. Aliquots of this guanidine extract were analyzed by SDS-PAGE. Tissue was washed with water, and the type II collagen was extracted with pepsin in 0.5 M acetic acid and 0.2 M NaCl. The pepsin concentration was 50 μg/ml, and all tissue was extracted at a ratio of 10 mg tissue/1 ml pepsin solution. After 48 h, the digest was centrifuged. The supernatant contained the type II collagen, which was precipitated with 0.8 M NaCl and brought up in 1 M NaCl, 50 mM Tris-HCl, pH 7.5 to neutralize the pepsin. The tissue pellet was extracted with 1 M NaCl, 50 mM Tris-HCl, pH 7.5 to obtain the type XI collagen (Burgeson and Hollister 1979).

Electrophoresis

Intact chains were analyzed by SDS-PAGE on 5% polyacrylamide Laemmli gels and were visualized by silver staining (Laemmli 1970; Oakley et al. 1980). Cyanogen bromide (CNBr) peptides were made according to a method described elsewhere (Bentz et al. 1983) and were analyzed on 10% acrylamide gels by a modified silver stain. To retain the lower-molecular-weight peptides in the gels, 10% methanol was included in all water washes.

HPLC

CNBr peptides were lyophilized and brought up in 2 M guanidine, 50 mM Tris HCl, pH 7.5, for analysis on a 7.5 × 600-mm BIO-SIL TSK 250 sieving column,

eluted under isocratic conditions with the same buffer (Miller et al. 1983), by using a flow rate of 1 ml/min with a Waters 840 HPLC system. Absorbance was monitored at 225 nm, by using a Spectra-Physics SP8440 variable-wavelength detector.

Amino Acid Analysis

Samples were brought up in 6 N HCl, hydrolyzed for 20 h at 108°C, and derivatized with PITC by the Waters PICO-TAG method. Samples were analyzed on two Waters PICO-TAG columns linked in tandem, by using the PICO-TAG method. The use of two columns results in good separation of the amino acids and allows quantitation of the hydroxylysine and hydroxyproline peaks. The system was controlled with a DEC Pro 350 computer using Waters software Expert REV 3.0. Buffer A was 17.86 g sodium acetate trihydrate/liter, 0.5 ml triethylamine/liter in 0.6% acetonitrile, adjusted to pH 6.4 with acetic acid; buffer B was 60% acetonitrile in H_2O. Initial conditions were 0% B at time 0, with a flow rate of 1 ml/min. From 0 to 20 min, buffer B was increased to 46%, by using a convex curve (curve 5) to form the gradient. The subsequent gradients were linear (curve 6): 100% B from 21 to 24 min with a flow rate of 1 ml/min; 100% B at 24 min with a flow rate of 1.5 ml/min.; 0% B at 25 min with a flow rate of 1.5 ml/min. The flow rate was changed to 1 ml/min at 41 min prior to the next run. Elutions were conducted at 50°C. Elution order of amino acids is the same as that for the standard Waters PICO-TAG method. Pierce CH-hydrolyzed collagen standards were used as amino acid standards. The amount of each amino acid in the samples was calculated manually by the following formula: area of sample amino acid peak multiplied by (amount of standard amino acid loaded/area of standard amino acid peak). Residues per thousand were calculated on analyses that contained between 10 and 50 nmol amino acids. Controls and experimentals were derivatized and eluted on the same day.

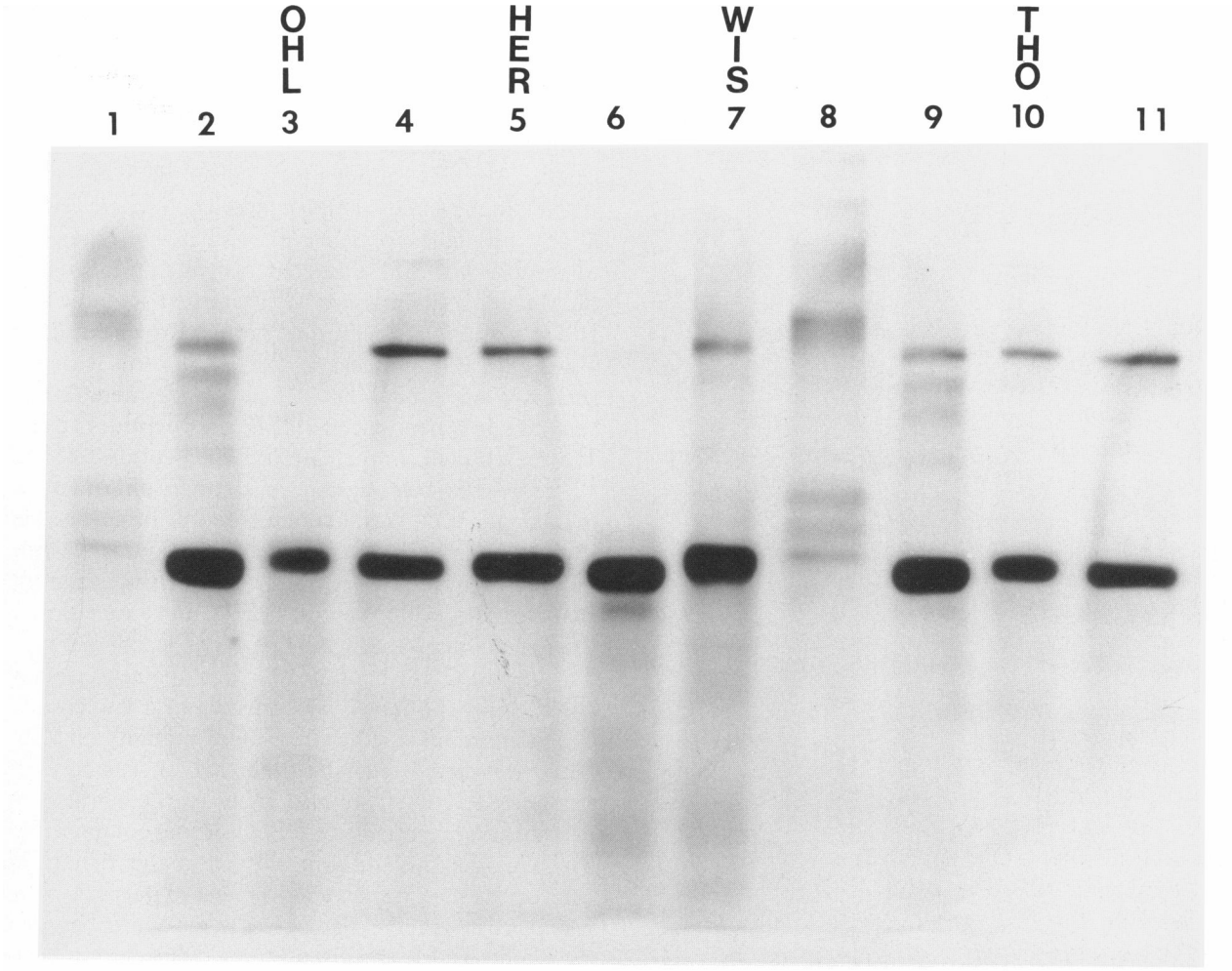
Results

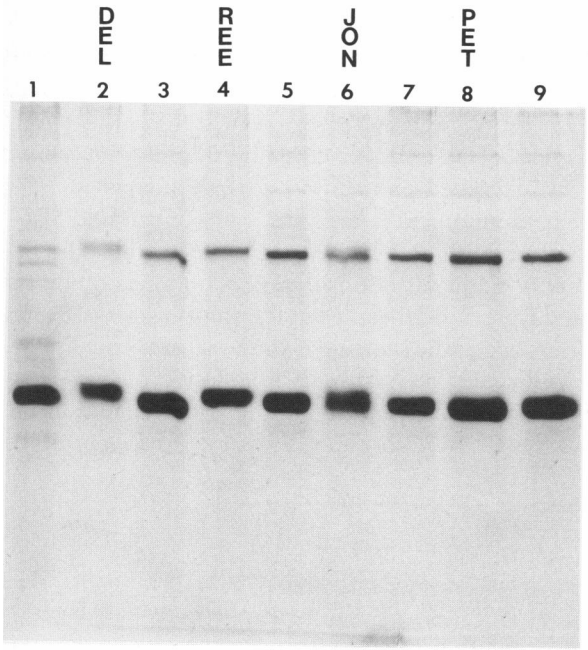
We have analyzed cartilage from a total of 12 patients with various forms of SED, including SED congenita and SEMD Strudwick. Preliminary results from six cases (OHL, ROB, DUN, HER, WIS, and IST) are presented elsewhere (Murray and Rimoin 1988). Here we present further results from these cases, plus six new cases (PET, JON, BRO, THO, DEL, and REE). We have also analyzed type II collagen from two cases of lethal SED (MAN and KIR) in a study dealing with achondrogenesis-hypochondrogenesis (Murray et al., submitted). Only SED cases that survived the neonatal period are presented here.

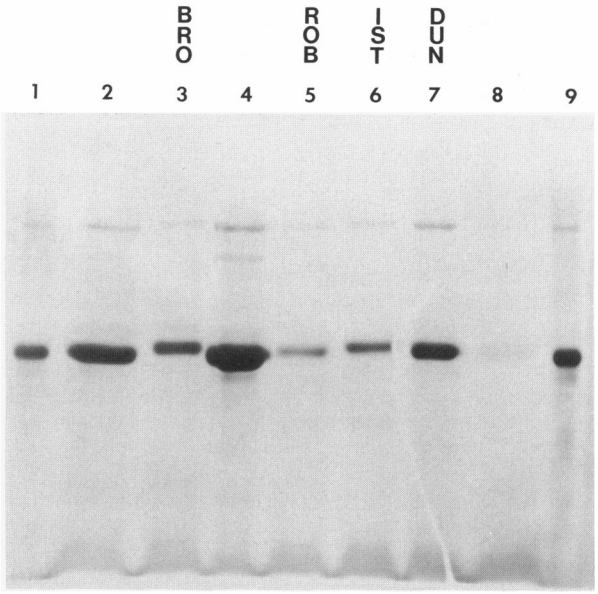
All of the patients with SED had normal 4-M guanidine extract profiles on analysis by SDS-PAGE (results not shown). All had normal type XI collagen as analyzed by SDS-PAGE (results not shown). However, almost all of the SED-patient type II collagen exhibited slower mobility on SDS-PAGE when compared with age- and site-matched control type II collagen that was extracted at the same time (figs. 1*A*–1*C*). The difference in mobility between control type II collagen and type II collagen from cases such as WIS (fig. 1*A*, lane 7), DEL (fig. 1*B*, lane 2), and IST (fig. 1*C*, lane 6) was quite noticeable. However, the mobility differences in other cases, such as HER (fig. 1*A*, lane 5) and THO (fig. 1*A*, lane 10) were subtle. While most of the type II collagen from the SED cases migrated more slowly, collagen from PET (fig. 1*B*, lane 8) appeared to migrate slightly more rapidly. The type II collagen band from JON was unusual in that it always appeared fuzzy (fig. 1*B*, lane 6) and, in some silver-stained gels, appeared to be a doublet.

The collagens were cleaved with CNBr and were analyzed by SDS-PAGE. The CNBr peptide patterns of the SED type II collagen were electrophoretically abnormal (figs. 2*A*, *B*). Many of the peptides exhibited slower mobility, particularly those closest to the amino terminus, such as peptides 12 and 11 (fig. 2*A*). The order

Figure 1 SDS-PAGE of pepsin-extracted, intact type II collagen. Type II collagen from patients was analyzed on 5% polyacrylamide silver-stained gels and was compared with control type II collagen that was extracted at the same time by using the same procedure and with purified type XI collagen. Controls were age matched as closely as possible. Patients are identified by a three-letter designation. The mobility of the SED patient type II collagen is slower than that of control type II collagen, with WIS being the most affected. Panels *A*, *B*, and *C* represent three different gels. *A*, Lane 1, purified type XI collagen standard; lane 2, normal control, newborn; lane 3, OHL, age 8 years; lane 4, normal control, age 9 years; lane 5, HER, age 7 years; lane 6, normal control, newborn; lane 7, WIS, age 2 years; lane 8, purified type XI collagen standard; lane 9, normal control, newborn; lane 10, THO, age 13 years; lane 11, normal control, age 9 years. *B*, Lane 1, normal control, fetus; lane 2, DEL, age 22 mo; lane 3, control, diastrophic dwarf, age 14 mo; lane 4, REE, age 5 years; lane 5, normal control, age 5 years; lane 6, JON, age 7 years; lane 7, normal control, age 9 years; lane 8, PET, age 7 years; lane 9, normal control, age 16 years. *C*, Lane 1, normal control 1, newborn; lane 2, normal control 2, newborn; lane 3, BRO, age 12 mo; lane 4, normal control 3, newborn; lane 5, ROB, age unknown; lane 6, IST, age 8 years; lane 7, DUN, age 2 years; lane 8, normal control, age 34 years; lane 9, normal control 1, newborn.


OHL
HER
WIS
THO
1 2 3 4 5 6 7 8 9 10 11


DEL
REE
JON
PET
1 2 3 4 5 6 7 8 9


BRO
ROB
IST
DUN
1 2 3 4 5 6 7 8 9

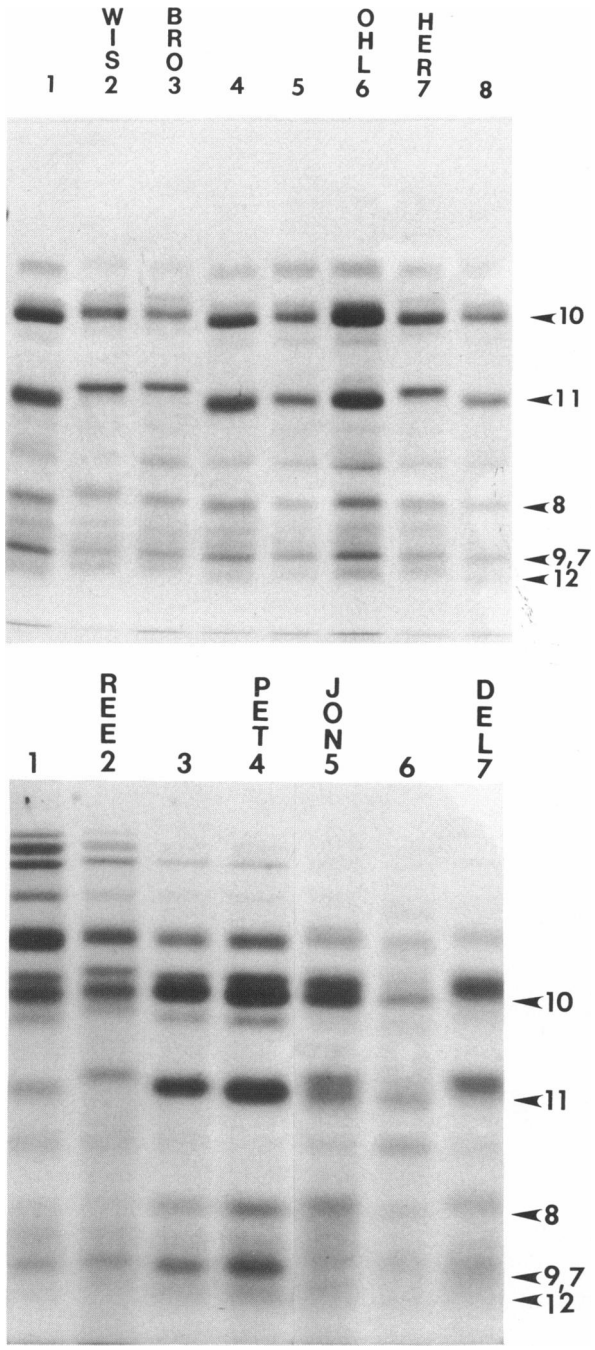


Figure 2 SDS-PAGE of CNBr peptides. Cyanogen bromide peptides of type II collagen from SED patients were compared, on 10% polyacrylamide silver-stained gels, with those of normal type II collagen. There are differences in the peptide patterns from one patient to another, and all differ from control peptide patterns. Peptides 12 and 11, those closest to the amino terminus, have slower mobility for WIS, BRO, OHL, and HER than for controls. Migration of peptide 11 is slower in REE and HER, but faster in PET, when compared with those in controls. Peptides 10 and 11 appear as doublets in JON. *A*, Lane 1, normal control, newborn; lane 2, WIS; lane 3, BRO; lane 4, normal control, newborn; lane 5, normal control, age 9 years; lane 6, OHL; lane 7, HER; lane 8, normal control, age 9 years. *B*, Lane 1, normal control, age 5 years; lane 2, REE; lane 3, normal control, age 16 years; lane 4, PET; lane 5, JON; lane 6, normal control, fetus; lane 7, DEL.

of these CNBr peptides, from the amino to the carboxyl terminus, is 12, 11, 8, 10,5, and 9,7 (see fig. 4) (Miller and Lunde 1973; Miller et al. 1973). The other, smaller peptides cannot be visualized by SDS-PAGE. As seen in the eight examples shown here, there is variability among the patients in the degree of abnormal migration. While these differences in migration are subtle, the experiments have been repeated several times and the results are reproducible. CNBr peptide 11 is always the most noticeably affected. In some patients, such as WIS and BRO (fig. 2*A*), all peptides were slightly retarded. In others, such as HER, only peptides 12 and 11 were affected. In the case of PET, peptides 11, and 12 seem to be faster migrating in comparison with those in controls. JON was different from the other cases in that peptides 10,5 and 11 appeared as doublets, while peptides 8 and 9,7 were thick and indistinct. In most of these cases, CNBr peptides 12, 11, and 8 were slower than the comparable CNBr peptides of control type II collagen. CNBr peptide 10,5 was affected in many cases, and peptide 9,7 was affected in a few cases.

To determine whether the slowed mobility on SDS-PAGE reflected a true size difference or was an electrophoretic artifact, we also analyzed the CNBr peptides of several patients by HPLC sieving columns (fig. 3). Experimentals and controls were analyzed in triplicate, on the same day. Retention times of the peaks were very consistent and varied by less than 0.1 min between the triplicates for any given sample. The elution positions of peptides 10,5, 11, 8, 9,7 and 12 from the SED cases were consistent with a higher molecular weight. An increase in levels of glycosylation of the hydroxylysines would result in higher molecular weight of the peptides. However, the position of peptide 6 is unaltered. CNBr peptide 6, a small peptide near the amino terminus, lacks lysine (Butler et al. 1976) and thus cannot be hydroxylated and subsequently glycosylated.

The amino acid composition of control and SED-patient type II collagen was compared (table 1). Because of slight variations in results obtained on different days, all of these samples were hydrolyzed and derivatized at the same time and were run sequentially, in triplicate. The level of hydroxylation of prolines is moderately increased from 42% hydroxylated in the controls to 47% for WIS, 45% for HER, and 44%

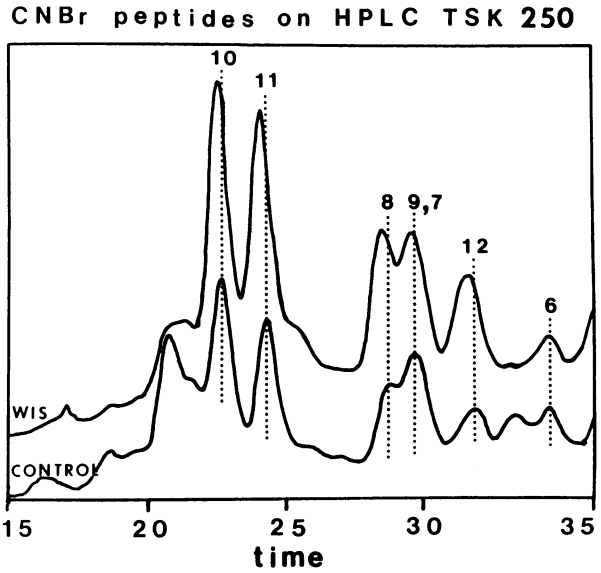


Figure 3 Comparison of CNBr peptides on an HPLC sieving column. Cyanogen bromide peptides of a normal control and of WIS were analyzed on a sieving HPLC column, eluted with 2 M guanidine, 50 mM Tris HCl, pH 7.4. These runs were made on the same day. Three runs each of the control and the SED patient were made, giving results identical to those shown here. All of the peptides from patient WIS had faster retention times than did those of the controls, except for CNBr peptide 6.

for OHL and IST. The lysines also show an increased degree of hydroxylation, from 45 and 44% for controls 1 and 2, respectively, to 52% for WIS and OHL, 54% for HER, and 56% for IST. Other compositional features were normal. Not enough material was available for carbohydrate analysis.

The patients were ranked by degree of clinical severity, as based on radiographic, histologic, and clinical criteria (table 2). Patients were also evaluated for height and were compared with a normal growth curve for SED patients (Horton et al. 1982). Patients who were more severely affected clinically, such as patients BRO, DEL, and WIS, showed greater alterations in mobility of the CNBr peptides, both in degree of retardation and in number of peptides that were altered. These patients fell below the mean height for patients with SED congenita. Conversely, patients who had milder forms of the disorder, such as patients HER and PET, showed less alteration of the peptides and were above the mean height. In severely affected patients, all peptides were altered (except for CNBr peptide 6), while in milder cases, only CNBr peptides 12 and 11 were altered. There was varying degree of alteration of peptides in the moderately affected cases, perhaps owing to position of the mutation within the peptide or to varying degrees of folding delay due to differences in the amino acid that was substituted. This suggests that there is a correlation between the position of the mutation in the type II collagen molecule and the degree of clinical severity. This is presented graphically in figure 4, which shows the relative positions of the CNBr peptides and the areas of proposed overmodification.

Discussion

Our results suggest that mutations similar to those occurring in type I collagen in some forms of perinatal lethal osteogenesis imperfecta (OI type II) are occurring with type II collagen in patients with a variety of forms of SED. In OI type II, a substitution of a glycine by an arginine or cysteine in either the alpha 1 or alpha 2 chain of type I collagen results in electrophoretically abnormal collagen chains (Byers et al. 1984; Prockop and Kivirikko 1984). This electrophoretic abnormality is the result of overmodification, i.e., excess carbohydrate addition onto hydroxylated lysines. The excess carbohydrate is thought to be the result of the delayed helix formation that occurs when a bulky amino acid

Table 1

Amino Acid Analysis of Type II Collagens from Control and SED Patients

Amino Acid	Control 1	Control 2	WIS	IST	OHL	HER
asp	37	34	29	38	38	35
glu	88	85	82	89	87	87
4hypro	99	95	106	93	93	99
ser	27	28	33	40	33	33
gly	339	344	340	342	338	352
his	2	2	3	3	3	3
arg	49	50	49	49	49	49
thr	19	20	20	23	23	21
ala	99	101	97	96	96	100
pro	137	129	119	118	118	121
tyr	1	2	9	4	2	4
val	16	20	20	22	22	20
met	6	6	7	5	7	6
ile	10	11	11	12	15	11
leu	27	27	29	27	29	24
hylys	14	14	17	14	17	13
phe	12	13	13	12	14	11
lys	17	18	16	11	16	11

NOTE.—Values are presented as residues/1,000. Control 1 values are the average of two runs, while control 2, WIS, OHL, and HER are the averages of three runs. Values for IST are from a single run. Control 1 and control 2 are different neonate individuals.

Table 2

Comparison of Clinical and Biochemical Severity

Case	Severity	Diagnosis	Height[a]	12	11	8	10,5	9,7
PET	Mild	SED	+	X	X	O	O	O
HER	Mild	SED congenita	+	X	X	O	O	O
DUN	Moderate	SMED	+	X	X	X	X	O
OHL	Moderate	SED	–	X	?	X	X	O
JON[b]	Moderate	SED	+	X	X	X	X	X
IST	Severe	SED Strudwick	–	X	X	X	X	X
REE	Severe	SED congenita	–	X	X	X	X	?
BRO	Severe	SED congenita	–	X	X	X	X	X
WIS	Severe	SED congenita	–	X	X	X	X	X
DEL	Severe	SED congenita	–	X	X	X	X	X

NOTE.—Cases were ranked on the basis of clinical severity, using criteria of height and histologic and radiographic features and were assigned labels of mild, moderate, or severe. Cases were also assessed for biochemical severity on the basis of the number of peptides that were altered and the extent of modification of the CNBr peptides. In most cases, several SDS-PAGE gels were used to assess altered mobility for each individual.

[a] A plus sign (+) indicates that the patient was taller than the mean height for patients with SED; a minus sign (–) indicates that the patient was shorter than the mean height for patients with SED.

[b] Patient JON was unusual in that his type II collagen appeared as doublets, with one band comigrating with normal type II collagen and one band migrating more slowly than normal.

is substituted for a first-position glycine in the gly-X-Y sequence. Only the small, hydrogen atom–side chain of glycine can fit into the center of a collagen helix. Another amino acid in its place is thought to delay helix formation. In the endoplasmic reticulum, helix formation by newly synthesized collagen chains begins at the carboxyl termini after completion of translation (Bachinger et al. 1981; Bruckner et al. 1981). Collagen alpha chains are posttranslationally modified by the lysyl and prolylhydroxylases and by the glycosyltransferases only prior to helix formation (Kivirikko and Myllyla 1982; Blumenkranz et al. 1984). In osteogenesis imperfecta type II, helix formation is slowed from the site of the mutation toward the amino terminus, resulting in overmodification of that portion of the molecule that lies between the substitution and the amino terminus (Byers et al. 1984; Steinmann et al. 1984; Bonadio and Byers 1985). There is evidence for both overhydroxylation of lysines and overglycosylation of hydroxylysines in osteogenesis imperfecta type II (Trelstad et al. 1977; Kirsch et al. 1981*a*, 1981*b*; Bateman et al. 1984, 1987). The severity of the disorder seems directly related to proximity of the mutation to the carboxyl terminus, depending on the type of gene mutation (Byers et al. 1984, 1988; Cohn et al. 1986). In osteogenesis imperfecta type II, there seems to be a positional correlation for substitutions of another amino acid for glycine, but if the mutation is an insertion or deletion, then there is no positional correlation. Our results suggest that the extent of overmodification of type II collagen, i.e., the position of the implied mutation, correlates with clinical severity of the SEDs and that substitutions of glycine by another amino acid in the type II collagen molecule may be responsible for some forms of SED.

An explanation for the altered electrophoretic and HPLC patterns that we observed is that overglycosylation of the hydroxylysines has occurred. Although we have no direct evidence, owing to lack of sufficient material for carbohydrate analysis, we have found evidence that is consistent with the hypothesis of overglycosylation. As in osteogenesis imperfecta, if helix formation is delayed by an alteration in the primary amino acid sequence, such as a substitution of a glycine by another amino acid, portions of the molecule between the substitution and the amino terminus could be overmodified. Our amino acid compositional studies suggest that there is a greater degree of hydroxylation of the lysines and prolines in the type II collagen of SED patients than in that of controls. The slower electrophoretic mobility is consistent with higher molecular weight, as is earlier elution from the HPLC sieving column. Overglycosylation of the type II collagen would result in higher molecular weight. Finally, CNBr peptide 6, which is closest to the amino terminus, cannot be overglycosylated be-

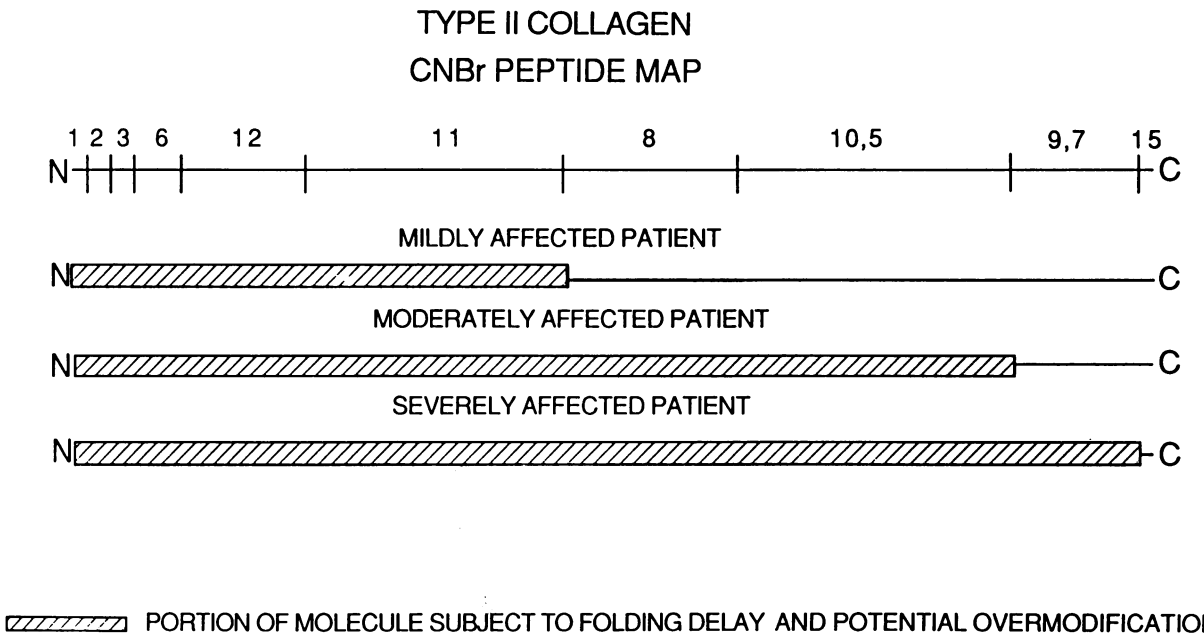


Figure 4 Correlation of position of the proposed mutation and overmodification of the CNBr peptides. This figure shows the relative positions of the CNBr peptides and indicates the portion of the molecule that might be subject to folding delay and potential overmodification. The extent of overmodification is correlated with clinical severity.

cause it lacks lysine and therefore lacks sites of potential glycosylation. HPLC analysis of peptides from an SED patient showed that CNBr peptide 6 was normal, while the other peptides were abnormal. These results suggest, but do not prove, that the altered mobility of the type II collagen from SED patients is due to overglycosylation.

The order of the major CNBr peptides, from the amino to the carboxyl terminus, is 6, 12, 11, 8, 10,5, and 9,7. We found that in the type II collagen from SED and SEMD patients, peptides 12, 11, and 8 were abnormal in almost all patients; peptide 10 was abnormal in some patients, and peptide 9,7 was abnormal in only the more severely affected patients. The extent of alteration of the molecule correlated positively with severity of the disease.

Given equal numbers of normal and mutant chains forming triple-helical molecules, it can be seen that there are eight possible combinations of molecules for the altered type II collagen molecules. One eighth of the molecules will contain all normal alpha chains, and the remainder will contain one, two, or three defective chains. Presumably, all of those molecules with at least one defective chain will fold more slowly, and both the altered and the normal chains will be overmodified in those molecules. We did not detect a normal population of molecules. The one-eighth portion that are normal molecules might be below the level of detection by the methods used here. Since there was no family history of dwarfism in the cases reported here, we feel that a new dominant mutation is the most likely explanation for most cases. However, the Strudwick form of the disorder (patient IST) is reportedly inherited as an autosomal recessive trait (Anderson et al. 1982). This may represent gonadal mosiacism in one of the parents, rather than a recessive disorder (Byers et al. 1988). In most cases, we observed only altered chains and no normal chains. However, in one case (JON), we observed doublets for the intact chain and for some CNBr peptides, correlating with a normal population of peptides, and an overmodified population of peptides. An insertion would be the most likely explanation for this finding, with one-eighth of the population consisting of all normal length molecules, one-eighth of the population consisting of all altered length molecules, and three-quarters of the population consisting of heteropolymers of normal and abnormal length molecules. We would expect the heteropolymeric molecules to be degraded intracellularly. The one-eighth part of the population that is normal might result in a milder

phenotype than would be expected from the mutational position. Further results from this patient will be reported elsewhere.

We conclude that some of the SEDs and SEMDs are the result of mutations in type II collagen that occur between the carboxyl terminus and CNBr peptide 11. The CNBr peptides from each of the patients exhibit slightly different mobilities, suggesting that each case is the result of a mutation in a slightly different location. The extent of the molecule that is overmodified—and thus the proximity of the mutation to the carboxyl terminus—correlates positively with clinical severity. Molecular studies of the type II collagen gene in these cases can now be attempted, based on the predicted site of the defect in the molecule.

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