Rough Endoplasmic Reticulum Abnormalities in a Patient with Spondyloepimetaphyseal Dysplasia with Scoliosis, Joint Laxity, and Finger Deformities

Frederic Shapiro
Departments of Orthopaedic Surgery and Pathology, Children’s Hospital Boston, Boston, Massachusetts, USA

Howard Mulhern
Department of Pathology, Children’s Hospital Boston, Boston, Massachusetts, USA

Mary Ann Weis and David Eyre
Department of Orthopaedics and Sports Medicine, University of Washington, School of Medicine, Seattle, Washington, USA

ABSTRACT
Iliac crest growth cartilage biopsy in spondyloepimetaphyseal dysplasia (SEMD) showed an endoplasmic reticulum storage disorder of epiphyseal and physeal chondrocytes. Biochemical analyses of iliac crest cartilage extracellular matrix showed no signs of deficits in any of the structural collagens types II, IX, or XI. The physis was abnormal by light microscopy with chondrocyte columnation replaced by clone-like cell accumulations surrounded by widened acellular cartilage septae. The rough endoplasmic reticulum (RER) of most chondrocytes was dilated. In some cells the RER contained homogeneous material but in most there were abnormal electron-dense accumulations. In some the material was seen in small amounts adjacent to the edge of the RER. In others, increasingly large amounts were seen that were randomly oriented and diffusely marginated. In many cells, assembly had progressed to well-marginated collections of wavy rod-like structures with a circular orientation parallel to the outer edges of the RER. The electron-dense accumulations measured from 34 to 40 nm in diameter. Mutations have prevented normal processing of collagen such that exit from the RER is abnormally slowed and abnormal self-assembly occurs within the dilated cisternae.

KEYWORDS
epiphyseal morphology, rough endoplasmic reticulum storage disorder, spondyloepimetaphyseal dysplasia

Spondyloepimetaphyseal dysplasia (SEMD) refers to a group of skeletal dysplasias with short stature where vertebrae and long-bone epiphyses and metaphyses are radiographically abnormal. The term encompasses considerable clinical, radiographic, and molecular heterogeneity. There are several subgroups with variable features such as joint laxity, multiple dislocations, scoliosis, and finger abnormalities [1–4]. We report an iliac crest
growth cartilage biopsy in a patient with SEMD/scoliosis–joint laxity–abnormal fingers demonstrating epiphyseal chondrocytes with markedly dilated rough endoplasmic reticulum containing uniquely patterned electron-dense inclusions. Biochemical analyses of cartilage tissue extracellular matrix showed no signs of deficits in any of the structural collagens, types II, IX, or XI.

**METHODS**

**Tissue Preparation for Light and Transmission Electron Microscopy**

Iliac crest biopsy was performed at 10 years of age. The iliac crest growth cartilage was dissected and fixed immediately in the operating room. Tissue slices were removed by scapel dissection passing from the outer fibrous layer of the iliac crest apophysis through the cartilage layer to metaphyseal bone. Tissue for light microscopy (LM) was fixed in 10% neutral buffered formalin, decalcified in 7.5% EDTA, infiltrated in JB4 solution, embedded in Poly Bed 812 (Polysciences, Inc.), sectioned at 0.25-μm thickness, and stained with 1% toluidine blue. Following removal of the outer fibrous layer and persisting spicules of metaphyseal bone, the epiphyseal cartilage was cut into small segments approximately 1 × 0.5 × 0.5 mm in dimension for transmission electron microscopy (TEM). The cartilage was fixed in modified Karnovsky solution (1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 3 h at 4°C, washed twice for 10 min each time in 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide for 3 h, washed in 0.1 M cacodylate buffer, dehydrated in increasing concentrations of ethanol from 70 to 100%, infiltrated and embedded in Epon, sectioned at 1 μm thickness, and stained with 1% toluidine blue. Once regions for ultrastructural assessment were identified by LM, the blocks were trimmed, sectioned at 60 nm, stained with lead citrate and uranyl acetate, and examined on a Philips 300 TEM at 60 kvs.

**Preparation of Collagen**

Carefully dissected hyaline cartilage from the iliac crest biopsy was washed extensively at 4°C with saline containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 5 mM N-ethylmaleimide, and 10 mM benzamidine). The tissue was extracted in 4 M guanidine–HCl, 0.05 M Tris–HCl, pH 7.0, to remove proteoglycans and other matrix proteins. The latter were profiled by SDS-PAGE. Portions of the insoluble matrix were aliquoted for analysis of component collagens II, IX, and XI molecular properties as follows.

Collagens II and XI were solubilized by pepsin digestion from another cartilage sample. Diced tissue, washed after 4 M guanidine–HCl extraction, was stirred in 3% acetic acid with pepsin (1:10 tissue dry weight) for 24 h at 4°C. Solubilized collagen was freeze-dried and run on SDS-5%PAGE and stained with Coomassie blue to detect the collagen α-chains [5].

Collagen IX was profiled by a peptide-mapping technique using a polyclonal antiserum and Western blotting to reveal major CNBr−derived peptides in the tissue digest [6]. Briefly, about 5 mg cartilage (wet weight) after 4 M guanidine–HCl extraction was washed in water, dried, and digested with CNBr in 70% (v/v) formic acid. Peptides were resolved by reverse-phase HPLC, followed by SDS-PAGE of the resulting serial fractions, transblotted to PVDF membrane, and detected by a rabbit polyclonal antiserum recognizing multiple epitopes in all three collagen IX chains. Another aliquot of the CNBr-digest was run directly on SDS-10%PAGE, stained with Coomassie blue, to seek evidence of post-translational overmodification of the type II collagen matrix pool.

**Clinical and Radiographic Findings**

The patient was female. Her major clinical problems from the early years of life were marked bilateral genu valgum, patellar dislocations, and knee instability. She also had severe subluxation of the radial heads, dorsal subluxation of the distal ulnae, mid-face hypoplasia, and tracheobronchomalacia. Moderate thoracic scoliosis developed but there was no interpedicular narrowing in the lumbar spine. Characteristic metaphyseal striations were seen most extensively on radiographs of distal femur and proximal tibia. (Figure 1A). Asymmetric and premature physeal fusion was common. Bracketing was prominent in the epiphyses of metacarpals 1–5 and of the proximal and middle phalanges of each hand during growth (Figure 1B). Many of the long-bone epiphyses demonstrated irregular shapes of the secondary centers and misshapen (primarily flattened)
RESULTS

Light Microscopy

Many of the epiphyseal chondrocytes were large and contained increased cytoplasmic inclusions. The chondrocytes were surrounded by denser staining matrix in the pericellular regions compared with the interterritorial areas. The matrix appeared homogeneous with no evidence of fibrous tissue collections. The physis was abnormal. Proliferating zone cells were not well aligned. Groups of chondrocytes were undergoing some of the characteristic hypertrophic changes of this region but they were clustered into clone-like accumulations separated by wide acellular septae rather than demonstrating the normal linear, columnar sequence of changes. The cartilage matrix at the lower end of the physis was surrounded by bone, and endochondral bone formation occurred but bone was synthesized around large accumulations of cartilage rather than in linear array around single trabecular cores (Figure 2). Osteoclast resorption and marrow cavi- tation were normal. The physeal–metaphyseal junc- tion was irregular and collections of chondrocytes and thick acellular cartilage septae persisted in the metaphysis, a finding consistent with the characteristic radiographic metaphyseal striations of this disorder.

Transmission Electron Microscopy

Ultrastructural abnormalities were most marked in the epiphyseal chondrocytes but some changes were also seen in physeal chondrocytes. In the proliferating zone physeal chondrocytes the RER was markedly dilated with the accumulations containing a mildly electron-dense, homogeneous uniform appearing substance (Figure 3Ai and Aii). Similar findings characterized upper hypertrophic zone chondrocytes. Both epiphyseal and physeal cartilage matrix appeared normal.

There were increased amounts of dilated rough endoplasmic reticulum in virtually all the epiphyseal chondrocytes. In many cells the RER was massively increased in amount and degree of dilation. A progressive shift in the appearance of the contents of the RER was seen. These ranged from isolated focal electron-dense accumulations with poorly outlined margins at the periphery of the RER (Figure 3Bi and Bii), to random but still poorly marginated accumulations filling entire sections of RER (Figure 3C), to well-marginated circular or wavy rod-like accumulations filling entire sections of RER (Figure 3D, E). In some chondrocytes membrane-bound regions of dilated RER with mildly electron-dense homogeneous collections of material were juxtaposed with dilated RER containing the well-marginated wavy, linear electron-dense accumulations in circular or spiral array (Figure 3E). In those cells with the best defined electron-dense inclusions, the RER often had fat body inclusions while the rest of the cyto- plasm was markedly degenerated (Figure 3D). Some

FIGURE 1 (A) Radiograph of knees at 6 years 7 months of age shows metaphyseal radiodensity and striations in distal femurs and proximal tibias. (B) Antero-posterior radiograph of metacar- pals and phalanges at 10 years 10 months of age shows bracket- ing of epiphyses (atypical peripheral bone formation).
cells had undergone cell death with advanced degeneration seen. Many chondrocytes had glycogen inclusions.

The earliest least discrete and poorly marginated electron-dense accumulations measured in the 40- to 50-nm-wide range. Most of the better structured, well-marginated rods filling entire segments of the RER measured from 34 to 40 nm in width in cross sections and longitudinal-sections.

Matrix Biochemistry

SDS-PAGE analysis of 4 M guanidine–HCl-extracted matrix proteins showed no obvious abnormalities in comparison to similar extracts of control cartilage samples (a spectrum of fetal to adult human samples). No new or more pronounced protein bands were evident, for example, that might originate from the RER inclusion bodies.

The gel electrophoretic analyses of pepsin-solubilized collagen showed α1(II) chains of normal mobility and no evidence of the post-translational overmodification that characterizes SED cases caused by COL2A1 mutations [5]. Similarly, collagen XI chains as far as they were investigated also appeared to be normal in amount and mobility. The collagen IX fingerprint was normal, with cross-linked peptides present, indicating normal polymerization in the collagen heteropolymer in contrast, for example, to the profile in an MED case caused by a COL9A3 mutation [7].

DISCUSSION

The structural abnormalities of the epiphyseal chondrocytes in this study of SEMD define an endoplasmic reticulum storage disorder (ERSD) [8]. The rough endoplasmic reticulum is the intracellular organelle where synthesis of extracellular matrix proteins occurs prior to their passage to the Golgi complex and eventual extrusion into the extracellular matrix. The molecular activities accompanying RER function are being increasingly defined [9–11]. Mildly dilated RER is an ultrastructural sign of a cell actively involved in protein synthesis. Massively dilated RER, however, is an ultrastructural indication of improper processing associated with a disorder where a mutation prevents proper folding and extrusion of protein products. Improperly folded proteins in the RER are normally transported back into the cytosol by a process referred to as retro-translocation where they undergo degradation by the ubiquitin–proteasome system [9]. In some cases, the abnormal protein accumulates in the RER and is there for a sufficiently long period of time that it begins to aggregate, forming differing patterns of electron-dense inclusions, such as the one we have identified here. This further complicates cell function since it appears to overwhelm the normal synthesis-degradation mechanisms. The aggregates eventually fill the interior of the cell, leading to cell degeneration and death. In this study those chondrocytes where the dilated RER is packed with electron-dense inclusions are
FIGURE 3  (Ai) Physeal chondrocyte with markedly dilated RER filled with mildly electron-dense homogeneous material. Surrounding cartilage matrix is normal in appearance. Bar = 500 nm. TEM, x11,000. (Aii) Higher-power view of dilated RER with homogeneous material is shown. Bar = 500 nm. TEM, x36,000. (Bi) Electron micrograph of portion of an epiphyseal chondrocyte shows electron-dense material at periphery of dilated rough endoplasmic reticulum in 2 separate areas at top and bottom. Bar = 500 nm. TEM, x14,000. (Bii) Electron micrograph shows deposition of electron-dense material at higher magnification at upper peripheral part of dilated RER. Most of the material in the dilated RER is still homogeneous and minimally electron dense. Bar = 500 nm. TEM, x36,000. (C) Epiphyseal chondrocyte with dilated RER filled with electron-dense material, which is diffusely margined and randomly arrayed. Bar = 500 nm. TEM, x28,000. (D) Dilated segment of RER in degenerating epiphyseal chondrocyte contains a wavy, rod-like electron-dense material dispersed throughout. The electron-dense material is more abundant, discrete, and organized than when it is seen initially in (Bi), (Bii), and (C). Three fat inclusions are also present in the RER. Bar = 500 nm. TEM, x14,000. (E) Electron micrograph of epiphyseal chondrocyte shows several dilated membrane-bound segments of RER, two of which contain discrete wavy short rod-like or circular structures that are reasonably well margined. Some of the inclusions are dense and circular, which is consistent with a rod-like structure cut in cross section. In the larger section of RER at the periphery, adjacent to the membrane, there is a circular or spiral-like alignment of the rods parallel to the curvilinear membrane. Other segments have dilated RER with mildly electron-dense material, which remains homogeneous. Bar = 500 nm. TEM, x10,500.
often degenerated while those with definite but less extensive accumulations are still structurally intact.

The ultrastructural abnormalities were more prominent in epiphyseal cartilage chondrocytes than in physeal chondrocytes. The rough endoplasmic reticulum was abnormal in both groups of chondrocytes. In the proliferating zone physeal chondrocytes the RER was markedly dilated with the accumulations containing a mildly electron-dense, homogeneous, uniform-appearing substance. Similar findings characterized hypertrophic zone chondrocytes. In the epiphyseal chondrocytes, however, the RER was invariably markedly dilated with irregular, electron-dense accumulations noted in varying stages of formation. These ranged from focal electron-dense accumulations with poorly outlined margins at the periphery of the RER (Figure 3Bi and Bii) to well margined rod-like accumulations filling

FIGURE 3 Continued.
entire sections of RER (Figure 3D and E). In some chondrocytes membrane-bound regions of dilated RER with mildly electron-dense homogeneous collections of material were juxtaposed with dilated RER containing the well-marginated, wavy, linear, electron-dense accumulations in circular or spiral array (Figure 3E).

The fact that epiphyseal chondrocytes were more affected than physeal chondrocytes, in terms of having large concentrations of electron-dense accumulations of aggregated material, may be due to a temporal effect since they turn over more slowly, allowing the abnormal protein inclusions to be transformed or self-assembled to ultrastructurally evident forms. The pattern of initial aggregate appearance at focal spots along the RER membrane, followed by eventual presence throughout large dilated sections of RER, may indicate that the RER does not process materials uniformly throughout its entire expanse but that certain reactions occur at certain parts of the RER. The presence within the same chondrocytes of membrane-bound dilated RER with electron-dense aggregates immediately adjacent to dilated RER with a uniform homogenous substance also supports this interpretation.

Abnormalities of endoplasmic reticulum processing in other disorders have been defined. Kim and Arvan [8] reviewed RER storage disorders in relation to endocrinopathies, but few ultrastructural examples were given for bone and cartilage and most of those showed only dilated RER with uniform, homogenous content. Since extracellular matrix protein mutations are prominent in the skeletal dysplasias, it is not surprising that ultrastructural findings can accompany gene and molecular abnormalities. We demonstrated dilated RER inclusions in chondrocytes in a case of multiple epiphyseal dysplasia (MED 3) with a mutation in type IX collagen (alpha 3 chain) [7]. The RER contained alternating parallel bands of electron-dense and electron-lucent material, an appearance different from findings in this case. Epiphyseal chondrocytes also showed the phenomenon clearly while physeal chondrocytes were characterized by dilated RER without aggregates. Previously, chondrocytes from patients with pseudoachondroplasia, now known to be due to COMP mutations, were shown to contain dilated RER with spirally oriented bands of electron-dense and electron-lucent material [12,13]. This observation in pseudoachondroplasia has been made by several different groups, which supports its specificity as a marker for this disorder. Menger et al. [14] reported a similar electron microscopic finding of RER inclusions to our case in physeal hypertrophic chondrocytes in a case of SEMD with extreme short stature. In other skeletal dysplasias, chondrocytes have been shown with massively dilated RER containing homogeneous material, for example, spondyloepiphyseal dysplasia congenita and Kniest dysplasia, both type II collagenopathies [15–17]. Osteoblasts in patients with osteogenesis imperfecta have moderately dilated RER with homogeneous material [17].

The biochemical data showed no signs of abnormalities characteristic of spondyloepiphyseal dysplasia (SED) caused by COL2A1 mutations [18,19] or other evidence of chondrocyte mishandling of cartilage collagen gene products. There have been reports of gene mutations and molecular abnormalities in the SEMD group but the variability is wide. Strudwick–type SEMD is due in some instances to collagen type II mutations. Tiller et al. [20] identified differing mutations in αI(II) collagen underlying SEMD in 3 unrelated patients: a Gly 709 Cys substitution in the triple helical domain in one, a Gly 304 Cys substitution in the second, and a Gly 292 Val substitution in the third. Kaitila et al. [21] found Gly 154 Arg mutations in 2 unrelated patients with SEMD with similar clinical appearances. A mutation in PAPSS2 (phosphoadenosine-phosphosulfate-synthase 2) has been identified in Pakistani-type SEMD [22–24]. A homozygotic substitution (973T>A) in the gene encoding for Matrilin 3 (MATN3), a cartilage specific matrix protein, was found in 5 affected people from an Arabic family with SEMD [25]. The phenotype was characterized by short stature, lower extremity bowing, radiographic epiphyseal–metaphyseal changes, but normal hands and no scoliosis.

A molecular-pathogenetic classification of the skeletal dysplasias has been outlined [26]. The structural data presented here and elsewhere show considerable value in also using histologic and ultrastructural studies of growth cartilage to diagnose and categorize dysplasias [7,8,12–17,27]. Many of the cell and matrix changes are either pathognomic of particular dysplasias or highly informative of where mutations act in the matrix synthesis pathway.
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