Reversible Metaphyseal Dysplasia, a Novel Bone Phenotype, in Two Unrelated Children with Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy: Clinical and Molecular Studies

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We report the association of an undescribed, reversible metaphyseal dysplasia (RMD) with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in two patients, one homozygous and one heterozygous for a 13-bp deletion in exon 8 of the autoimmune regulator (AIRE) gene. One patient also had a novel deletion in exon 6, resulting in a frameshift mutation and introduction of a STOP codon in exon 10. Their APECED phenotypes differed, but both patients developed progressive skeletal deformities and growth failure from early childhood. Radiological examination suggested a generalized abnormality of endochondral ossification, with irregular, flared, radioopaque regions in the metaphyses, subjacent to the growth plates. Histopathology in patient 1 showed islands of calcified cartilage within bone, consistent with impaired coupling of cartilage resorption with vascular invasion and ossification. Despite discordance for puberty, both patients experienced radiological resolution of their bone disease in their mid-teens, with improvement in histopathology in patient 1. RMD may constitute a rare phenotypic variation of APECED, possibly resulting from autoimmune directed against skeletal proteins. We also demonstrated AIRE expression in chondrocytes derived from human fetal growth plates, primary culture of human chondrocytes, and two chondrosarcoma cell lines, suggesting a potential role for abnormal AIRE expression in the development of RMD. (J Clin Endocrinol Metab 88: 4576–4585, 2003)

AUTOIMMUNE POLYENDOCRINOPATHY-candidiasis-ectodermal dystrophy (APECED) is a rare, autosomal recessive, autoimmune, multiorgan disease associated with mutations in the AutoImmune REgulator gene (AIRE) (1). At least 45 mutations of the AIRE gene have been reported in association with APECED; however, despite the apparently monogenic etiology, there is no genotype/phenotype correlation even within families (2). Most commonly, the disease is characterized by the progressive development during childhood of mucocutaneous candidiasis, hypoparathyroidism, and Addison’s disease; however, the autoimmune process can affect a variety of endocrine and nonendocrine organs, and manifestations can develop throughout life. Skeletal abnormalities have not been described in association with APECED.

The AIRE protein is thought to act as a transcription factor based on its structure, its nuclear and cytoplasmic subcellular localization, and its trans-activating ability in transient transfection studies (2–5). Detection of AIRE gene expression in tissues such as thymus, fetal liver, spleen, lymph nodes, and peripheral blood lymphocytes (2) is consistent with the encoded protein playing a central role in modulating the immune response. AIRE gene expression has also been reported in organs that are not directly involved in immune function (4, 6), implying a broader biological role for the putative transcription factor. Its target genes, those regulating it and the mechanism by which altered expression results in such diverse features as autoimmune destruction of endocrine glands, ectodermal dystrophy, and susceptibility to fungal skin infections have not been elucidated.

This paper describes two unrelated individuals with differing phenotypes of APECED who during childhood developed a hitherto unreported, reversible abnormality of endochondral ossification. From the histopathological examination of serial bone biopsies carried out in patient 1, we postulate that the normal coupling of hypertrophic cartilage resorption with vascular invasion and ossification has been disrupted. Mutational analyses and expression studies were performed to explore the possibility that AIRE mutations could contribute to the bone phenotype seen in these patients.

Case Reports

Patient 1

The first patient is one of three affected children in a sibship of nine born to unrelated parents of differing European origins. Like her two affected brothers, she developed the classic triad of mucocutaneous candidiasis, hypo-
parathyroidism, and Addison’s disease from early to late childhood. She and her older brother have developed diverse, additional, known manifestations of APECED.

Mucocutaneous candidiasis developed during the first year of life and proved more difficult to control than in her affected brothers, but responded to a course of fluconazole at age 14 yr, 9 months. Hypoparathyroidism was diagnosed at 2.4 yr when she presented with seizures associated with hypocalcaemia. On presentation to our clinic at age 9.5 yr, her serum concentration of calcium was 1.53 mmol/liter [reference range (RR), 2.2–2.7 mmol/liter], and that of phosphate was 1.9 mmol/liter (RR, 1.1–1.8 mmol/liter) associated with an undetectable serum concentration of PTH. Over the past 8 yr, she has received 11–16 ng calcitriol/kg/d and approximately 2 g oral calcium daily (Sandocal 1000, Novartis Pharmaceuticals, East Hanover, NJ). During that time, hypercalcemia has not been detected with her serum concentrations of total calcium ranging between 1.53–2.52 mmol/liter (mean ± sd, 2.09 ± 0.2 mmol/liter; n = 46; associated with normoproteinemia). Urinary calcium/creatinine ratios have been normal (0.1–0.6 mm/mM; RR < 0.8), and there has been no evidence of nephrocalcinosis or ectopic calcification of other soft tissues. Addison’s disease was diagnosed when she was investigated for fatigue at age 3 yr, and she has responded well to hydrocortisone (10–12 mg/m2/d) and fludrocortisone (0.05 mg/d). Keratitis-uveitis was diagnosed at 10 yr, but has remitted. She has been investigated for episodes of malabsorption with exclusion of celiac disease and pancreatic insufficiency, and has had infrequent, transient, mild elevations of liver transaminases (2–4 times the upper limit of normal). She also has developed primary gonadal failure associated with a normal female karyotype. At 16.9 yr, her serum concentration of estradiol was in the prepubertal range (76 pmol/liter), and her gonadotropins were in the postmenopausal range (LH, 28.6 mIU/ml; FSH, 87.2 mIU/ml).

When first assessed in the Sydney Children’s Hospital Endocrine Clinic at 9.5 yr, she was complaining of pain on walking and had a waddling gait, marked genu valgum, and erosion of the ankles. Her intermalleolar distance was 9 cm, compared with 6 cm at 7.2 yr when she was evaluated for genu valgum by an orthopedic surgeon. A skeletal survey obtained at 9.5 yr demonstrated metaphyseal changes in all long bones, the digital phalanges, and the iliac crest. These comprised irregular, flared, radiopaque regions subjacent to the growth plates (Fig. 1). The extent of the radiopaque bands in the lower limbs had progressed in comparison with x-rays taken at 7.2 yr (Fig. 1). The diaphyseal bone had a normal architecture and density. There were no abnormalities in the bones of the skull. A bone scan showed increased metaphyseal uptake, but no other abnormality. Bone mineral density was above average for age and sex at the lumbar spine and average at the femoral neck. Her bone age was 2–3 yr delayed. Her serum concentrations of alkaline phosphatase and osteocalcin were normal.

To investigate whether this might be an inherited bone disease unrelated to APECED, the hands and wrists of her four older unaffected siblings and parents as well as her two affected brothers were radiographed. Her younger affected brother had very mild metaphyseal sclerosis affecting the distal radius (Fig. 2A); however, he has not developed musculoskeletal symptoms or impaired growth, and the observed radiological changes had resolved 3 yr later (Fig. 2B). The other family members had no clinical or radiological abnormalities.

Further investigations were suggestive of chronic inflammation. At age 9.5 yr, antinuclear antibodies were weakly positive (homogeneous pattern), as were antismooth muscle antibodies. Antimitochondrial and gastric parietal cell antibodies were negative. The patient is HLA-B27 negative. She had mild anemia (hemoglobin, 9.8 g/dl at 9.5 yr), which persisted for several years associated with a normal white cell count and differential. Her anemia had resolved by 16.9 yr. Her erythrocyte sedimentation rate was 75 mm/h (RR, 3–30) at 9.5 yr and was still raised when measured at 13.2 and 14.2 yr (41 and 42 mm/h, respectively). Plasma concentrations of IgG were initially more than 3 times the upper limit of the reference range, but decreased with time [9.8 yr, 46 g/liter (RR, 8.5–13); 10.2 yr, 31.5 g/liter (RR, 7–16); 16.9 yr, 22.6 g/liter (RR, 6.2–14.4)]. In contrast, her affected brothers had mild elevations of IgM only.

By age 11.4 yr, her ability to walk even short distances was limited by pain. She had an intermalleolar distance of 17 cm and mild bilateral knee contractures. The continued clinical and radiological progression of her skeletal pathology was associated with severe linear growth failure (Fig. 3). Thyroid function was normal. She was GH sufficient, with a peak serum concentration of 45 mU/liter in response to stimulation with glucagon and clonidine at 11.4 yr, and IGF-I was normal for age (16 nmol/liter; RR, 15.7–63). She did not respond to a course of somatotropin (22 IU/m2/wk) between 11 yr and 12 yr.

To try to limit further deformity, bilateral knee stapling was performed at 11.4 yr, and an open bone biopsy was obtained from the distal right femur. In addition to histopathology, cultures for Candida albicans were performed and were negative. Knee stapling failed to halt the progression of her lower limb deformity. She had a left femoral osteotomy and repeat bone biopsy at 12.6 yr and a right femoral osteotomy at 13 yr.

X-rays of her knees at 15.3 yr showed improvement in the radiological appearance of the metaphyses. This was associated with some improvement in growth. To capitalize on this and to stabilize her knee joints, bilateral femoral screws were placed at age 15.4 yr, and a further biopsy was obtained from the distal right femur. Subsequent x-rays have continued to show regression of her disease (Fig. 1).

**Patient 2**

The second case is a male, now aged 20 yr. He is the only child affected with APECED born to unrelated parents of different European origin from those of patient 1. Chronic mucocutaneous candidiasis developed in the first year of life. Pernicious anemia was diagnosed at 9 yr. At 10 yr, he developed Addison’s disease and chronic hepatitis. Type 1 diabetes mellitus developed at 11 yr. At 17 yr he was found...
Bone histopathology

Bone biopsies in patient 1 were obtained from the distal right femoral metaphysis at ages 11.4 and 15.4 yr and from the distal left femoral metaphysis at age 12.6 yr. On each occasion, bone specimens were processed in two ways.

Method 1. Bone was fixed in 10% cold buffered formalin, decalcified in EDTA, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Sections from the biopsies at 12.6 and 15.4 yr were examined for Igs using antibodies to IgG, IgA, and IgM (DAKO, Glostrup, Denmark). Immunostaining was performed with EDTA antigen retrieval and an LSAB.2 detection kit (DAKO). Staining was visualized with dianaminobenzidine, resulting in a brown product, and counterstaining was performed with hematoxylin.

Method 2. Bone was fixed in 95% ethanol at 4°C for 48 h, dehydrated in 100% ethanol, and embedded in hydroxy-ethyl-methacrylate monomer. Subsequent polymerization was carried out at 4°C. Sections were cut and stained with 1% Toluidine Blue buffered to pH 7.2, von Kossa stain, alkaline phosphatase (Kit 86R, Sigma-Aldrich Corp., St. Louis, MO), and tartrate-resistant acid phosphatase (TRAP; Kit 580A, Sigma-Aldrich Corp.).

Mutational analysis

Blood samples for mutational analysis of the AIRE gene were obtained after informed consent was obtained from patient 1, her parents, her affected brothers, and her two youngest siblings and from patient 2. Genomic DNA was extracted from blood samples by the phenol-chloroform standard procedure (7).

PCR was performed in a total volume of 50 μl containing 250 ng template genomic DNA according to the APECED study group recommendations (8). To detect the APECED mutations, AIRE exons 6 and 8 were amplified by PCR with the use of primers located in the respective flanking intron. The PCR products were then purified with the Qia-Quik PCR Purification Columns Kit (Qiagen, Ontario, Canada) and submitted to direct sequencing using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Baie d’Urfé, Canada) according to the manufacturer’s instructions. The exon 6 PCR product was further subcloned into pBluescript II KS (Stratagene, La Jolla, CA). Cloned products containing either the normal or the mutant allele were then directly sequenced in the manner described above.

Materials and Methods

Bone histopathology

Bone biopsies in patient 1’s younger brother with APECED at ages 7.6 yr (A) and 11 yr (B). At 7.6 yr, radiopaque bands were evident in the metaphyses of the radius and ulna that had almost completely resolved by 11 yr. Unlike his sister, his growth plate was normal despite the metaphyseal changes, and there was no fraying of the metaphyseal margins. He had no growth failure or musculo-skeletal abnormalities.

to have acquired hyposplenia. He has not developed hypoparathyroidism.

Skeletal abnormality was noted at 5 yr when he presented with genu valgum. Radiological examination revealed findings similar to those described in patient 1, affecting the metaphyses of the femur, tibia, fibula, talocalcaneal joint, distal forearms, and shoulders. Investigations failed to reveal an etiology. The bony deformity progressed and was associated with growth failure. At age 13 yr further x-rays were obtained (Fig. 4A), and epiphysiodesis of both proximal tibias was performed. At age 17 yr repeat x-rays of the humerus, femur (Fig. 4B), talocalcaneal joint, and wrist showed regression of the metaphyseal abnormalities.

Short stature was noted at 13 yr, when his height was at the third percentile. He underwent spontaneous puberty at 13 yr; however, his growth proceeded below the third percentile. His final height was 163 cm compared with a mid-parental height of 182.5 cm.

Fig. 1. Progression and regression of the metaphyseal dysplasia in patient 1. Standing x-rays of the lower limbs were obtained at 7.2 yr (A), 11.2 yr (B), 12.4 yr (C), and 16.9 yr (D). There is progressive increase in the extent of the metaphyseal radioopaque bands in the long bones and the iliac crests between the ages of 7.2–12.4 yr as well as an increase in the genu valgum deformity. In the x-rays obtained at 16.9 yr (D), however, there is noticeable regression of the opacities, with a margin of bone of normal radiological density appearing subjacent to the growth plate in all of the long bones. This progression and regression are more obvious in x-rays of the left wrist obtained at 9.5 yr (E), 12 yr (F), and 16.4 yr (G). These x-rays also show that at 9.5 and 12 yr, the growth plates were widened, and the metaphyseal margins were frayed. These changes had resolved by 16.4 yr. X-rays of the left humerus were obtained at 9.5 yr (H), 15.3 yr (I), and 16.9 yr (J). The metaphyseal opacity had progressed between 9.5 and 15.3 yr, associated with significant deformity of the upper humerus. A fracture line is evident at 15.3 yr, although there was no associated pain. At 16.9 yr, the deformity was still present; however, the metaphyseal opacity was contracting away from the growth plate and had decreased in density. Although at all three sites, there was irregular sclerosis extending into the diaphysis (B–F and H–J), further down the shafts, the architecture of the diaphyseal bone remained normal.

Fig. 2. X-rays of the left wrist of patient 1’s younger brother with APECED at ages 7.6 yr (A) and 11 yr (B). At 7.6 yr, radiopaque bands were evident in the metaphyses of the radius and ulna that had almost completely resolved by 11 yr. Unlike his sister, his growth plate was normal despite the metaphyseal changes, and there was no fraying of the metaphyseal margins. He had no growth failure or musculo-skeletal abnormalities.

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Tissue expression of the AIRE gene

Fetal tissues (growth plates, thymus, and liver) were obtained courtesy of Dr. C. Goodyer after elective termination of pregnancies (13–18 wk) for reasons other than fetal disorder. Written informed consent was given by the mothers, and the use of the samples was approved by the institutional review board of the Hôpital Maisonneuve-Rosemont (Montréal, Canada). Tissue samples were frozen in liquid nitrogen and stored at −70 C until use.

RNA derived from various cell types was provided by Dr. Alain Moreau (Ste-Justine Hospital Research Center and Department of Biochemistry, Université de Montréal). These included first passage cultures of human chondrocytes established from patients with osteoarthritis and obtained at the time of orthopedic procedures after informed consent was given, as detailed previously (9), and two chondrosarcoma cell lines (Hs819.T and SW 1353, ATCC CRL-7891 and HTB-94, respectively). Chondrocytes and Hs819.T cells were grown in DMEM containing 10% heat-inactivated fetal bovine serum, and SW1353 cells were grown in Leibovitz’s L15 medium until RNA isolation from confluent cultures.

Total RNA was extracted from fetal tissue samples and cells using TRizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RT-PCR was performed using 2 μg total RNA in the presence of 1 mM deoxy-NTP, 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2, 40 U Moloney murine leukemia virus reverse transcriptase, and 500 nM random hexamer primers in a final volume of 20 μl. Samples were incubated for 10 min at 65 C, 10 min at 25 C, and 1 h at 37 C.

Five microliters of newly transcribed cDNA were amplified by PCR for 35 cycles at 94 C for 15 sec, 65 C for 30 sec, and 68 C for 30 sec in the presence of 1X pfx Amplification Buffer, 200 μM deoxy-NTP mix, 0.5 U pfx DNA polymerase, 5% dimethylsulfoxide, and 0.3 μM of each primer (10): sense, exon 12: 5'-GATCCTGCTCAGGAGACGTGACCC-3'; and antisense, exon 14: 5'-CACCAGGCAAGGAGAGGCTCCCGG-3'. Amplification of β-actin cDNA (233 bp) was used as an internal standard. The sequences of oligonucleotide primers were GGAAATCGTGCGT-GACAT for the sense and TCATGATGGAGTTGAATGTAGTT for the antisense. The specificity of the RT-PCR products was verified by gel electrophoresis and restriction enzyme digestion. All reagents were purchased from Invitrogen.

Results

Bone histopathology and immunocytochemistry in patient 1

Similar abnormalities were demonstrated in the biopsies from the right and left femoral metaphyses at 11.4 and 12.6 yr, respectively. Islands of hyaline cartilage containing hypertrophic chondrocytes identical to those normally found in the degenerating hypertrophic zone of the metaphyseal

![Graph](image-url)
FIG. 5. Histopathology from bone biopsies of the right distal femoral metaphysis at ages 11.4 yr (A–C) and 15.4 yr (D and E) in patient 1. A and B show mature lamellar bone (b), woven bone (w), and islands of hyaline cartilage containing degenerating hypertrophic chondrocytes (d). The intertrabecular spaces (shown in B and C) are characterized by a lymphoid cellular infiltrate including abundant plasma cells (arrowed) and loose fibrovascular tissue (fv) with many blood vessels (v). The presence of islands of degenerating chondrocytes enclosed in bone suggests that vascular invasion of the growth plate was abnormal. D, In contrast, at 15.4 yr there is recognizable metaphyseal growth plate, with normally oriented zones of proliferating (p), hypertrophic (h), and degenerating hypertrophic (d) chondrocytes, with TRAP-positive chondroclasts (cc) resorbing the degenerating hypertrophic zone and normal vascular invasion identified by yellow-staining blood cells (r) contained in blood vessels. E, Remnants of cartilaginous matrix (m) contained within lamellar bone. The intertrabecular spaces contain normal-appearing marrow consisting of clumps of adipocytes as well as myeloid and erythroid precursors with an occasional plasma cell. A, B, C, and E are taken from sections stained with hematoxylin and eosin. D is from a section stained with TRAP and counterstained with methyl green. Scale bar, 40 μm.
growth plate (Fig. 5A) were trapped within mature lamellar bone and some patches of woven bone (Fig. 5B). The presence of large islands of degenerating chondrocytes suggests that vascular invasion of the growth plate was abnormal; however, because the biopsies at 11.4 and 12.6 yr did not include growth plate, vascular invasion of the growth plate cannot be commented on directly.

Osteoblasts lining the osseous trabeculae were numerous. TRAP-staining osteoclasts were present in Howship’s lacunae along trabecular surfaces; however, they were scarce and were counted at 6.4 cells/mm² in the biopsy at 11.4 yr. No osteoclasts were observed adjacent to or resorbing the cartilaginous remnants trapped in bone. When juxtaposed with hematoxylin and eosin and Toluidine Blue stains, the von Kossa stains showed that these cartilaginous foci were calcified. Calcification of the bone was normal, but the osteoid seams looked broad, measuring between 50–75 μm. The intertrabecular spaces contained fibrovascular tissue (Fig. 5B) and an infiltrate of lymphocytes among which plasma cells were prominent (Fig. 5C).

The biopsy obtained at 15.4 yr included metaphyseal growth plate and metaphyseal bone from the distal right femur. The metaphyseal growth plate appeared thin, but showed normal differentiation and vascular invasion (Fig. 5D). TRAP-positive giant cells (chondroclasts) were resorbing the degenerating hypertrophic zone in the expected manner (Fig. 5D). TRAP-positive cells (chondroclasts and osteoclasts) were counted at 15/mm² in the metaphyseal zone. The osteoid seams of the osseous trabeculae appeared normal and measured 25–40 μm in thickness. Some trabeculae contained normal remnants of cartilaginous matrix; however, the islands of hypertrophic chondrocytes that characterized the earlier biopsies were no longer present (Fig. 5E). The intertrabecular spaces were occupied by normal-appearing hemopoietic marrow (Fig. 5E). The plasma cell infiltrate that characterized the earlier biopsies was not observed.

Immunocytochemistry performed on sections from the biopsy of the distal left femoral metaphysis at 12.6 yr showed that occasional plasma cells were positive for IgA and IgM; however, most were positive for IgG. In contrast, at 15.4 yr no cells stained positively for IgA, IgM, or IgG (data not shown).

**AIRE mutational analyses**

Patient 1 and her two affected brothers were homozygous for the 13-bp deletion mutation in exon 8 (1094–1106), one of the two most common AIRE mutations (2, 11). This deletion was confirmed by sequence analysis on two separate DNA samples. Both parents and the two youngest siblings were found to be heterozygous carriers for this mutation. This 13-bp deletion results in a frameshift and produces a 371-amino acid truncated AIRE protein with the loss of at least one of the two plant homeo domain zinc finger domains.

Patient 2 was found to be a compound heterozygote. One allele contained the 13-bp deletion mutation in exon 8 as described for patient 1. This deletion was confirmed by sequence analysis on two separate PCR products. Sequence analysis of several exon 6 cloned PCR products also revealed a novel mutation in the second allele (Fig. 6). A cytosine deletion at nucleotide 909 in exon 6 was detected that results in a frameshift mutation which changes the amino acid sequence from position 264 onward. In addition to changing the amino acid sequence downstream of the deletion, this mutation introduces a new termination codon at position 1250 in exon 10. The resulting protein lacks the SAND and both plant homeo domain zinc finger domains. This mutation abolishes Hin6I and Bsp143II restriction enzyme digestion sites.

**AIRE mRNA expression**

RT-PCR analysis of total RNA from human fetal tissues, chondrosarcoma cell lines, and human chondrocyte primary cultures revealed that AIRE is expressed in fetal growth.

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**Fig. 6.** Novel deletion mutation in exon 6 revealed by sequencing of cloned alleles obtained as described in Materials and Methods. The cytosine at position 909 (GenBank accession no. Z97990) was deleted in patient 2, resulting in the mutation 789delC according to the nomenclature suggested by den Dunnen and Antonarakis (23). This mutation results in a frameshift and introduction of a STOP codon in exon 10.

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**C** | **G** | **A** | **T** | **C**
---|---|---|---|---
**Mutated allele**
**C** | **C** | **C** | **C** | **C**
**G** | **T** | **C** | **G** | **G**
**G** | **G** | **G** | **A** | **C**

**C** | **G** | **A** | **T** | **C**
---|---|---|---|---
**Normal allele**
**C** | **C** | **C** | **C** | **C**
**C** | **G** | **T** | **G** | **G**
**G** | **G** | **A** | **C**

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plates (knee) from fetal wk 15.5–18, in human chondrocyte primary cultures, and in the two human chondrosarcoma cell lines tested (Fig. 7).

**Discussion**

The two patients with APECED described differ in their genotype and phenotype, but share a hitherto undescribed abnormality of endochondral ossification. The combined clinical, radiological, and histopathological features suggest that the metaphyseal radioopaque bands represent an accumulation of calcified cartilage. The association between disordered endochondral ossification and APECED may be coincidental, although the skeletal abnormalities described do not resemble any known metaphyseal dysplasia. Alternatively, the skeletal pathology could represent a rare manifestation of APECED resulting from either an autoimmune process directed against a growth plate-associated antigen or as a consequence of decreased expression of the AIRE gene within the growth plate. We have termed this novel skeletal phenotype, reversible metaphyseal dysplasia (RMD).

RMD appears to be a novel form of metaphyseal dysplasia. Extensive review of the literature and circulation of the radiographs and histopathology slides to local and international experts in bone disease failed to find similarities between RMD and recognized abnormalities of bone development. Moreover, the clinical, radiological, and histopathological features of RMD provided no clues for investigation of a second genetic abnormality independent of the AIRE mutations identified. Although collagen gene mutations have been implicated in a number of osteochondrodysplasias, looking for known gene defects in dysplasias with differing phenotypes has not proved fruitful (12, 13). Regression of metaphyseal abnormalities has been described in metaphyseal anadysplasia (14) and metaphyseal chondrodysplasia Schmid type (15), but both conditions differ significantly from RMD and from each other. Metaphyseal chondrodysplasia Schmid type is associated with mutations in COL10A1, the gene encoding type X collagen; however, the genetic abnormality appears to be phenotype specific, as mutations of COL10A1 have not been found in metaphyseal anadysplasia or other forms of metaphyseal dysplasia (13, 14).

Several lines of evidence suggest that the abnormal skeletal phenotype in RMD is due to an abnormality of endochondral ossification resulting in the accumulation of unresorbed calcified cartilage in the metaphyses. The radiological changes were limited to the metaphyseal aspect of the growth plates of the appendicular skeleton. Elsewhere, the radiological appearance of the bone was normal, excluding a generalized abnormality of osteoclast function, such as that associated with osteopetrosis. Calcified cartilage has a denser radiological appearance than bone (16), but does not have its biomechanical strength. The accumulation of calcified car-

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**Fig. 7.** Amplification of *AIRE* and β-actin mRNAs from human fetal thymus, human fetal knee, first passage chondrocyte cultures, and chondrosarcoma cell lines by RT-PCR. The RT-PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. The predicted sizes for the amplification bands using primers specific for *AIRE* and β-actin were 457 bp for *AIRE* (A) and 233 bp for β-actin (B). MM, 100-bp molecular markers; lane 1, fetal thymus; lane 2, 15.5-wk fetal knee; lane 3, 18-wk fetal knee; lanes 4 and 5, first passage chondrocyte primary cultures; lane 6, Hs819.T chondrosarcoma cell line; lane 7, SW 1353 chondrosarcoma cell line. C, Negative control (no reverse transcriptase).
tilage in the metaphyses thus would explain both the hyperdense areas subjacent to the growth plates and the debilitating deformities of the weight-bearing long bones that developed in both children. The early biopsies in patient 1 confirmed the presence of islands of calcified cartilage in the metaphysis, whereas these were not evident in the biopsy taken at 15.4 yr when radiological resolution was taking place.

Impaired vascular invasion may underlie the uncoupling of chondrogenesis and osteogenesis at the metaphyses. During endochondral ossification, chondrocytes proliferate, differentiate, and undergo apoptosis (17). Apoptosis of the hypertrophic chondrocytes is tightly linked with vascular invasion, resorption of the extracellular matrix, and bone formation. The early biopsies in patient 1 were characterized by islands of hypertrophic chondrocytes surrounded by bone, without evidence of vascular invasion or chondroclasts on the surface of cartilage. It thus would appear that chondrocytes were undergoing normal proliferation and maturation, but subsequent cartilage remodeling and ossification were delayed. In contrast, at 15.4 yr when radiological resolution was taking place, vascular invasion was evident in the biopsy that also showed correctly oriented proliferating, hypertrophying, and degenerating chondrocytic zones and resorption of the degenerating chondrocytes by chondroclasts (Fig. 5D).

The cooccurrence of this newly described bone disease with the relatively rare APECED in two unrelated individuals with different genotypes suggests that it is more than a chance association. Against a link with APECED, skeletal manifestations independent of RMD have not been reported (1), and we did not find radiological evidence of RMD in an additional four unrelated children with APECED between 7–16 yr of age, three of whom were heterozygous for the AIRE exon 8 deletion. Against a second independent gene defect, however, among patient 1’s extensive family, only her younger brother, who also had APECED, had radiological features suggestive of RMD. He had metaphyseal radiopaque bands in the distal radius and ulna that resolved spontaneously and were never associated with musculoskeletal symptoms. This suggests that there may be a spectrum of severity associated with RMD, with the milder form going undetected without radiological examination. That the three children with APECED in patient 1’s family were not equally affected with RMD would be consistent with the variation in phenotype within kindreds typical of APECED, the basis for which is unknown (1).

There could be an indirect link between RMD and APECED, with RMD developing as a consequence of other APECED manifestations or as a side-effect of treatment. The phenotypes for patients 1 and 2 were discordant, sharing only the commonest manifestation of APECED (1), mucocutaneous candidiasis, before the development of RMD. Although cultures of patient 1’s initial bone biopsy were negative for Candida albicans, radiological resolution of RMD was temporally related to successful treatment of her candidiasis with fluconazole, and a contribution of early-onset mucocutaneous candidiasis to the development of RMD cannot be ruled out. Metaphyseal osteosclerosis has been described in association with vitamin D intoxication and hypoparathyroidism (18). Discordance for hypoparathyroidism between patients 1 and 2, and the low normal calcium levels documented in patient 1 make it unlikely that abnormalities of calcium metabolism underlie the genesis of RMD. Similarly, as patient 1 remains prepubertal, whereas patient 2 underwent normal puberty, pubertal hormones are unlikely to have been important in facilitating the apparent resolution of RMD noted in their mid-teens.

An autoimmune basis for RMD, consistent with its association with APECED, is suggested by some of the findings in patient 1. The prominent inter trabecular plasma cell infiltrate in the early histological sections was no longer demonstrable at a time when there was radiological and histological regression of her disease. She also had markedly raised plasma concentrations of IgG, in contrast with her two brothers with APECED, and the plasma cell infiltrate was strongly positive for IgG. A variety of autoantibodies has been reported in the serum of patients with APECED, including antibodies against SOX 9 (19), a transcription factor important in skeletal development. This suggests that proteins expressed within the skeleton may represent targets for an autoimmune response in APECED. Proteins involved in angiogenesis at the growth plate, such as vascular endothelial growth factor (VEGF) and gelatinase B, would be candidates in view of patient 1’s histopathology. Administration of antibodies against VEGF to immature monkeys (20) produced metaphyseal dysplasia with histological changes similar to those seen in patient 1 that were reversible when treatment with the anti-VEGF antibodies was ceased. Similar changes were described in mice with homozygous null mutations of the matrix metalloproteinase 9 (gelatinase B) gene (21). Other mechanisms appeared able to compensate for the loss of gelatinase B because after the third postnatal week, aberrant apoptosis, vascularization, and ossification started, resulting ultimately in a normal skeletal appearance. Similar mechanisms may underlie the reversible nature of RMD.

Expression of AIRE mRNA and/or protein has been variably detected using different techniques, including Northern blot, RT-PCR, in situ hybridization, and Western blot, in a wide range of tissues (2). Our demonstration by RT-PCR of AIRE expression in fetal knee, in cultured chondrosarcoma cell lines, and in primary cultures of human chondrocytes broadens the scope of known tissue expression and raises the possibility that decreased expression of the AIRE gene within the growth plate contributes to the development of RMD. To our knowledge, AIRE expression in human chondrocytes has not been studied previously (2), although AIRE immunostaining has been described in adult mouse tracheal cartilage (6) in both undifferentiated and differentiating chondroblasts. A role for AIRE in skeletal development is supported by the demonstration of an interaction between AIRE and the common transcriptional coactivator cAMP response element-binding protein-binding protein in vitro (3). Long et al. (22) have shown that cAMP response element-binding protein family transcriptional activators are required for endochondral osteogenesis. AIRE protein structure and function will have been severely disrupted in our patients, because both the 13-bp deletion in exon 8 and the novel exon 6 frameshift mutation would be predicted to result in altered nuclear localization and abolition of transcriptional activator
capacity (4, 5). The diverse phenotypes associated with APECED, however, are not predicted by either the genotype or the known tissue expression of AIRE mRNA and/or protein (2). The genetic interactions and/or environmental influences presumably responsible for this phenotypic diversity remain to be defined. Although the functional significance of AIRE mRNA expression in chondrocytes deserves further study, the rarity of RMD in APECED suggests that decreased or abnormal expression of AIRE in the growth plate is insufficient alone for the development of RMD.

As similar bone pathology has not been described independent of APECED, we propose that RMD constitutes a further example of the phenotypic variation associated with APECED. We postulate that the pathological basis of RMD involves temporary impairment of vascular invasion of the metaphyseal growth plate, perhaps secondary to autoimmune targeting of a skeletal protein involved in angiogenesis. The demonstration of AIRE expression in chondrocytes, however, raises the possibility that decreased expression of AIRE in the growth plate may be contributing to the development of RMD.

Acknowledgments

Received January 17, 2003. Accepted July 10, 2003.

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This work was supported by the Sydney Children’s Hospital Foundation (to M.H.) and by la Fondation Léon Fredériç à Liége (to O.K.).

M.H. and O.K. are equal first authors.

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