Introduction

The familial forms of the calcium crystal-induced arthropathies, specifically calcium pyrophosphate dihydrate (CPPD) deposition disease and the basic calcium phosphate disorders, occur in almost all ethnic groups and are frequently characterized by early onset and severe clinical manifestations. Although these inherited disorders are relatively rare, they can often provide a means of identifying critical constituents of biochemical pathways that are important to the development of the familial as well as idiopathic varieties of these disorders. Here we will review the current status of gene discovery in chondrocalcinosis and describe the putative role of these genes in the development of these diseases.

Phenotype and genetic analyses

The history of the non-urate, calcium crystal arthropathies dates to 1958 when Zitnan and Sitaj presented case studies of 27 patients, most of whom were members of five families, with what they referred to as articular chondrocalcinosis1,2. The nature of the crystal deposition in affected patients was clarified by McCarty and Hollander3 who studied two cases of non-urate associated crystal deposition in the joints of patients thought to have gout. Radiographic examination of the joints in these and other patients revealed distinctive and abnormal calcifications in and around articular hyaline cartilage and fibrocartilage. Following the initial description of chondrocalcinosis in the five Czech families, multiple ethnic series of affected families were reported4-20. Most familial cases appeared to be inherited in an autosomal dominant manner with precocious onset and variable clinical expression, with articular cartilage deposition of calcium-containing crystals occurring before the development of frank degenerative joint disease (see Fig. 1). A peculiar type of osteoarthritis with numerous and large subchondral cysts was also observed. The primary crystal types that are observed in chondrocalcinosis include CPPD and basic calcium phosphate including hydroxyapatite. With few exceptions (see below) family studies of chondrocalcinosis demonstrate the presence of CPPD crystals in synovial aspirates. The mechanisms responsible for the deposition of the CPPD crystals are not known, although some studies have reported that structural changes in articular cartilage extracellular matrix might promote such a process23,24, thus prompting an exploration of genes encoding cartilage extracellular matrix proteins as candidate genes for chondrocalcinosis.

In a large family from the Chiloe Islands with a clinical phenotype of severe osteoarthritis, late-onset spondyloepiphyseal dysplasia, and chondrocalcinosis in multiple joints and fibrocartilages, a heterozygous mutation in the COL2A1 gene that resulted in an Arg to Cys substitution at amino acid 75 in the type II collagen molecule was identified25,26. However, it is likely that the chondrocalcinosis phenotype in this kindred was a secondary consequence of the advanced and severe osteoarthritis.

In addition to extracellular matrix proteins as potential candidates for familial CPPD disease, numerous studies of a chondrocyte nucleoside triphosphate pyrophosphohydrolase (NTPPPH) have suggested that the biochemical pathway responsible for the generation of inorganic pyrophosphate (PPI) may play a role in the crystal deposition27-29. Increased levels of intracellular PPI have been observed in cultured fibroblasts and lymphoblasts of patients affected with familial CPPD disease30,31 and in synovial fluids from an affected British family18, thus...
prominent calcification of the articular cartilage is clearly visible on the tibial plateau and femoral condyle at the early stage of disease in this individual; however, prominent calcification of the articular cartilage is clearly visible (indicated by arrow).

perpetuating the notion that abnormalities in pyrophosphate metabolism may give rise to abnormal crystal deposition in these families.

GENETIC LINKAGE ANALYSES IN FAMILIES WITH CPPD DISEASE

The availability of numerous families presenting with CPPD disease as a Mendelian trait has permitted the use of parametric methods of linkage analysis to define potential disease loci. A study of a large family from Maine, in which the CPPD disease phenotype was associated with severe, non-dysplastic osteoarthritis, excluded linkage to the COL2A1 locus; in this family, genetic linkage was demonstrated between the disease phenotype and a locus on the long arm of chromosome 8, now referred to as the CCAL1 locus32. The locus on chromosome 8q, although statistically significant, was broad and spanned a genetic interval of approximately 30 cM, or a physical distance of over 25 Mbp. Without additional family members from the original kindred, or the availability of other families whose CPPD disease phenotypes are linked to the CCAL1 locus, further exploration of the genetic basis for linkage of CPPD disease to chromosome 8q is limited.

Linkage analysis on a British CPPD disease family subsequently identified a second chondrocalcinosis locus on the short arm of chromosome 533 and was confirmed in genetic studies of two other families from France and Argentina34. All of the families presented with typical symptoms of CPPD disease and the large kindred from the Alsace region of France described by Andrew et al., like the British family reported by Hughes et al.33, had also been extensively characterized with respect to abnormalities in PPI metabolism30-31. The chromosome 5p15 locus, referred to as CCAL2, has now been shown to be linked to the CPPD disease phenotype in five apparently unrelated families, confirming the fact that CCAL2 is an important locus for familial chondrocalcinosis.

THE STATUS OF CANDIDATE GENES
FOR FAMILIAL CPPD DISEASE

The chromosome 5p15 locus contained a number of positional genes that could serve as viable candidates for familial CPPD disease. However, in a timely intersection of research efforts, a gene for an animal model of aberrant calcification was identified on mouse chromosome 15 in a region of the chromosome that was syntenic to human chromosome 5p33. The animal model was the progressive ankylosis (ank) mouse, a naturally occurring autosomal recessive mutant whose phenotype included the deposition of hydroxypapatite in articular tissues and synovial fluid. In affected animals, disease progression includes joint space narrowing, cartilage erosion and formation of osteophytes that cause joint immobility and eventual fusion. Complete rigidity and death occurs at around 6 months of age36-40.

Although the phenotype of the mouse model was considerably different from that seen in human chondrocalcinosis, the abnormal articular calcification and the fact that the human homologue of the gene, referred to as ANKH, was located at the CCAL2 locus, made it a viable positional candidate gene. Furthermore, Ho et al. presented evidence that the gene product of ank functions to regulate PPI levels in cells (see below). Mutational analyses of ANKH detected four mutations in the five families in which linkage to the CCAL2 locus had been confirmed. In the family described by Hughes et al., a heterozygous base substitution at position –11 of the 5′ untranslated region (UTR) introduced a new methionine (ATG) codon. Pendleton et al.41 performed in vitro translation studies and mass determination of the resultant protein by electrospray ionization mass spectrometry to demonstrate that the upstream ATG sequence was recognized as a new translational start site. In the Argentinean and French families, heterozygous missense mutations were observed in highly conserved amino acids in the first and second exons, respectively41,42. Two US CPPD disease families have mutations at the same amino acid position as that observed in the Argentinean kindred; however, the sequence variants in the two US families are transversion mutations, while that in the Argentinean family is a transition mutation42,43. Haplotype analyses of microsatellite and single nucleotide polymorphic markers in all three families demonstrate that they are not related, suggesting that the mutations at the same amino acid arose independently of each other, and that this site may represent a “hot spot” for mutations in CPPD disease families (see Table I for compilation of ANKH mutations in familial CPPD disease).

To evaluate the relevance of mutations in ANKH to idiopathic CPPD disease, Pendleton et al. studied 95 CPPD disease patients from the UK. One patient, who presented with late-onset CPPD deposition in several joints, displayed a 3 bp in-frame deletion in exon 12 that eliminated a glutamic acid at amino acid position 490. This change, like those observed in the familial mutations, was not observed in any controls. The same change was observed...
in the sister and nephew of the patient, although CPPD disease could not be confirmed in these individuals.41,44 Recently, Zhang et al.44 sequenced ANKH in a small cohort of sporadic CPPD patients and performed follow-up studies of detected sequence variants in a larger cohort of sporadic CPPD disease patients (n = 104) and controls (n = 500). They detected a significant association between sporadic CPPD disease and a polymorphism in the 5’ UTR of ANKH at position –4 bp with respect to the A of the ATG initiation of translation codon.

### GENETIC HETEROGENEITY IN FAMILIAL CPPD DISEASE

Interestingly, not all families with CPPD disease are linked to the CCAL1 or CCAL2 loci that have been identified by linkage analyses. For example, the multi-generation family described by Eshel et al.18 failed to exhibit linkage to either loci when genotyped with markers selected from the two candidate regions, CCAL1 and CCAL2 (Baldwin C and Williams CJ, unpublished data). These findings suggest that another uncharacterized locus may be responsible for the familial CPPD disease phenotype in this kindred.

### THE FUNCTION OF ANK IN CPPD DISEASE

Studies of ank function in cells from the progressive ankylosis mouse and in COS cells transfected with normal and mutant ank suggested that the protein may regulate transport of PPI. Ho et al.35 showed that intracellular PPI levels in fibroblasts from mutant mice were increased about twofold over that of wild type controls, and that extracellular levels of PPI were dramatically reduced in fibroblast cultures derived from mutant ank mice compared to wild type controls. Since PPI is a potent inhibitor of hydroxyapatite deposition in cartilage and bone, reduction in extracellular levels of PPI could account for the excessive calcification phenotype seen in ank/ank mice. Further experiments by Ho et al. showed that fibroblasts from mutant mice could be restored to normal levels of intracellular and extracellular PPI when transfected with wild type ank. They also showed that over-expression of wild type ank in COS cells resulted in a dramatic decrease in intracellular PPI levels and a concomitant increase in extracellular PPI levels. Finally, the decreased levels of intracellular PPI resulting from over-expression of ank in COS cells could be restored by the addition of probenecid, a non-specific anion transport inhibitor, suggesting that ank functioned via an ion channel transport mechanism.

In order to evaluate the impact of dominant mutations in ANKH observed in two families on the function of the ANK protein, Pendleton et al. transfected COS cells with constructs containing the INS + 4 and Met48Thr mutants. Interestingly, the familial mutations did not significantly alter intracellular PPI levels. To explain this result, Pendleton et al.41 hypothesized that, in contrast to the ank mouse mutant, the human CPPD disease mutations might act as gain-of-function alleles which would moderately increase extracellular PPI levels over time, leading to subtle abnormalities that have a minor and cumulative impact on articular cartilage homeostasis.

Recent studies of a familial ANKH mutation (Pro5Leu42) and two sporadic ANKH mutations (−4 bp, 5’ UTR44 and deletion of Glu49041) in an immortalized human chondrocyte cell line demonstrated an increase in the transcription and translation of ANK. Furthermore, transfection of the −4 bp (5’ UTR) ANKH variant into the CH-8 cell line resulted in elevated levels of extracellular PPI. However, other ANKH mutants, specifically the Pro5Leu and Met48Thr, and delGlu490 mutations, had divergent effects on the elaboration of extracellular PPI in this cell system44. Our own studies have examined the impact of three familial missense mutations in ANKH identified by us (Met48Thr, Pro5Leu and Pro5Thr) on the elaboration of extracellular PPI, as well as ecto-nucleotide pyrophosphohydrolase and alkaline phosphatase activity in stably transduced, chondrogenic ATDC5 cells. Our results demonstrate statistically significant increases in extracellular PPI in all mutants examined, thus confirming the gain-of-function hypothesized by Pendleton et al. [Zaka R, Stokes D, Dion AS, Kusnier A, Han F, and Williams CJ, manuscript submitted].

Figure 2 illustrates the proposed structure and function for the ANK protein. Under conditions of homeostasis, ANK facilitates the transport of PPI, thus ensuring appropriate levels of extracellular PPI to inhibit calcification of articular cartilage. However, in the ank/ank mouse, in which a recessive mutation in the ank gene produces non-functional ank protein, there is a decrease in the efflux of PPI from the cell, resulting in the deposition of hydroxyapatite in articular cartilage. Finally, in the case of familial CPPD disease mutations, there is increasing evidence that the ANKH mutations result in an excess of extracellular PPI and may promote the deposition of CPPD crystals in articular cartilage.

### Familial hydroxyapatite deposition disease: phenotype and genetic analyses

There are few reports of the deposition of hydroxyapatite and other basic calcium phosphate crystals as a heritable disorder in the medical literature, and the earliest descriptions were of calcific periarthritis in multiple joints of identical twins45, and in relatives of a proband presenting with intervertebral disc calcification46. In a case where the primary crystal type was determined to be basic calcium phosphate, the phenotype mainly involved the dorsolumbar spine, with intervertebral disc calcification primarily in the nucleus pulposus, as well as the peripheral joints with periarticular calcific deposits in the hand joints. This phenotype was displayed by the family described by Marcos et al. in 198147 in which no family members displayed calcific deposits in the knees, pubic symphysis, or triangular ligament of the carpus, thus distinguishing the hydroxyapatite arthropathy in this family from the condition seen in patients with familial CPPD disease.

Caspi et al.48 described three members of another family in which periartthritis of multiple joints was observed, but with an exclusive crystal type of octacalcium phosphate, as confirmed by Fourier transform infrared spectrophotometry of an open biopsy of a calcification of a proximal interphalangeal joint. This crystal type often accompanies
deposits of carbonate substituted hydroxyapatite, but the finding of octacalcium phosphate alone in a biopsy specimen is unique to this family. Another unusual observation in this kindred was the fact that affected individuals displayed low serum alkaline phosphatase activity—a finding not seen in other families with basic calcium phosphate arthropathies. No follow-up genetic studies have yet been performed on this kindred.

A detailed description of an erosive arthritis of the shoulder, known as "Milwaukee shoulder" was published by McCarty et al.49. In this disorder, apatite crystal deposition in the shoulder begins with limited joint mobility and stability, accompanied by joint effusion, and progresses to degenerative changes to the scapula or humeral head and the acromioclavicular joint, and calcification of the rotator cuff. Rotator cuff tear is common. A family of four members with calcific periartthritis of the shoulder was described by Hajiroussou and Webley in 198350 but a more detailed description of a large Italo-Argentinean kindred with Milwaukee shoulder was recently reported51. This family displayed an unusual type of osteoarthritis with secondary intraarticular and periarticular calcification in numerous joints, along with Milwaukee shoulder in the most severely affected elderly members and evidence of superior shoulder subluxation in younger members. Examination of synovial fluid from the shoulders of two affected family members showed the presence of both hydroxyapatite and CPPD crystals. Some genetic studies of this family were performed and are described below.

**GENETIC LINKAGE ANALYSES AND THE STATUS OF CANDIDATE GENE ANALYSES IN FAMILIES WITH HYDROXYAPATITE DEPOSITION DISEASE**

Because of the rarity of familial hydroxyapatite/basic calcium phosphate arthropathy, genetic analysis of kindred afflicted with these disorders has been limited. Studies of the Italo-Argentinean kindred described above indicated that the disorder was inherited in an autosomal dominant manner. While the phenotype in the family was not consistent enough to warrant a genome-wide search for linkage to a putative disease-causing locus, it was sufficient for analysis of potential candidate loci. The loci that were targeted included the chondrocalcinosis loci on chromosomes 8q and 5p15, and the COL2A1 locus on chromosome 12q. These loci were definitively excluded in the family. Several other loci that have been implicated in normal skeletal patterning and cartilage differentiation, the HOX A, B, C, and D gene cluster and the PAX 1 and 9 genes, were also analyzed. These loci were either excluded or were

---

**Fig. 2. Putative structure and function of ANK.** Panel A: The transmembrane prediction algorithm, TMPred152, suggests that ANK, the 492 amino acid gene product of ANKH, is a multipass transmembrane protein with 10 transmembrane helices. The positions of mutations in familial CPPD disease, relative to the position of the naturally occurring recessive mutation in the ank/ank mouse (which occurs at amino acid 440, changing a glutamic acid residue to a stop codon) are shown. Symbols: ◣ indicates potential N-glycosylation site; ◤ potential phosphorylation sites by protein kinase C or cAMP/cGMP dependent protein kinase. Panel B: Proposed impact of mutations on PPI generation. ANK appears to act as a channel or transporter for the inorganic anion, PPI. Under normal conditions, there is presumably a homeostasis in PPI transport such that extracellular PPI inhibits calcification of articular cartilage. A recessive mutation in the ank/ank mouse produces a decrease in extracellular PPI permitting deposition of hydroxyapatite. Dominant mutations in humans affected with CPPD disease may lead to excess efflux of PPI (Zaka et al., manuscript submitted); excessive extracellular PPI may ultimately result in the deposition of CPPD crystals in articular cartilage.
uninformative in terms of their linkage to the disease phenotype of Milwaukee shoulder/subluxation in the kindred.

Finally, observations of mineralization defects in the ank mutant mouse indicated that hydroxyapatite deposition was the crystal type deposited in articular spaces. Therefore, two other families displaying autosomal dominant hydroxyapatite deposition disease that have not been reported in the literature but have been studied by us were screened for mutations in ANKH. No sequence variants in the coding regions of the ANKH gene were observed in these families (Kingsley D and Williams CJ, unpublished observations).

Conclusions

This review has attempted to recapitulate the development of the genetic studies of the inherited non-urate crystal arthropathies. The progress in our understanding of these disorders is in large part due to the identification of families in which the calcium crystal-associated arthropathies are inherited in a Mendelian manner, thus permitting the use of traditional methods of parametric linkage analysis to establish loci that are linked to the phenotype in these families. Also, the availability of high-throughput techniques for genotype analyses and candidate gene analysis has significantly increased the speed with which suitable kindreds can be analyzed. Furthermore, animal models presenting with skeletal abnormalities associated with pathological mineralization have proven to be an outstanding resource for providing suggestions of potential candidate genes that may warrant analysis in families suffering from crystal arthropathies. At this time, there is every reason to believe that population-based studies of susceptibility genes for the crystal arthropathies will, likewise, contribute to our understanding of the complexity of inheritance of these disorders. Clearly, close cooperation between clinical and basic science researchers will hasten the identification of genetic loci whose gene products contribute to those biochemical pathways that are responsible for the pathology of the non-urate crystal disorders.

References


