Rickets, from history to molecular biology, from monkeys to YACS

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Introduction

Rickets has long been of interest in Britain, since the original monographs of Whistler and of Glisson, Bate and Regemorter. Both of those works were in Latin but Whistler rapidly had an English translation made which shows that the title of his thesis was ‘Inaugural Medical Disputation of the Children’s Disease of the English which the inhabitants idiomatically called ‘the rickets’, which, God aiding him, Daniel Whistler, Eastern Anglo-Saxon, with the authority of the most noble and magnificent rector Dr Johannes Polyander a Kerkchoven, doctor of Holy Theology and Principle Professor of the same faculty in the illustrious Academy of Leyden in Holland pro- pounded there for dispute for the Degree of Doctor and its entailed high privileges in Medicine on the 18th day of October at the accustomed time and place’. His thesis was printed in 1645, a few years before Glisson’s book (1650), but even so there were suggestions that he had committed plagiary.

Little progress was made over the next two centuries though there were reports from the Royal Infirmary in Manchester that cod liver oil could heal rickets. Then a series of papers were presented by John Bland Sutton, (initially Lecturer on Comparative Anatomy and later Surgeon at the Middlesex Hospital in London) in The Proceedings of the Zoological Society ‘On the diseases of monkeys in the Society’s gardens’. Writing of rickets, he recorded ‘this disease is extremely frequent in monkeys living in captivity in London. Nearly half the total number of monkeys introduced into the Zoological Society’s garden die rickety, provided they live a few months after reaching London. The changes in the skeleton develop so rapidly that a capuchin monkey, apparently in good health and thriving well, when introduced into the cages died horribly deformed by rickety changes in four months’. There was debate as to whether what was being described was in fact rickets, since it would appear that the picture is rather different in monkeys from what is seen in humans and other animals, and changes in the vertebral column and compression of the spinal cord seemed to be features seen in monkeys. In the last of these papers, Bland Sutton in 1889 wrote ‘that the bulk of the material has come under observation during my attendance at the prosector’s room of the Zoological Society, London during the last seven years’. That final paper included observations on lion
cubs and contained the remarkable statement ‘it may be mentioned that some rickety cubs, which early manifested signs of rickets, were promptly fed on bone dust and cod liver oil, made a good recovery and were alive and active, presenting no signs of paralysis two years afterwards’. Those observations were, of course, made before vitamin D had been discovered and it is interesting to note that though the Monkey House was light and airy, it was enclosed by glass that would not have let through UV (Fig. 1a).

A major landmark in the studies of rickets in humans was made by Harriet Chick and colleagues in 1922 who were members of the Medical Research Council team that went to Vienna after the First World War and described their findings in a paper in the Lancet in 1922. In Vienna,
at that time, rickets was remarkably prevalent but its cause was unknown. The possibility that rickets might be caused by something in the water or a feature of the diet, or something in the air, or possibly an infection, were all considered. Effectively, children with rickets at the Kinderklinic in Vienna were divided into four groups. Two of these groups were kept in the ward and the other two groups were kept out on the balcony which must have been very cold in the Viennese winter. In the ward, one group was given the normal diet only while the other group was also given supplementary cod liver oil. The rickets in the latter group of children was healed as demonstrated radiologically, while the first group remained sick. On the veranda (Fig. 1b) one group was kept well covered while the other group of children, wearing remarkably little clothing, was exposed to sunlight. This second group got better. Thus it was shown that cod liver oil and exposure to sunlight both healed rickets. It was not clear at that time whether the effectiveness of cod liver oil was due to vitamin D or to vitamin A, both of which had been discovered by that time.

Vitamin D deficiency became virtually extinct in the western world but recurred in the late 1960s and early 1970s in the United Kingdom, predominantly in Asian immigrants to the country. The fact that this was due to vitamin D deficiency was established in two ways. One depended on showing that it responded to administration of small amounts of vitamin D (Pietrek et al. 1976) and the other depended on direct measurement of 25-hydroxyvitamin D. The feasibility of the second method stemmed, of course, from advances in the understanding of the metabolism of the vitamin with the demonstration that vitamin D, from the diet or made in the skin, was converted into 25-hydroxyvitamin D in the liver and then, critically, to 1,25-dihydroxyvitamin D in the kidney. The importance of 1,25-dihydroxy-cholecalciferol was shown almost simultaneously by the groups of Kodicek, De Luca and Norman and its mode of action, the hormonal form of vitamin D, is reviewed by Haussler et al. (1997). In vitamin D deficiency the concentration of 25-hydroxyvitamin D is low and the measurement of this compound probably gives the best estimate of vitamin D status (Preece et al. 1975). In untreated cases the concentration of 1,25-dihydroxyvitamin D is also low. However, the concentration of the latter is remarkably labile and small vitamin D supplements can produce dramatic and sustained changes in the circulating concentration (see Fig. 2). With administration of quite small doses, the concentration of 1,25-dihydroxy-cholecalciferol can reach the normal range within 24–48 h and become supranormal within a few days, before the concentration of 25-hydroxyvitamin D has changed greatly. Subsequently, the concentration of 1,25-dihydroxyvitamin D in previously vitamin D-deficient subjects remains high for many months, before falling gradually back to normal (Papapoulos et al. 1980). In fact, it remains elevated until the metabolic bone disease has healed, as reflected by the normalisation of alkaline phosphatase, which can take several months, and the associated healing of the radiological changes of rickets or its adult equivalent, osteomalacia. The reason for the sustained supranormal concentrations of 1,25-dihydroxyvitamin D in this situation is unknown. The renal 1α-hydroxylase is stimulated by parathyroid hormone, low calcium and low phosphate but it is not clear that these adequately explain the situation. Possibly, the recent cloning of the renal 1α,25-dihydroxyvitamin D hydroxylase from animals may help elucidate the basis for this (see Glorieux 1997).

The discovery of vitamin D and the ability to assay its biological activity made it possible to confirm that most cases of rickets responded to small concentrations of vitamin D and were due, therefore, to vitamin D deficiency. However, that left a group of cases that could be attributed to resistance to the action of vitamin D and also revealed a form of rickets that was not due to an abnormality of vitamin D metabolism per se but due to phosphate depletion resulting from a renal tubular leak. Burnett et al. (1964) analysed cases of vitamin D-resistant rickets in twenty-four pedigrees with hereditary and sporadic forms of disease. These were cases of hypophosphataemic rickets. An important feature of this study was the use of a measurement of serum phosphate as a determinant of the phenotype, which made it possible to demonstrate that this was inherited as an X-linked dominant condition and was not, as previously thought, an autosomal dominant disease.

At about the time that Dent and co-workers were clarifying the mode of inheritance of X-linked hypophosphataemic rickets, they also described children with another form of rickets associated with hypercalciuria (Dent & Friedman 1964); this can also be associated with nephrocalcinosis, renal stone formation and impairment of renal function. It became apparent that this condition was also inherited in an X-linked dominant manner and subsequently a gene abnormality has been identified in families with this condition. Rather surprisingly, the structure of this gene implies that its product is involved in regulation of chloride transport (Wrong et al. 1994, Fisher et al. 1995, Lloyd et al. 1996) and the name 'Dent's Disease' has been given to the condition. In different families the phenotypic manifestation of mutations in this gene vary and the manifestations of a single mutation can vary quite markedly even within a single kindred.

Vitamin D resistance

Over the years a number of other forms of rickets have been identified. One of these is the so called Prader rickets (Prader et al. 1961), also known as 'pseudo vitamin D
Figure 2 The changes in circulating 1,25-dihydroxy-cholecalciferol (1,25-(OH)\textsubscript{2}D\textsubscript{3}) and calcium in a vitamin D-deficient subject treated with small doses of the vitamin, showing the resulting sustained rise in the concentration of 1,25-dihydroxy-cholecalciferol.

deficiency. In this condition the concentration of 25-hydroxyvitamin D is normal but that of 1,25-dihydroxyvitamin D is low. This resembles vitamin D deficiency, but is an hereditary condition and while not responding to physiological doses of vitamin D, it heals completely with the administration of large amounts of vitamin D. It also responds to physiological doses of 1,25-dihydroxyvitamin D and it is assumed that this represents an abnormality of the renal 1α-hydroxylase and is discussed further by Glorieux (1997). Rickets due to end-organ resistance to the action of 1,25-dihydroxyvitamin D has also been identified, and found to be associated with elevated concentrations of 1,25-dihydroxyvitamin D (Brooks et al. 1978, Rosen et al. 1979, Liberman et al. 1980). Some of these cases were associated with total alopecia and it has been suggested that the
presence of alopecia indicates that the disease is more severe, but fatal cases can occur even in the presence of normal hair growth. A remarkable feature of this condition is that it can be healed completely by intravenous calcium administered over a long period of time. This form of treatment was pioneered by Balsan et al. (1986) and described more fully by Hochberg et al. (1992). Figure 3a and b shows the radiological healing obtained in a patient with this condition given calcium intravenously over a twelve-month period (Lin & Uttley 1993). The efficacy of intravenous calcium implies that the primary defect is in calcium absorption in the intestine and that if this is overcome, then bone can mineralise normally. This also indicates, of course, that the action of vitamin D on bone itself is not so important. The molecular basis for the end-organ resistance is described by Haussler et al. (1997) and need not be described in any great detail here. In most cases in whom mutations are sought to explain this resistance, changes are found in the gene encoding the vitamin D receptor (Hewison & O’Riordan 1994, Hawa et al. 1996, Haussler et al. 1997). However, there is one case in whom the phenotype (of rickets and alopecia) was present and the rickets responded to treatment with 1α-hydroxy-cholecalciferol and calcium supplements but no mutation in that gene for the receptor could be found; an alternative mechanism must exist to explain the hormone resistance (Hewison et al. 1993).

It is possible to analyse the effects of these mutations of the vitamin D receptor in terms of the crystal structure of other members of the steroid hormone receptor family. The DNA-binding domain of the glucocorticoid receptor, bound to target DNA, has been crystallised (Luisi et al. 1991), as has the corresponding region of the oestrogen receptor–DNA complex (Schwabe et al. 1990). These DNA-binding domains, with their zinc fingers, combine as homodimers and part of the receptor molecule fits into the major groove of the double helix of DNA. Using crystallography and molecular modelling techniques, it is possible to define the regions that have particular roles, for example, in contributing to an α helix of the receptor protein, residues that make hydrogen bonds with DNA or those that make specific base contacts (see Fig. 4). This interpretation (Rut et al. 1994) of the effects of mutations that cause the rickets phenotype is possible because the
mutated amino acid residues are common to vitamin D and glucocorticoid receptors and have identifiable roles in the receptor–target gene interaction. The effects of two of these mutations are shown in Fig. 5; it should be noted that the numbering system used in that illustration is from Rut et al. (1994) and differs from that used by Haussler et al. (1997) giving figures that differ by three amino acids. For consistency, the residue number used in this text will be that of Haussler et al. (1997) followed by the previous notation in parentheses. The effect of changing a lysine to glutamic acid at residue 45 (lys 42 to glu in figure) is illustrated in the upper part of Fig. 5. In this case the normal lysine residue makes contact and hydrogen bonds in the major groove of the DNA. Mutation of this to glutamic acid, apart from changing the charge, would not allow these contacts of hydrogen bonds to form and so this could provide a basis for understanding the genesis of vitamin D resistance. The effect of the second mutation is shown in the lower part of Fig. 5. In the normal wild type, phenylalanine residue 47 (residue 44 in figure) is shown between two helices, in a hydrophobic region. In a patient in whom this was mutated to isoleucine, the new residue would not fill this space fully and the ensuing changes presumably limit the ability of the receptor to react with the target gene. The scale of this analysis can be understood when it is realised that the dimension of the α helix shown in these pictures is of the order of 3–5 angstroms.

The effects of changes in the ligand binding domain can now also be considered in the light of the crystal structure of other members of the superfamily of nuclear hormone receptors. The crystal structures of the ligand binding domain of the thyroid hormone receptor (Wagner et al. 1995) and of the retinoic acid receptor (RAR) (Bourguet et al. 1995, Renaud et al. 1995) have recently been established. In the case of the thyroid hormone receptor binding of the ligand induces changes so that the thyroid hormone is enclosed within a pocket which is closed, rather like a trapdoor, when ligand is present. In general, the structure of the RAR ligand binding domain is similar and in this case a region important for dimerisation has been established. We have localised a mutation in one of the patients with vitamin D resistance to this region, namely a change of glutamine to proline (Q259P) (Cockerill et al. 1977), and this has been produced previously in vitro (Whitfield et al. 1995). Since this glutamine residue in the vitamin D receptor is in a position corresponding to an identical residue in the RAR molecule that is important for dimerisation (Fig. 6), it is likely to be the cause of hormone resistance.

**Regulation of phosphate and the genesis of X-linked hypophosphataemic rickets**

Phosphate homeostasis is clearly essential for adequate mineralisation of bone and the deposition of hydroxyapatite, which contains both calcium and phosphate.
Figure 5 Models of normal and mutant vitamin D receptor (VDR) showing the possible effects of substitutions at residues 42 (a and b) and 44 (c and d) as designated by Rut et al. (1994). Models were prepared from the structure of the GRdbd/DNA complex (Luisi et al. 1991). The recognition helix is indicated with an arrow and zinc atoms are shown as spheres. The zinc atom shown in the upper left of the illustration is the one in the amino terminal zinc module, while that in the lower half of the illustration is from the second, more carboxy terminal zinc module. (a) Lys42/DNA interactions in wild type. The axis of the recognition α-helix lies in the plane of the page and the helix is seen from the side. On the hydrophilic surface of the recognition helix, guanine (G) is shown hydrogen-bonded (broken line) to Lys42. His32, in the peptide loop of the zinc module, forms a hydrogen bond to the phosphate backbone. The interactions of Lys and His with the DNA are proposed to be conserved in the steroid/nuclear receptor superfamily. (b) The view is the same as in (a) and shows the effects of Lys42 → Glu substitution. The hydrogen bond to the guanine is lost, and the hydrogen bond from His32 could be displaced as shown. (c) This shows Phe44 in the conserved hydrophobic core in wild-type VDR. The packing of residues in the hydrophobic cluster is illustrated. The view is at right angles to that in (a) and (b). The α-helix that is seen from the side is the one on the carboxyl end of the second zinc module. (d) Orientation is as in (c) and shows the effect of the Phe → Ile substitution. A gap is introduced by this change in the hydrophobic core. (Reproduced with permission from Rut et al. 1994.)

Relatively little, however, is known about the regulation of phosphate, as opposed to the extensive information available on calcium homeostasis. However, studies of X-linked hypophosphataemic rickets have thrown light on the situation. With markers on the X-chromosome, it was shown that there was linkage of this condition to the region Xp22.1, on the short arm of the X-chromosome (Read et al. 1986, Thakker et al. 1987). Gradually, with...
the availability of more markers, it became possible to
narrow the region that contained the disease locus from
10–15 centiMorgans down to about 0.3 centiMorgan
although this still left a region with over 300,000 base pairs
(Rowe et al. 1996a). Further advances depended on using
Yeast Artificial Chromosomes (YAC) to establish a YAC
contig (that is a series of overlapping pieces of cloned
DNA) across the disease locus (Francis et al. 1994) and the
subsequent creation of other contigs, cosmids and PACs
spanning the same region (see Fig. 7). Within these contigs
were two markers, designated CAP32 and DXS1683 that
spanned the Hyp locus (Econs et al. 1994, Rowe et al.
1996a). Therefore, the phosphate regulating gene causing
X-linked hypophosphataemic rickets had to fall within
these markers and be present in one or more members
of the contig.

Identification of the precise localisation of the gene
depended on the demonstration, with one of these
cosmids, of a large deletion in a patient with X-linked
hypophosphataemic rickets. That deletion was subse-
quently shown to be at least 55 kilobases long. Having
identified a deletion in one patient it was then feasible
to use the same cosmid to screen a number of other
families so that five mutations were found relatively
quickly (the Hyp Consortium 1995). This made it possible
to characterise the gene, which had a totally unexpected
structure since the predicted protein had homology with
endopeptidases. It was given the name PEX, an acronym
reflecting the fact that it was Phosphate regulating with
Endopeptidase features on the X-chromosome. The com-
plete structure of the coding region of the human PEX
gene has now been established, together with short regions
of the 5' and 3' non-translated sequences. The coding
region of PEX as determined from cDNA analysis contains
2483 base pairs, arranged within 22 exons, and encoding
746 amino acids. These include a single transmembrane
Figure 6 A model of the crystal structure of RAR modified from Renaud et al. (1995) to show the expected position of a mutation in the vitamin D receptor that causes resistance. The mutation is in a region that is important for dimerization.
domain, close to the amino terminus, with a short, presumably intracellular tail (Fig. 8). The rest of the molecule contains a single zinc-binding domain, as would be expected in a metalloproteinase. In these features, PEX resembled neprilysin (also called neutral endopeptidase or NEP, CALLA, CD10, gp100 and enkephalinase), endothelin converting enzyme, and Kell antigen. Overall the amino acid sequence homology between PEX and these others is between 30 and 60%, the similarity being closest to neprilysin. The predicted PEX protein would seem to be a member of the MA clan of metalloproteinases with thermolysin and neprilysin, and the M13 family which includes neprilysin but not thermolysin.

The first deletion that was detected in a patient with X-linked hypophosphataemic rickets resulted in the loss of exons 1, 2, 3, 4 and 5 (Rowe et al. 1997). A second kindred with the deletion of exons 2, 3, 4 and 5 was then found, as well as a number of single exon deletions in other families (see Fig. 8). A range of other single point mutations has also been detected including eight different mutations leading to the introduction of stop codons and mutations causing a frame shift and, therefore, nonsense coding in four families. Mutations giving rise to single amino acid change have also been detected including a change from cysteine to serine in exon 3, leucine to proline in exon 4, proline to lysine in exon 15 and glycine to arginine in exon 17 (Rowe et al. 1997). From these data, it is clear that the mutations causing the phenotype of X-linked hypophosphataemic rickets can be associated with changes at varying points along the length of the molecule and there is no indication that the phenotypic appearance varies with the nature of the mutation. Using SSCP and PCR to screen for mutations in all 22 exons, it is possible to detect changes in 80% of affected families. About 100 families have been screened in this way and it seems reasonable to conclude, therefore, that a change in the PEX gene is likely to be the cause of X-linked hypophosphataemic rickets in the vast majority, if not all, of the families. From the nature of the changes, it is

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Figure 7 A contig of YACS and other constructs containing cloned DNA from regions of the short arm of the X human chromosome spanning the Hyp gene. (Modified from Francis et al. 1994.)

Figure 8 A schematic representation of PEX cDNA showing (upper part) the regions derived from the 22 exons and the parts encoding the transmembrane domain and the zinc binding region and (below) some of the regions of PEX found to be mutated in patients with X-linked hypophosphataemic rickets. (Based on Rowe et al. 1997.)
possible to use model systems to predict the effects of these mutations on the secondary structure of the protein, its glycosylation or phosphorylation as well as changes in the catalytic site where substrate is bound within a cleft associated with the zinc-binding domain of the protein; this is described elsewhere (Rowe et al. 1997).

The complete amino acid sequence of PEX in mice has also been determined (Du et al. 1996, Strom et al. 1997) and shows 98% homology between the murine and human forms of PEX. Determination of the sequence of the gene in mice is important because there are two murine homologues of X-linked hypophosphataemic rickets. One of these is a spontaneous mutation in the so-called Hyp mouse (Eicher et al. 1976) while the other, in the Gyr mouse, is produced by X-irradiation (Lyon et al. 1986). In Hyp it has been shown that there is deletion of the last seven or eight exons whereas in Gyr there is deletion of the first three exons (Strom et al. 1997). The Gyr mouse differs phenotypically from the Hyp mouse in that the animal gyrates and it is not known why this should be, though it is possible that another neighbouring gene is affected by the deletion, since the 5′ extent of the deletion has not been identified.

A possible mode of action of PEX

Since the predicted PEX protein has homology with other endopeptidases, it has to be assumed that it is an enzyme, but there is difficulty in establishing with certainty what tissues are expressing this gene. Expression in bone has been shown (Du et al. 1996) but its potential presence in other tissues may also be important. The key questions have to be what is the substrate for this protein and what is the mechanism whereby PEX is regulating phosphate homeostasis? An earlier possibility that X-linked hypophosphataemic rickets might be due to an abnormality in the sodium/phosphate co-transporter can be excluded since the gene for that has been cloned and localised to chromosome 5 (Kos et al. 1994). Alternatively, it is reasonable to suppose that the substrate for PEX is acting in a hormonal manner since there is evidence from parabiosis and renal cross-transplantation studies in animals that a humoral mechanism is involved (Meyer et al. 1989, Nesbitt et al. 1992). These studies have indicated that the Hyp mouse, for example, produces a circulating factor that can affect renal tubular phosphate handling in a normal kidney. A corollary of these observations is that the hypophosphataemic factor, producing phosphaturia, is not inactivated by PEX in a normal animal so that the hypophosphataemic factor per se is not a substrate or is not accessible to the enzyme.

Further evidence for the involvement of a circulating factor in the genesis of hypophosphataemia due to a renal tubular leak is given by the existence of the syndrome of ‘oncogenic osteomalacia’. In this syndrome (Hewison et al. 1992), hypophosphataemic, phosphaturic osteomalacia develops in the presence of a tumour and there is also a low concentration of circulating 1,25-dihydroxyvitamin D. The tumours are often small and difficult to find: they are frequently, but not always, described as hamangio-pericytomata. They may occur anywhere including the soles of the feet, the vaginal wall, and the anterior abdomen; they may be in the nasal sinuses or even intracranially. The metabolic disorder may be recognised for many years before the presence of a tumour is detected; sometimes this is because the lesion has been overlooked. Usually it is possible to gain a remission by treatment with a combination of phosphate and a 1α-hydroxylated compound, either calcitriol (1,25-dihydroxy-cholecalciferol) or alphacalcidol, but the condition can relapse even though the treatment continues. A remarkable feature is the improvement that occurs when the tumour is removed. Within 48 h the concentration of 1,25-dihydroxy-cholecalciferol can rise from undetectable to supra-normal and the serum phosphate rises more slowly to normal, within 4 or 5 days. The concentration of 1,25-dihydroxy-cholecalciferol then remains elevated for several months, while the bone disease is healing. If the tumour recurs then the biochemical features also return with relapse of the bone disease. These features would imply that the tumour is producing a factor that is affecting vitamin D metabolism and phosphate handling, causing a renal tubular phosphate leak. The name ‘phosphatonin’ has been given to this unknown factor (Econs & Drezner 1994). It has proved difficult to purify the substance but it seems likely that it is a peptide though estimates of its size vary considerably, from less than 5000 up to 60 000 (Miyauichi et al. 1988, Cai et al. 1994, Rowe et al. 1996b). By analogy with other tumour-related endocrine syndromes, it is reasonable to suppose that this factor, phosphatonin, is one that is normally involved in the endocrine system, though in this case its nature, and its mode of action are still unknown.

It is possible to create a theoretical model to explain the action of PEX, assuming that phosphatonin is the substrate. In the normal kidney, phosphate is filtered in the glomeruli and then largely reabsorbed in the renal tubules. The absorption process involves the renal tubular sodium/phosphate co-transporters. As a result most of the phosphate that has been filtered is reabsorbed. It is assumed that phosphatonin is inhibiting renal tubular phosphate reabsorption and so leading to increased phosphate excretion possibly by modulating the Na/Pi transport system (see also Collins & Ghishan 1996). A second assumption is that PEX is acting on phosphatonin leading to its inactivation. A defect in the PEX gene would then lead to an excess of phosphatonin and a renal tubular phosphate leak just as overproduction of phosphatonin by a tumour leads to phosphaturia. The challenge now is to characterise the phosphatonin and so advance our understanding of the genesis of hypophosphataemic rickets as
well as, of course, the regulation of normal phosphate handling.

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