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Editorial

Vitamin A and the developing embryo

It was just before the First World War when vitamin A was chemically identified as “fat soluble A” and in the 1920s studies were conducted on laboratory rats to see what happened when this component was left out of the diet.¹ A now familiar series of hypovitaminosis A changes occurred—widespread keratinisation of epithelia, decreased immune function, anaemia, xerophthalmia, and blindness. In the human population even subclinical deficiency of vitamin A leads to high levels of childhood morbidity and mortality. Astonishingly the ancient Egyptians knew that liver in the diet or the juice of cooked liver put into the eyes cured night blindness, but it took us about 5000 years to rediscover this medical fact.

Vitamin A deficiency and the embryo

Soon after, in the 1930s, similar dietary deprivation studies were performed with a view to asking what happens to the embryo when vitamin A is removed. In fact vitamin deficiencies, and most dramatically deficiencies of vitamin A, were the first dietary means of producing congenital malformations of the embryo. Most of these experiments were done with farm animals and the first report of this type of experiment was that a litter of pigs were born with no eyes at all.² Subsequently it was shown that a wide range of embryonic defects were apparent in the vitamin A deficient embryos of sheep, cattle, rabbits, rats, and humans.³ These defects include the central nervous system (hydrocephalus, spina bifida), eyes (anophthalmia, microphthalmia), face (harelip, cleft palate), dentition, ear (accessory ears, otosclerosis) limb, urinogenital system (cryptorchidism, ectopic ovaries, pseudohermaphroditism, renal defects), skin (subcutaneous cysts), lungs (hypoplasia), and heart (incomplete ventricular septation, spongy myocardium, aortic arch defects, aorticopulmonary septal defects, valvulus communis). Clearly, the developing embryo crucially requires vitamin A for the proper development of a whole range of its organ systems.

Vitamin A excess and the embryo

It was not long after these experiments that the opposite ones were done—feeding vitamin A metabolites, and retinoic acid in particular, in excess to pregnant mammals.⁴ This too produced a spectrum of abnormalities, which in many respects showed remarkable similarities to those generated by a lack of vitamin A. These defects include the central nervous system (hydrocephalus, anencephaly, exencephaly, spina bifida), eyes (anophthalmia, microphthalmia, defects of the retina), face (harelip, cleft palate, brachygnathia, hypoplastic maxilla), dentition, ear (absent or deformed), limb (phocomelia), urinogenital system

(hypoplastic kidney, polycystic kidney, absent/hypoplastic genitalia), heart (incomplete ventricular septation, transposition of the great vessels, double aortic arch, hypoplastic aortic valves), thyroid gland (hypoplasia), and the axial skeleton (vertebral and rib fusions, extra vertebrae and ribs, hypoplastic tail). Several of these abnormalities have also been seen in humans due to the taking of Accutane (13-*cis*-retinoic acid) while inadvertently being pregnant.⁵

In the last 10 years these sorts of detailed analyses of abnormal embryos have been revisited for two reasons. The first is because we now have a range of anatomically specific molecular markers which we can use to find out precisely what has changed in embryos which are either vitamin A deficient or have been treated with excess vitamin A. The results of these experiments are very surprising and exciting. Secondly, we have learned a great deal about the molecular biology of how vitamin A is made and how it acts within cells, and the defects caused by vitamin A deprivation have now been recapitulated in mice made mutant for the retinoic acid receptors.

How does vitamin A work?

The parent molecule on which the actions of vitamin A are based is called retinol and the family of molecules derived from retinol is the retinoids. Vitamin A is stored as retinyl esters, mainly in the liver, but also in the lungs and bone marrow. Cells of the body that require retinol receive it from the bloodstream where it circulates bound to retinol binding protein. Inside the cell, retinol is converted firstly to retinal (the molecule used in the visual cycle) by alcohol or retinol dehydrogenases and then to retinoic acid by retinaldehyde dehydrogenases.⁶ Retinoic acid is further metabolised to supposedly inactive compounds by the action of a cytochrome P450 enzyme called CYP26. Retinoic acid is the biologically active metabolite of retinol because it acts at the level of the nucleus to activate specific retinoic acid responsive genes. This it does by binding to two classes of ligand activated transcription factors known as the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). There are three members of each class of receptor and these are known as α , β and γ , thus giving a RAR α , RAR β , RAR γ and RXR α , RXR β , and RXR γ . In addition there are several isoforms of each of these genes which are generated by differential promoter usage.^{7,8} To act on a responsive gene one of the RARs heterodimerises with one of the RXRs (giving for example a RAR α /RXR β heterodimer) and this dimer binds to a specific sequence of base pairs in the upstream promoter region known as the retinoic acid response element (RARE) of the gene in question. In fact there are two forms of retinoic acid,

all-*trans*-RA (tRA) and its isomer, 9-*cis*-RA and tRA binds to both the RARs and the RXRs whereas 9-*cis*-RA only binds to the RXRs.

A return to excess and deficiency studies

We now know that both excess retinoic acid and a deficiency of retinoic acid causes both the ectopic induction and the down-regulation of many genes as a prelude to changing the anatomy of the embryo. For example, excess retinoic acid causes the chick limb bud to duplicate such that instead of the normal three digits a six digit, double posterior limb is produced. A host of gene changes precede the appearance of the precisely organised extra digits—the induction of a nested set of *Hox* genes, the *fibroblast growth factor-4* gene, the *bone morphogenetic protein-2* gene, the *sonic hedgehog* gene, all of which with others form interacting networks of genes to control outgrowth in the three axes of the limb.⁹ Conversely, quail embryos deficient in retinoic acid have down-regulated genes such as *sonic hedgehog*, *fibroblast growth factor-4*, *engrailed*, but also ectopically induced genes such as *Wnt-7a* whose up-regulation is presumably due to the down-regulation of a repressor.¹⁰ These types of complicated analyses are clearly telling us a great deal about the genes involved in limb development.

Another profoundly affected area of the embryos is the developing central nervous system, and in particular, the hindbrain. The normal hindbrain consists of a series of seven segments known as rhombomeres (numbered 1–7) each with a distinct set of gene expressions, notably of the *Hox* genes and each giving rise to discrete motoneuronal populations and cranial sensory ganglia.¹¹ It was found that after retinoic acid administration to mouse embryos the hindbrain *Hox* genes were all induced in an anterior direction and then they regressed leaving behind an altered expression pattern which resulted in an altered anatomy. Thus instead of the normal rhombomeric sequence of 1, 2, 3, 4, 5, 6, 7, retinoic acid caused rhombomeres 2 and 3 to transform into 4 and 5 respectively producing the sequence of 1, 4, 5, 4, 5, 6, 7.¹² This means that instead of the trigeminal ganglion appearing adjacent to rhombomere 2 and the facial ganglion adjacent to rhombomere 4, the trigeminal ganglion had gone and a duplicated facial ganglion appeared next to the transformed rhombomere 4 in addition to the normal one. Once again, the converse situation of a deficiency of retinoic acid also highlights the developing hindbrain. In retinoic acid deficient quail and rat embryos the posterior part of the hindbrain (rhombomeres 4–7) is either missing or fails to form rhombomere boundaries.^{13 14} This means that the entire myelencephalon (pons and medulla) has gone or is abnormal along with its associated cranial ganglia and the posterior branchial arches.

Knockouts of receptors and enzymes

With the ability to make mice mutant for any gene of choice, so called knockout mice, it became possible to determine the function of each of the six receptors and their isoforms. Considering the very high level of conservation of sequence between species, one would have expected an individual function for each receptor isoform. The first results were therefore surprising. Knockouts of individual isoforms gave no phenotype. Even more surprising, knockouts of all isoforms of any one receptor also gave minor embryonic phenotypes (with one exception). Thus as adults, *RARα* mutants display homeotic transformation of some cervical vertebrae, male sterility and growth deficiency; *RARβ* mutants are normal except for increased numbers of lung alveoli and impaired spatial learning and

memory defects caused by defective hippocampal functioning; *RARγ* mutants are very similar to *α* mutants; *RXRα* mutants die at day 15 due to defective ventricle wall development; and *RXRβ* mutants display defective spermatogenesis as adults.^{15 16}

However, double RAR mutants (*RARα/RARβ*, *RARα/RARγ*, and *RARβ/RARγ*) recapitulate all the defects characteristic of vitamin A deficiency in embryos, demonstrating redundancy between RARs that was artefactually generated in the knockouts. Furthermore, double mutants between RARs and RXRs also recapitulate most of the abnormalities of the deficiency syndrome and in fact it is the *RXRα* which is functionally the most important RXR during development (amazingly, the triple mutant *RXRα^{+/-}/RXRβ^{+/-}/RXRγ^{+/-}* does not exhibit any overt developmental abnormalities). The double mutant defects are quite precise. For example, respiratory tract hypoplasia and absence of the oesophagotracheal septum are found in *Aα/Aβ2* mutants or *Aα/Xα* mutants; heart outflow tract, aortic arch and neural crest abnormalities in *Aα/Aβ* mutants or *Aα/Xα* mutants; kidney abnormalities in *Aα/Xα* mutants; thinner retina in *Aβ2/Aγ2* mutants; other ocular abnormalities in *Aβ2/Aα*, *Aα/Aγ* or *Xα* mutants. The defects are also very precise in terms of the effect within a tissue. For example shortening of the ventral retina, no effects on dorsal retina; agenesis of the left lung, not the right lung; agenesis of the anterior chamber of the eye, not the posterior chamber; homeotic transformations of specific vertebrae either in a posterior or anterior direction.

In addition, a range of abnormalities have been found in these double knockouts which are not associated with vitamin A deficiency in embryos: exencephaly, homeotic transformations of cervical vertebrae, lens agenesis, thymus, thyroid and parathyroid abnormalities, kidney hypoplasia. This probably reflects the difficulty of achieving by dietary means complete vitamin A deficiency which is compatible with pregnancy.

Expression and knockouts of enzymes

Further surprises have come from studies of the RALDHs, the enzymes which metabolise retinal to retinoic acid and CYP26, the enzyme which breaks down retinoic acid. There are three RALDHs that have been studied and they each show amazingly precise expression patterns in the embryo. For example, RALDH1 is expressed in the dorsal half of the developing eye, RALDH3 is expressed in the ventral half of the developing eye, and in between these two is an equatorial stripe of CYP26.¹⁷ RALDH1 is further expressed in a part of the foregut and in neuroepithelial cells in the base of the mesencephalon which become the substantia nigra. RALDH3 is further expressed in Rathke's pouch, the developing isthmus which becomes part of the cerebellum and the posterior somites. RALDH2 is expressed in the somites right from the start of development and later in the developing motoneurons of the spinal cord. CYP26 is further expressed at the anterior end of the developing neural plate in tissue fated to become the forebrain and midbrain. When the two domains of expression of CYP26 at the anterior end of the neural plate and RALDH2 from the level of the first somite backwards were put together then it was realised that the hindbrain develops in the gap.¹⁸ The idea has therefore developed that there is a gradient of retinoic acid across the developing hindbrain with a high point, a source, at its posterior end (at the level of the first somite) and a low point, a sink, at the anterior end caused by its breakdown by CYP26. Individual rhombomeres in the hindbrain may be specified according to the precise concentration of retinoic acid to which they are exposed.¹⁹ This explains why the hindbrain

is so sensitive to disturbance by retinoic acid during development (see above). These ideas have been strongly supported by the phenotype of the RALDH2 knockout mouse. This mutant phenocopies many of the defects seen in vitamin A deficient embryos and the posterior hindbrain is completely missing.²⁰

Conclusion

These data from the molecular approach to vitamin A function using receptor and enzyme knockouts has provided fascinating insights into which specific parts of the embryo require retinoic acid. So too have data using the classical approach of depriving embryos of vitamin A. Together these experiments show how important this molecule is for embryonic development and the dramatic effects of disturbing the retinoic acid signalling pathway. From the human perspective this tells us that it is very important to have the correct balance of vitamin A in the maternal diet for a successful developmental outcome, but it may also be telling us that even reduced levels of vitamin A or a slight reduction in its signalling properties could be harmful to the embryo in more subtle ways than we have searched for in these gene expression analyses. Although the hindbrain has been highlighted and may explain subtle abnormalities in humans such as facial nerve palsies in Accutane exposed infants, what if moderately reduced levels of retinoic acid signalling affected some aspect of forebrain development resulting in schizophrenia in later life?^{21 22} And, of course, one could ask the same question of many of the other organ systems in the body.

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