Commentaries

See article on page 246

Endothelins, pseudo-obstruction and Hirschsprung's disease

It has been known since the 1950s that the enteric nervous system is formed from cells that arise from the neural crest.[1] The enteric neurones mainly arise from the vagal neural crest of the developing hind brain and colonise the gut in a rostro caudal migration but some seem to arrive in the hind gut from the lumbosacral level via a caudo rostral wave of colonisation. The neural crest cells that migrate and colonise the gut are committed to become neuroblasts or neuronal support cells, glioblasts; however, differentiation into neurones and glial cells seems not to take place until they have reached their final resting places in the gut. Movement through the gut mesenchyme, survival in the gut and differentiation into mature cells is strongly influenced by contacts with the microenvironment which consists of other cells in the mesenchyme, neural crest, and the extracellular matrix. The extracellular matrix components provide directional clues to migrating neural crest cells and together with neighbouring cells provide some of the signals for crest cell differentiation. For example, the appearance of neural crest cells in the gut is preceded by expression of extracellular matrix molecules[2] and other factors such as glial derived neurotropic factor (GDNF) ensure survival of committed neuroblasts.[3] Thus defects of the neural crest cells themselves or alteration of the microenvironment of the migratory pathway may result in defects of development of the enteric nervous system. In humans this disordered development results in the most commonly presenting forms of chronic idiopathic intestinal pseudo-obstruction, congenital enteric neuromuscular disease. In Hirschsprung's disease defects in at least two different cell signalling systems, ret/GDNF and endothelin-3/endothelin B receptor,[4] cause the aganglionosis.

The endothelin system's important role in the development of the enteric nervous system has become apparent in the past four years or so when mice with targeted disruption of endothelin B receptor (ETR-B) and endothelin-3 (ET-3)[5–6] were found to have congenital distal intestinal aganglionosis.

The endothelins are a family of three peptides, endothelin-1, -2 and -3, coded for by distinct but related genes and act on cells via two G protein coupled receptors ETR-A and ETR-B. The endothelins are synthesised as much larger proproteins which are cleaved by an endothelin converting enzyme (ECE-1) to produce the active 21 amino acid peptide.[7] Of the three endothelins it is ET-3 which is so important in the enteric nervous system and binding of ET-3 to ETR-B on vagal neural crest cells is required for colonisation of the hind gut.

Mutations of either ETR-B or ET-3 have been identified in several naturally occurring animal models of Hirschsprung's disease, the piebald lethal mouse and the lethal spotted mouse[4] respectively. The ovaro-lethal white foal also has a significant mutation of ETR-B with a single amino acid substitution in the first transmembrane spanning domain of the ETR-B gene. The lethal spotted mouse carries a mutation in its ET-3 gene which prevents proteolytic activation of the peptide. Mutation analysis of children with Hirschsprung's disease has shown that about 10% carry mutations of either ETR-B, ET-3 or ECE-1. The effects of these genetic defects is to curtail neural crest migration in the distal colon and this is associated with localised overexpression of extracellular matrix molecules.[2] Using transgenic lines of mice which are either ETR-B deficient or ET-3 deficient, Kapur and colleagues[8] have shown that in ETR-B deficient mice enteric nervous system precursors can colonise the murine hind gut when they are surrounded by wild type enteric nervous system precursors. Further wild type enteric nervous system precursors will fail to colonise the hind gut when surrounded by ETR-B deficient ones. This strongly suggests that the enteric nervous system precursors signal ETR-B activation to those nearby and that when this signal is of sufficient intensity an ETR-B deficient crest cell can develop normally. It is thus clear that the interaction between the migrating neural crest cells and the mesenchymal environment of the hind gut is of critical importance in achieving normal innervation of the colon. The mechanism of the terminal aganglionosis that occurs either in the absence of ET-3 or ETR-B however remains unclear.

Despite the increasing understanding of the role of endothelins in the developing enteric nervous system, little work has been done in normal mice or men regarding the timetable of activity or the spatial orientation of these molecules in the developing embryonal gut. On page 246 of this issue Leibl *et al* describe the temporal and spatial expression of ET-3 and ETR-B in CD1 mouse embryos. They show clearly that ETR-B is confined to migrating neural crest cells and ET-3 to mesenchymal cells initially of the caecum but with a gradient extending rostrally into the small intestine and caudally into the proximal colon. Interestingly by 14 days postcoitum the ETR-B mRNA signal in the colon was stronger than in the more proximal part of the gut at this or earlier stages, perhaps, suggesting that ETR-B is expressed by both vagal and sacral neural crest cells.

The present results add to the growing body of work emphasising the importance of the gut mesenchyme in determining regional identity along the gut primordium and also in the regulation of region specific innervation of the gastrointestinal tract. The mechanisms that regulate expression of ET-3 and ETR-B genes are currently unknown. It is clear however that the rostro caudal specification of the gastrointestinal tract is likely to involve a spatial, temporal and combinatorial patterns of expression of homeobox genes, the so called enteric hox code. In chick embryos there is clearly overlapping expression of the genes Hox A-9, -10 and -11 and we have recently produced some preliminary data demonstrating specific spatial, temporal and combinational expression patterns of hox genes A4, B4, D4, A5 and C5[9] in developing murine gut. The relation between caecum specific hox gene expression and ET3 and ETB-R is currently unknown but they are certainly candidate downstream molecules for these developmental control genes. A number of transgenic animal models provide evidence of the importance of homeobox genes in the control of morphogenesis of the gut and these include the "knock out" of ENX, causing increased innervation of the hind gut,[10] and over expression of hox A4, resulting in megacolon.[2] Thus this family of genes and their

downstream targets are of importance within the genetic hierarchy of gut morphogenesis. Delineation of the genes comprising the enteric hox code, their downstream targets and their spatiotemporal patterns of expression is an essential and integral part of understanding the molecular events underlying the devastating diseases which cause pseudo-obstruction and Hirschsprung's disease in humans. Such knowledge may enable antenatal diagnosis in some families and will be essential for the development of neuronal transplant strategies for the treatment of enteric neuropathic diseases.

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See article on page 231

Back to the whale bone?

Most doctors with any practical experience of achalasia would be willing to admit that the disorder often provides considerable professional satisfaction. Firstly, it can be very satisfying to make the diagnosis. Far too often, patients will have suffered from gradually worsening dysphagia for many years and the diagnosis will have been missed at earlier consultations. The second moment of satisfaction can be enjoyed when the symptoms are relieved immediately after a relatively simple procedure such as pneumatic dilatation.

Malfunction of the lower oesophageal sphincter (LOS) plays a key role in the genesis of dysphagia in achalasia, in which it usually maintains an abnormally high resting tone. More importantly, however, the LOS does not relax sufficiently on swallowing, causing a persistent barrier to food boluses. In addition, the oesophageal body lacks normal propagation of contractions.

Presently there are four therapeutic options in achalasia, all of which are directed at lowering the tone of the LOS.

In what is considered to be the first report on treatment of achalasia, Sir Thomas Willis in 1672 described the successful dilatation of the sphincter using a whale bone.[1] Since then several types of dilating instruments have been used, but today endoscopically guided pneumodilatation with a low-compliant polyethylene balloon is preferred by most gastroenterologists. The occurrence of gastro-oesophageal reflux disease (GORD) after pneumodilatation is rare. Pneumodilatation with modern balloons is associated with a perforation rate of 0–4%, the perforations rarely requiring surgical intervention.[2]

The surgical approach, consisting of longitudinal myotomy of the LOS, is named after Heller. Either a thoracic or an abdominal approach can be used, the latter being associated with a higher incidence of GORD. For this reason, Heller's myotomy through an abdominal route is often combined with fundoplication.[3] More recently thoracoscopic and laparoscopic techniques for LOS myotomy have been described. Surgical myotomy carries a mortality risk approaching zero. Studies comparing dilatation and surgical myotomy have shown that the efficacy of these procedures is comparable.[4]

The third therapeutic option in achalasia consists of adminstration of a calcium channel blocker such as nifedipine. This treatment is generally considered to be less effective than surgery and dilatation,[2] and is therefore used mainly for short periods—for example, while the patient is on a waiting list for a more definitive procedure.

Since 1994, a fourth option has emerged, namely intrasphincteric injection of botulinum toxin type A, a toxin produced by *Clostridium botulinum* that inhibits acetycholine release from nerve endings.[5] This approach was shown to lead to short term symptom relief in up to 90% of patients.[6] By six months, 20 (65%) of the 31 patients treated were still in remission. The endoscopic injection procedure is simple and no major complications have been reported as yet. A question that remained unanswered in the early studies with botulinum toxin concerns the duration of its effect in comparison with surgery and pneumodilatation.

In this issue (see page 231) Vaezi *et al* describe a randomised study in which they compared the immediate and long term efficacy of botulinum toxin with pneumatic dilatation. Their study is the first that formally compares these two treatment modalities in a prospective way. The most important result of the study, from a clinical point of view, is that botulinum toxin injection resulted in a significantly lower remission rate at 12 months than did pneumatic dilatation (32% compared with 70% of patients in complete remission). As an explanation for the apparent difference between the outcome of their study and those of some previous reports, the authors rightly highlight the fact that in previous studies of botulinum toxin in achalasia, repeat injections were given to patients who relapsed shortly after the initial injection. In an earlier study that compared botulinum toxin injection with pneumatic dilatation, only patients who did not respond to botulinum toxin treament were pneumodilated, rendering the comparison unfair.[7]

Vaezi and colleagues also examined the response of a number of objective parameters to treatment with botulinum toxin and pneumodilatation. Changes in diameter and length of the barium column at radiographical examination of the oesophagus paralleled changes in symptom scores. Somewhat surprisingly, however, botulinum toxin, in contrast to pneumatic dilatation, did not have a statistically significant effect on LOS pressure. Even at one month after botulinum injection no reduction in LOS pressure was found. This finding is in contrast with observations made in earlier studies. The possibility that

measurement of mid-expiratory rather than of end-expiratory LOS pressure might have obscured an effect of botulinum toxin is discarded in the discussion section of the paper, although the authors do not provide us with the measurements.

The results of the study by Vaezi and colleagues raise more doubts on the clinical value of botulinum toxin treatment in achalasia than hitherto expressed. It is important to those actively involved in the treatment of patients with achalasia to consider the results of Vaezi *et al*'s study carefully. As the aim of treatment in achalasia, a life-long disease, is to reduce symptoms with a minimum number of interventions during the patient's lifetime, a new treatment that has to be repeated frequently is likely to be less satisfactory, both to the patient and to the doctor, than the existing range of potential treatments.

As has been the case with many other new therapeutic options for various other diseases, the initial enthusiasm for botulinum toxin treatment in achalasia may have been too great. It seems that the pendulum is swinging back again. It is highly unlikely, though, that it will swing back to the whale bone approach!

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See article on page 259

Genes means pancreatitis

Identifying the molecular mechanisms responsible for acute and chronic pancreatitis in humans is one of the most difficult problems in modern science. Major obstacles include the inaccessibility of the human pancreas to observation, the unpredictability of disease onset, the non-specific nature of abdominal pain early in the course of acute pancreatitis, an inability to biopsy the pancreas safely, difficulty in distinguishing initiating events from the concomitant inflammatory response, and the obvious problems of investigating a tissue that self-destructs during the disease process. Even fundamental questions as to whether pancreatitis begins in the acinar cell or through pathology related to the pancreatic ducts continue to be debated.[1 2] Animal models also fail to provide critical insights, partly because of the artificial methods used to induce pancreatitis.[3–5]

The discovery of the mutations in the cationic trypsinogen gene responsible for hereditary forms of pancreatitis in American and European kindreds[6 7] provided tremendous insights into the mechanism of acute and chronic pancreatitis in these families. It was hypothesised that the cationic trypsinogen R117H mutation eliminates a key hydrolysis site on the chain connecting the two globular domains of trypsin that is part of a fail-safe trypsin inactivation mechanism. Rather than being autolysed, prematurely activated mutant trypsin remains active within the pancreas, activates all other digestive enzymes, leads to acinar cell autodigestion and, therefore, acute pancreatitis. The second major insight was that the chronic pancreatitis commonly seen in patients was associated with mutations in trypsinogen. This observation suggests that recurrent acute pancreatitis may lead to chronic pancreatitis.[2 6 7] Families with the cationic trypsinogen R117H and N21I mutations have now been identified in Caucasians throughout the United States and Europe.

In this issue, Nishimori *et al* (see page 259) report the presence of the same two cationic trypsinogen gene mutations in Japanese kindreds with hereditary pancreatitis as seen in Caucasians. Additional polymorphisms in the cationic trypsinogen gene were also reported, but they either fail to result in an amino acid substitution or segregate with the panceatitis phenotype. Thus, this report expands the observation of pancreatitis causing cationic trypsinogen mutations to Asians and further defines the limits of pancreatitis causing mutations to cationic trypsinogen R117H and N21I. As hereditary pancreatitis is an autosomal dominant disorder, mutations that cause loss of function would not cause the syndrome. Furthermore, as hereditary pancreatitis is relatively rare and a number families have been investigated, it is unlikely that many additional gain-of-function mutations, such as the R117H mutation, will be identified.

The question of why the cationic trypsinogen N21I mutation predisposes individuals to pancreatitis was also tackled. Computer analysis of the N21I substitution suggests that the mutation changes the secondary structure in the region of the N21I mutation from a "turn" into a "sheet" conformation. The implications of the predicted secondary structural changes on the tertiary structure and trypsin biology may be important, but remain speculative. Other hypotheses on the role of the N21I mutation have also been offered[6] and could be consistent with these predictions. However, proving the actual structural changes caused by the mutation and determination of the mechanism through which the function of trypsin is altered will require further work.

Identification of the same two mutations in the cationic trypsinogen gene in kindreds with hereditary pancreatitis of both Caucasian and Asian ancestry, combined with the finding that potent trypsin inhibitors prevent pancreatitis associated with endoscopic retrograde cholangiopancreaography in humans,[8] provides us with strong evidence that cationic trypsinogen plays an important role in human acute pancreatitis. This represents a major conceptual breakthrough. Now, attention can be focused on experimental models of acute pancreatitis with premature trypsinogen activation, on mechanisms of premature trypsinogen activation and trypsin stabilisation, and on strategies to limit these processes in susceptible individuals.

Another interesting note is that the only mutations identified to date in patients with hereditary pancreatitis are in the human cationic trypsinogen gene. No pancreatitis associated mutations have been identified in anionic trypsinogen, nor in any of the other digestive enzymes. Indeed, human cationic trypsinogen is relatively unique among members of the trypsin family in its ability to

autoactivate.[9] Humans may differ from experimental animals in that acute pancreatitis in animals may require lysosomal hydrolases, such as cathepsin B, to activate trypsinogen.[10] Thus, in experimental animals, conditions must be met that allow trypsinogen and cathepsin B to co-localise, whereas in humans trypsinogen activation may occur is a variety of locations under relatively milder conditions. However the conditions that initiate excessive trypsinogen activation and pancreatitis in hereditary and non-hereditary pancreatitis require further investigation.

A final important finding in Nishimori *et al*'s report was that four of the six families with hereditary pancreatitis did not have mutations in the cationic trypsinogen genes. This observation suggests that at least one additional gene mutation is associated with hereditary pancreatitis. Discovery of this new gene may provide further insights into the mechanisms of acute and chronic pancreatitis.

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See article on page 168

Cross-reacting antibodies in coeliac disease?

Most patients with coeliac disease have antibodies to wheat gliadin, reticulin, and endomysium. In 1997 a seminal paper showed that at least a substantial fraction of anti-endomysial antibodies (EMA) recognises the endogenous enzyme tissue transglutaminase (tTG).[1] However, antibodies recognising other antigens can also be found and in this issue (see page 168) Krupičková and colleagues attempt to characterise these antibodies.

Anti-gliadin antibodies (AGA) were isolated from coeliac serum samples using (semi)purified α-gliadin as the substrate. These antibodies were tested for specificity using a synthetic α-gliadin peptide competition assay. This important study is the first of its kind. An interesting finding is that the antibody responses are directed towards a limited set of epitopes. These epitopes do not overlap with peptides recognised by small intestinal HLA-DQ restricted T cells,[2 3] but our current knowledge is too limited to judge whether this is important. One of the epitopes was the VLPVQQQQF peptide, which corresponds to α-gliadin residues 22–30. The glutamins were important, as substitution with glutamic acid removed the inhibitory function of VLPVQQQQF. Conversion of Qs to Es (deamidation) by tTG has been implicated recently in the pathogenesis of coeliac disease.[4 5] However, tTG acts on T cell epitopes of gliadin by specific deamidation of only some of the glutamins and it probably would have been more realistic if only one or some of the Qs in the 22–30 peptide had been replaced.

Although recent studies have focused on coeliac antibodies recognising tTG, other antibody specificities have been reported. Mäki *et al* described several antigens recognised by EMA but which were not recognised by AGA.[6] Börner *et al* isolated various components from different animal tissues using serum from patients with coeliac disease.[7] It is unlikely that the antigens reported in these two studies are tTG. As mentioned by Krupičková and colleagues, antibodies cross-reacting with gliadin, enterocytes and calreticulin can also be found. Interestingly, these authors have shown that some of the same peptides that interfere with AGA binding to α-gliadin also effect binding to enterocytes and calreticulin. By doing a sequence similarity search they identified corresponding sequences in α-gliadin and calreticulin. However, it would have been reassuring to see whether synthetic calreticulin peptides could also inhibit binding of AGA to α-gliadin, and further details of the calreticulin preparation used are essential. As with any new and unexpected finding it would also be good to see the cross-reactivity between gliadin and calreticulin reproduced by other investigators.

Can one now conclude that coeliac disease is an autoimmune condition directed against enterocytes and calreticulin? There are some difficulties in accepting such a hypothesis. Calreticulin is an abundant Ca^{2+} binding protein which is expressed in every cell in higher organisms.[8] It has a retrieval signal for endoplasmic reticulum (ER) and the ER is considered to be the major cellular site of localisation. Small amounts of calreticulin may also be present at extra-ER sites including the cell surface. At any rate, an immunological cross-reaction would presumably manifest itself in different organs. Coeliac disease is seen more frequently in IgA deficient individuals, so at least IgA antibodies are not necessary for the disease. If the cross-reactivities were attributable to IgG antibodies, they could give rise to the complement activation known to be present in coeliac lesions.[9] We know that IgG-AGA are not specific as they can be found both in healthy subjects and those with coeliac disease. However, this may not necessarily reflect what is going on in the small intestine. Conversely, cross-reactive AGA could have an effect during the first phases of disease pathogenesis directly following α gliadin challenge,[10] where the observed phenomenon might fit with a rapid antibody recognition event. Whether cross-reactive antibodies recognising enterocytes are capable of inducing apoptosis is still an open question.

Finally, a comment can be made on the usage of the "molecular mimicry" model as an explanation for the putative autoimmune component in coeliac disease. As Michael Bevan defined it, molecular mimicry is important for the induction phase of autoimmunity, where an infectious agent triggers an autoimmune loop, which persists even after the infection has been cleared. The complete remission seen in almost all coeliac patients after

withdrawal of cereal proteins from the diet is difficult to reconcile with this concept.

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