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NEW PATHOGENESIS OF THE COBALAMIN-DEFICIENT NEUROPATHY

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SUMMARY

Subacute combined degeneration (SCD) is considered the neurological counterpart of pernicious anaemia because it is the paradigmatic neurological manifestation of acquired vitamin B12 (cobalamin (Cbl)) deficiency in adulthood. Hitherto, the theories advanced to explain the pathogenesis of SCD have postulated a causal relationship between SCD lesions and the impairment of either or both of two Cbl-dependent reactions. We have identified a new experimental model, the totally gastrectomised (TGX) rat, to reproduce the key morphological features of the disease, and found new mechanisms responsible for the pathogenesis of SCD. We have demonstrated that the neuropathological lesions in TGX rats are not only due to mere vitamin withdrawal but also to the overproduction of the myelinolytic tumour necrosis factor(TNF)-a, nerve growth factor, the soluble(s) CD40:sCD40 ligand dyad, and the reduced synthesis of the neurotrophic agents, epidermal growth factor and interleukin-6. Cbl replacement treatments normalised all of these abnormalities.

In 1849, Thomas Addison, a physician at the Londoner Guy's Hospital, published his classical description of a group of patients with a mortal anaemia[1]. Subsequently, this anaemia came to be known as Addisonian anaemia, and when Biermer called it *perniciöse Anämie* (PA), the term was generally accepted[2]. In 1893, Nonne argued that neuropathy by cobalamin (Cbl) deficiency

Key words: Cobalamin deficiency - Subacute combined degeneration - Total gastrectomy

should be considered the neurological equivalent of PA, rather than a secondary state dependent on the haematopoietic disease, although the lack of a correlation between the degree of anaemia and the severity of neuropathy became clear later on[3]. In 1900, Russell et al. published the paper in which they first suggested the term ''subacute combined degeneration'' (SCD) to define the neuropathy observed in association with PA[4]. The reasons for this definition were the rapid course of this neurological disease, which usually affects the peripheral nerves and some of the columns of the spinal cord (SC), leading to a combination of tract degeneration.

Animal models that recapitulate human neurological diseases are needed to enable a detailed analysis of their pathogenetic mechanism(s). Many years ago, we believed that experimental total gastrectomy (TG) would make rats immediately deficient in intrinsic factor and, over time, Cbl-deficient (Cbl-D)[5]. TG provides a surgical paradigm of PA in rats (similar to autoimmune gastritis in humans) by removing gastric intrinsic factor, which is essential for the intestinal absorption of Cbl. The totally gastrectomised (TGX) rat is currently the animal model that mimics the key aspects of the pathogenesis of Cbl deficiency that are typical of PA and, consequently, also reproduces its main haematological and neurological symptoms. Notably, the central nervous system (CNS) of Cbl-D rats shows two histopathological features of SCD: astrogliosis and the most-severe degree of vacuolation in the white matter of the SC[5,6], especially in its thoracic segment[7,8]. It is worth noting that almost all of the morphological damages and biochemical abnormalities observed in TGX rats have also been found in rats made Cbl-D through prolonged feeding with a Cbl-D diet, although the time required to observe them is inexplicably much longer[7,9].

In mammalian cells there are only two known Cbl dependent enzymes. *L*-Methylmalonyl-coenzyme A(CoA) mutase (EC 5.4.99.2) requires adenosyl-Cbl and catalyses the conversion of *L*-methylmalonyl-CoA to succinyl-CoA. Methionine synthase (EC 2.1.1.13) requires methyl-Cbl and catalyses the simultaneous conversion of N_5-methyltetrahydrofolate to tetrahydrofolate and of

homocysteine (HCYS) to methionine. The metabolites methylmalonic acid (MMA) and HCYS accumulate when these two enzymatic reactions are impaired by Cbl deficiency. Other metabolites that commonly accumulate as the result of deficiency of the vitamin include cystathionine (CYSTA)[10].

We have measured the levels of the above metabolites in sera and in kidney, liver and SC at different times after TG, to better characterise the evolving Cbl deficiency and we have determined whether the serum levels of these metabolites would return to normal when the deficiency was corrected by chronic *in vivo* Cbl administration[11]. To prevent the post-TG rise in serum MMA levels we have also injected the rats with antibiotics by the chronic peroral, in an attempt to suppress the production by the gut flora of propionate, the major precursor of the MMA which accumulates in Cbl deficiency in rats and humans[12,13] and we have quantified, through morphometric analyses, the degree of the severity of the spongy vacuolation in the SC white matter at different times after TG, and the improvement, if any, of this neuropathy after the chronic administration of either Cbl or antibiotics[11]. MMA rose within 2 months and progressively increased thereafter until the end of the investigation period. HCYS was only clearly elevated much later than the serum MMA, i.e., 10 months after the operation[11]. The concentrations of MMA, and CYSTA were increased in kidney, liver, and SC of TGX rats at 10 months. Chronic treatment of TGX rats with Cbl greatly decreased the serum levels of all the metabolic indicators of Cbl deficiency[11]. Chronic peroral administration of the antibiotic lincomycin to TGX rats in an attempt to suppress the enteric flora markedly decreased serum MMA levels[11]. Only Cbl, however, given either for the first two months after TG or for the third and fourth month after TG (i.e., after SC abnormalities had already appeared) significantly decreased the severity of spongy vacuolation in SC white matter, although not completely preventing or repairing the neuropathological damage[11]. Therefore, neither the early impairment in TGX rats of the Cbl-dependent *L*-methylmalonyl-CoA mutase reaction nor the more delayed impairment of

the Cbl-dependent methionine synthase step, as reflected by changes in serum metabolite levels, seems to be causally related to the TG-induced spongy vacuolation in SC white matter[11].

Our findings also indicate that, regardless of its coenzyme functions, Cbl modulates the synthesis of some cytokines and growth factors in mammalian CNS. We have demonstrated that Cbl deficiency causes an increased synthesis of a neurotoxic agent, tumour necrosis factor(TNF)-α[14], and the reduced CNS synthesis of a neurotrophic agent, epidermal growth factor (EGF)[15]. However, we do not know the molecular bases of these phenomena. We have also demonstrated decreased interleukin(IL)-6 levels in the cerebrospinal fluid (CSF) of both types of Cbl-D rats whether TGX or fed a Cbl-D diet[16]. Interestingly, IL-6 regulates the proliferation and differentiation of haematopoietic cells[17,18], is present in glial cells[19], and belongs to a family of haematopoietic and neurotrophic cytokines that shares gp130, which is the signal-transducing chain of the IL-6 receptor complex[20,21]. We have also demonstrated that the levels of the soluble(s) CD40:sCD40 ligand(L) dyad[22], which belongs to the TNF-α:TNF-α-receptor superfamily, are significantly increased in the CSF, but not the serum of Cbl-D rats. Finally, we have recently demonstrated that Cbl deficiency increases nerve growth factor (NGF) levels in both rat SC and CSF[23].

All the cytokine and growth factor abnormalities in TGX rats, and all the morphological lesions and biochemical abnormalities, are substantially corrected by chronic postoperative Cbl treatment, thus leading to the conclusion that they are all specifically linked to Cbl deficiency. Interestingly, Cbl deficiency does not modify the CNS synthesis of other neurotransmitters and hormones in Cbl-D rats, such as vasoactive intestinal peptide (VIP), somatostatin (SS), or leptin[15,16]. The involvement of these growth factors and cytokines in the pathogenesis of experimental SCD in Cbl-D rats is further supported by the fact that: *(i)* intracerebroventricular (i.c.v.) microinjections of agents that antagonise TNF-α production (e.g. specific anti-TNF-α antibodies, IL-6 and transforming growth factor(TGF)-b_1) prevent SCD-like lesions in the SC white matter of

TGX rats, without modifying their Cbl-D status[14]; *(ii)* i.c.v. microinjections of EGF are as effective as Cbl in reducing intramyelinic and interstitial oedema in the SC white matter of TGX rats, without modifying their Cbl-D status[24]; *(iii)* i.c.v. microinjections of TNF-α[14] or anti-EGF antibodies (but not a non-immune serum)[24] cause typical SCD lesions in the SC white matter of normal rats (i.e. intramyelinic and interstitial oedema), without modifying their Cbl status; *(iv)* i.c.v. microinjections of TGF-b_1 normalised or significantly reduced the increased sCD40 and sCD40L levels in rat CSF, and the normal myelin ultrastructure of the SC was concomitantly restored[22]; *(v)* the ligation of CD40 by i.c.v. treatment with cross-linking anti-CD40 antibodies in TGX rats substantially prevented the onset of SCD-like lesions in the SC white matter[22]; (*vi*) the i.c.v. microinjections of anti-NGF antibodies were highly effective in preventing the onset of interstitial and intramyelinic oedema[23]; and (*vii*) NGF treatment during the third and forth month after TG markedly worsened the spread and severity of interstitial oedema[23].

Studies of the changes in the CSF proteome (i.e. proteins expressed by a genome at a given time[25]) of Cbl-D rats can confirm the existence of possible non coenzymatic effects of Cbl. Indeed, the proteomic approach enables the parallel evaluation of several components instead of one or just a few selected markers[26] because some CSF proteins are derived from the CNS[25] and also because no lesions of the blood-brain barrier have been observed in Cbl-D rats[26]. Chronic Cbl deficiency affects rat CSF proteome both qualitatively and quantitatively because the amount of proteome increases, and/or absent proteins under control conditions can be detected in the CSF of Cbl-D rats[26]. Cbl-replacement treatment in TGX rats also profoundly modifies the pattern of the rat CSF proteome[26], but CSF transthyretin is not influenced by Cbl deficiency, and CSF prostaglandin-D synthase (EC 5.3.99.2) is not influenced by Cbl-replacement therapy[26]. However, the changes in the CSF proteome of Cbl-D rats seem to occur mainly after the appearance of spongy vacuolation and intramyelinic and interstitial oedema (i.e. the histo-

logical and ultrastructural hallmarks of the CNS lesions of experimental SCD)[26]. This finding raises the question as to whether the overall changes in rat CSF proteome that are caused by chronic Cbl deficiency are connected with the maintenance of SCD-like lesions or represent a specific epiphenomenon of vitamin deficiency.

We questioned whether an imbalance in TNF-α and EGF levels similar to that observed in the CNS and/or CSF of TGX rats might also be present in the biological fluids of adult Cbl-D patients. We have demonstrated that there is an imbalance in TNF-α and EGF levels in the sera of humans with clinically confirmed severe Cbl deficiency (mainly caused by type-A autoimmune gastritis, partial gastrectomy or TG), but not in patients with severe iron-deficient anaemia[27], and this imbalance can be rectified by Cbl-replacement therapy[27]. This imbalance is also present in the CSF of SCD patients, where TNF-α levels are abnormally high and EGF levels abnormally low[28]. The percentage increase in TNF-α levels (83% versus 46.7%), and the percentage decrease in EGF levels (50.9% versus 30.4%) are significantly greater in SCD CSF than in Cbl-D serum[27,28]. These findings indicated that the opposite changes in TNF-α and EGF levels in the CSF of SCD patients are partially independent of the corresponding changes in their serum levels of adult Cbl-D patients[27,28]. They also indicate that the deranged TNF-a and EGF ratio in the serum and CSF of adult Cbl-D patients might reflect abnormal TNF-α and EGF synthesis in most, if not all, their tissues and/or organs, and that this imbalance is specifically linked to the Cbl-D status of the patients[27,28]. Therefore, the opposite modulation of TNF- α and EGF levels in biological fluids might mirror the Cbl-D status of adult patients in the same way as the increased levels of MMA and HCY[7,8,29,30].

Our studies have shown that Cbl modulates in opposite ways the synthesis of at least some cytokines and growth factors. Therefore, these cytokines and growth factors can be defined as new Cbl-dependent CNS proteins, regardless of whether their synthesis increases or decreases in the presence of Cbl. From this point of view, the neurotrophic action of Cbl resembles that of other vita-

mins such as vitamin K[31] and vitamin D_3[32,33] that also induce neurotrophic factors. However, our studies have raised more problems than they have solved and some key questions still remain unanswered. In particular, we do not know whether the effect of Cbl deficiency on the CNS production of cytokines and/or neurotrophic and neurotoxic growth factors is due to changes in the expression of their genes and/or in post-transcriptional and/or post-translational mechanisms, or in epigenetic mechanisms, such as the hypomethylation of promoter regions leading to gene overexpression[34,35]. However, the alterations in the synthesis of CNS cytokines and growth factors in Cbl-D rats do not go in the same direction: TNF-α[14], NGF[23] and sCD40:sCD40L dyad increase[22], EGF[15] and IL-6[16] decrease; and VIP, SS[15] and leptin[16] remain unchanged. However, in relation to the possibility of gene hypomethylation, EGF has been considered to be an epigenetic factor in the mammalian CNS[36] and a significant reduction in global DNA methylation in TGX rats has been reported[37]. Also, no changes were found in the total protein contents in the SC of Cbl-D rats in whichever way vitamin deficiency was induced. At this stage of Cbl research, there are insufficient reasons to include the broad spectrum of Cbl diseases among the human diseases of DNA methylation[38].

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