Further Evidence for the Involvement of Epidermal Growth Factor in the Signaling Pathway of Vitamin B$_{12}$ (Cobalamin) in the Rat Central Nervous System

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Abstract. In order to get further evidence for a mandatory involvement of epidermal growth factor (EGF) in the neurotrophic action of vitamin B$_{12}$ (cobalamin (Cbl)) in the central nervous system (CNS) of the rat, we observed the effects of repeated intracerebroventricular (ICV) microinjections of EGF in rats made Cbl-deficient through total gastrectomy. Morphometric analysis demonstrated a significant reduction in both intramyelinic and interstitial edema in the white matter of the spinal cord (SC) of totally gastrectomized (TGX) rats after treatment. Intramyelinic and interstitial edema are characteristic of Cbl-deficient central neuropathy in the rat. Similar lesions were also present in SC white matter of rats treated with repeated ICV microinjections of specific anti-EGF antibodies without any modification in their Cbl status. These results, together with those of a previous study showing the cessation of EGF synthesis in the CNS of TGX rats, demonstrate that: a) EGF is necessarily involved in the signaling pathway of Cbl in the rat CNS; and b) the lack of a neurotrophic growth factor EGF and not the mere withdrawal of Cbl, causes or at least contributes to neurodegenerative Cbl-deficient central neuropathy.

Key Words: Cobalamin; Epidermal growth factor; Myelinolysis; Subacute combined degeneration; Vitamin B$_{12}$.

INTRODUCTION

We have recently clarified some key aspects of the pathogenesis of the cobalamin (Cbl)-deficient (Cbl-D) neuropathy (classically termed subacute combined degeneration [SCD] (1)) induced in the central nervous system (CNS), particularly in the spinal cord (SC), of rats made Cbl-D as a result of total gastrectomy (TG) or chronic feeding with a Cbl-D diet. We have demonstrated that the myelin lesions of SCD, whose hallmark is intramyelinic edema in CNS white matter (2), are caused by the locally increased production of a neurotoxic agent, tumor necrosis factor (TNF)-α (3), combined with the locally decreased production of a neurotrophic agent, epidermal growth factor (EGF) (4). We have also demonstrated that the shift in physiological equilibrium between neurotoxic and neurotrophic agents in the CNS of Cbl-D rats in favor of the former is etiologically linked to their permanent Cbl-D status, since it has been substantially corrected in totally gastrectomized (TGX) rats by means of postoperative Cbl treatment (3, 4).

We thus identified new functions of Cbl in rat CNS, which are independent of its coenzyme function (5) and are hormone-like, since Cbl inversely regulates the expression of TNF-α and EGF genes (3, 4). This new role of Cbl in mammalian CNS seems to be of paramount importance, since accumulated clinical and experimental evidence has shown that an inappropriate expression and/or release of neurotrophic and/or neurotoxic agents in the mammalian CNS might be involved in the pathogenesis of different demyelinating or, broadly speaking, degenerative neurological disorders (6–10).

We have previously succeeded in reproducing SCD-like lesions in the CNS of our Cbl-D rats (1, 11, 12) and observed a widespread spongy vacuolation in the SC white matter (1, 11) due to intramyelinic edema (with the splitting of the lamellae) and interstitial edema (12). This neuropathological damage was substantially repaired or prevented by chronic postoperative Cbl treatment in the TGX rats (1, 11, 12). SC swelling and demyelination in Cbl-D patients with SCD have been detected using magnetic resonance imaging (MRI) (13–15); these MRI abnormalities typically diminished significantly or even disappeared after vitamin replacement therapy (13–15).

Our recent findings have compelled us to revise substantially the old theories advanced to explain the pathogenesis of SCD, which basically postulated a causal relationship between the impairment of either or both of the 2 Cbl-dependent reactions (i.e. L-methylmalonyl-coenzyme A mutase [EC 5. 4. 99. 2] and methionine synthase [EC 2. 1. 1. 12] (5)) and SCD lesions in Cbl-D animals or patients (16, 17). In this regard, we have previously shown that the accumulation of methylmalonic acid and/or homocysteine elicited by permanent Cbl deficiency in the serum and SC of TGX rats does not play any etiologic role in their SCD-like lesions (18).

Having demonstrated that EGF is the mediator of the neurotrophic action of Cbl in the rat CNS (4), we hypothesized that EGF might be as effective as Cbl in preventing the onset of SCD-like lesions in the CNS of TGX rats. We therefore administered EGF to TGX rats by means of repeated intracerebroventricular (ICV) microinjections in order to evaluate whether such treatment begun shortly after TG might ameliorate the SCD-like lesions in the SC white matter. In addition, we similarly...
administered specific EGF antibodies to normal rats in order to investigate morphological effects on SC white matter and compare them with the SCD-like lesions observed in the SC white matter of TGX rats. We used both light- and electron-microscopy to gauge the extent of intramyelinic and interstitial edema in SC sections from the treated rats in order to establish whether the injected material acted as a neuroprotective or neurotoxic agent, and to quantify morphometrically the in vivo effects of the treatments themselves.

MATERIALS AND METHODS

Surgical Procedures

Adult male albino non-inbred Sprague-Dawley rats (Charles River Italia, Calco, Italy) weighing 250 g at the beginning of the experiment were housed as previously described (1). Some rats underwent TG in order to induce experimental SCD, as previously reported (1). Body temperature was maintained at 37°C during all surgical procedures and during recovery from anesthesia (i.e. until normal locomotor activity was observed). To perform the ICV microinjections, a polyethylene guide cannula was stereotaxically placed also as previously described (3). In TGX rats the cannula was placed 1 wk after TG. Correct cannula placement was checked as previously reported (3).

Chemicals and ICV Drug Administrations

Normal control rats, laparotomized rats, and TGX control rats were given ICV microinjections of sterile pyrogen-free saline; normal rats and TGX rats were given ICV injections of murine EGF (Calbiochem, La Jolla, CA). Other normal rats received ICV microinjections of monoclonal antibodies to human EGF (R & D Systems Inc., Minneapolis, MN). Since there is a high interspecies homology for EGF (19), we used human specific anti-EGF antibodies. All of the in vivo treatments included 2 ICV microinjections weekly for 7 wk after implantation of the cannula using a 10 μl Hamilton syringe and a volume ranging between 5 and 7 μl. The dose per injection was 10 μg EGF or 8 μg anti-EGF antibodies.

Histological Processing, Staining, and Electron Microscopy

The animals were killed 48 h after the last ICV microinjection and all of the rats were the same age at death. The procedures involving the animals and their care conformed to institutional guidelines, in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication no. 86-23, 1985).

Under general ketamine anesthesia, the thorax was opened, a cannula was inserted into the left ventricle and the right auricle opened. After washing with 150–200 ml of 0.9% NaCl solution, the rats were perfused with 400–500 ml of a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.12 M phosphate buffer solution, pH 7.4. The SC segments were carefully dissected from the cervical3-4, thoracic10-11, and lumbar5-6 cord. From these segments, thin slices (0.5 mm) were fixed in osmium (1% in phosphate buffer) for 1 h at 4°C, dehydrated, and embedded in epoxy resin (12). Semithin sections (1 μm) were stained with toluidine blue for morphometric analysis at light microscopic level. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a CM 10 Philips electron microscope (12).

Morphometric Analysis

For each SC, 3 serially 10-μm-spaced semithin sections from each of the 3 SC levels were morphometrically analyzed (stereology-based measurements (20, 21)). With a camera lucida, a grid of 389 regularly spaced points was superimposed onto the microscopic field with a fixed magnification (objective lens: 63×). For each SC section, 2 samples of each funiculus (anterior, lateral, and posterior) were morphometrically analyzed. Therefore, the total number of points (observations) for each white matter of each SC was 21006. The percentage of points falling either on SC structures or empty SC vacuoles was quantified in the white matter only. The widespread but uneven spongy vacuolation (the so-called “field of holes” or “status spongiosus”) previously seen at optical microscopy in the SC of TGX rats (1, 11), is the hallmark of experimental SCD and is due to both intramyelinic edema and interstitial edema (12). All of the examinations of the semithin and ultrathin SC sections were made under blind conditions: i.e. without knowing to which experimental group the SC sections belonged.

Studies of Blood Components

Red cell counts, hemoglobin concentrations, and serum Cbl levels were determined in all 2-month TGX rats immediately before sacrifice as previously described (1, 18). Anemia and very low serum Cbl levels were observed in both the untreated (1, 11) and EGF-treated TGX rats (not shown). No changes in serum Cbl levels were observed in the normal rats treated with specific antibodies to EGF (not shown).

Assay of Cbl in SC and Cerebrospinal Fluid

The Cbl concentrations in the SC and cerebrospinal fluid (CSF) of the rats belonging to the different experimental groups was determined as previously described (4, 11).

Statistical Analysis

Our morphometric data were evaluated using equivalence testing (22). We evaluated the difference between each pair of means of the different treatments using analysis of variance (ANOVA) after appropriate weighting, at a 0.05 level of statistical significance. The p levels are reported in Table 1.

RESULTS

Morphometric Results: Evaluation of the Effects of the ICV Treatments on SC White Matter

The morphometric analysis of the quantitative changes in vacuolated, unstructured areas of the SC white matter of the normal and TGX rats in relation to the different treatments with anti-EGF antibodies or EGF is shown in Table 1. There was no significant difference between the SC white matter of the normal untreated rats and that of the normal saline-injected rats. Statistical analysis of the morphometric data showed a highly significant increase in the number of vacuolated areas in the SC white matter

of the rats treated with anti-EGF antibodies in comparison with both the intact and normal saline-injected rats. In contrast, statistical analysis of the morphometric data showed a significant reduction in the number of vacuolated areas in the SC white matter of the 2-month TGX-EGF-treated rats in comparison with both the untreated and saline-injected 2-month TGX rats (Table 1). It is worth noting that the therapeutic effect of EGF in the SC white matter of the TGX rats corresponded exactly to that previously observed with Cbl in the CNS of TGX rats (12). No ultrastructural pictures of the SC white matter of control laparotomized rats are shown here, since they have been reported previously (12).

Cbl Concentration in SC and CSF

From the data presented in Table 2, it appears that TG greatly decreased the Cbl content in both CSF and SC, in agreement with our previous results (4, 11), and that the ICV administration of EGF to TGX rats did not modify either of these Cbl levels. As expected, no changes were observed in the CSF or SC Cbl content of the normal rats given intracerebroventricularly either EGF or specific antibodies to EGF (Table 2).

DISCUSSION

Together with our earlier study (4), this study firmly supports the notion that EGF acts as a local mediator of the neurotrophic action of Cbl in the rat CNS. We have previously demonstrated that, however induced, a permanent Cbl deficiency selectively decreases EGF synthesis in the CNS and EGF levels in the CSF of the rat, and that both of these abnormalities are removed in TGX rats by chronic postoperative Cbl treatment (4). We here provide further experimental evidence of this concept: 1) repeated ICV administration of EGF to TGX rats substantially prevented the hallmark experimental SCD alterations, intramyelinic and interstitial edema; and 2) repeated ICV administrations of specific antibodies to EGF into normal rats did not modify Cbl content in the SC and CSF but nevertheless caused damage in SC white matter, which morphologically mimicked that observed in the SC of our Cbl-D rats (12). Both the cessation of EGF synthesis in rat CNS due to prolonged Cbl deficiency (4) and the inactivation of the normally occurring EGF in rat CSF by specific antibodies damages the interstitium and myelin sheaths of the SC white matter. At this stage of our research, however, it is exceedingly difficult to

TABLE 1

Morphometry of the Vacuolation in the SC White Matter of Normal or TGX Rats Given Different Drugs

<table>
<thead>
<tr>
<th>Group</th>
<th>ICV Treatment</th>
<th>Rat</th>
<th>Holes on 21006 points</th>
<th>R₁, R₂</th>
<th>R₁ mean</th>
<th>Holes (percentage)</th>
<th>Statistical Analysis (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>intact</td>
<td>520</td>
<td>530</td>
<td>525</td>
<td>2.5%</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Saline</td>
<td>normal</td>
<td>682</td>
<td>701</td>
<td>692</td>
<td>3.3%</td>
<td>NS vs A</td>
</tr>
<tr>
<td>C</td>
<td>Saline</td>
<td>LPT</td>
<td>666</td>
<td>654</td>
<td>660</td>
<td>3.1%</td>
<td>NS vs A</td>
</tr>
<tr>
<td>D</td>
<td>EGF</td>
<td>normal</td>
<td>590</td>
<td>618</td>
<td>604</td>
<td>2.9%</td>
<td>NS vs A and B</td>
</tr>
<tr>
<td>E</td>
<td>Anti-EGF Abs</td>
<td>normal</td>
<td>3144</td>
<td>3127</td>
<td>3136</td>
<td>14.9%</td>
<td>&lt; 0.000001 vs A and B</td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>TGX</td>
<td>2661</td>
<td>2638</td>
<td>2650</td>
<td>12.6%</td>
<td>&lt; 0.00001 vs A, B, and C</td>
</tr>
<tr>
<td>G</td>
<td>Saline</td>
<td>TGX</td>
<td>2897</td>
<td>2969</td>
<td>2933</td>
<td>14.0%</td>
<td>&lt; 0.000001 vs A, B, and C</td>
</tr>
<tr>
<td>H</td>
<td>EGF</td>
<td>TGX</td>
<td>661</td>
<td>701</td>
<td>728</td>
<td>3.3%</td>
<td>&lt; 0.00001 vs F and G; NS vs A and B</td>
</tr>
</tbody>
</table>

Abbreviations: Abs, antibodies; ICV, intracerebroventricular; LPT, laparotomized; NS, not significant; R, rat; TGX, totally gastrectomized.
establish whether the beneficial effect of EGF on the SC myelin sheaths of TGX rats occurs directly or indirectly (i.e. through a mediation of the CNS myelin-forming cells). EGF has been shown to govern oligodendrocyte development (23–25) and increase the levels of both oligodendrocyte markers (26), i.e. 2’,3’-cyclic nucleotide 3’-phosphodiesterase and myelin basic protein (MBP) (27), although there is a report showing a decrease in MBP synthesis by EGF (28). Furthermore, we previously observed ultrastructural and microscopic signs of activation of macroglial and microglial cells in the CNS of Cbl-D rats (11, 12) but no morphological changes in the neurons (11, 12), notwithstanding the fact that EGF synthesis is abrogated in a similar manner in both neurons and glia of the SC in prolonged Cbl deficiency (4).

The neurotrophic action of EGF on neurons and glia has been widely documented (29–32). Likewise, EGF has been shown to be neuroprotective from the toxicity of different agents in different types of cultured neuronal cells (33–36). Whether EGF plays a purely neuroprotective or a purely neurotrophic action with regard to the CNS damage brought about by chronic Cbl deficiency in the rat still remains a matter of speculation.

At present, no information is available as to the signal sequence set by Cbl in the mammalian CNS in order to achieve its neurotrophic effect. However, we can say that EGF is certainly required for the signal sequence of Cbl in the rat CNS. This makes it conceivable that EGF-receptors (EGFRs) of both neurons and glial cells might also mediate the biological signals of Cbl. Like EGF,
EGFRs have been shown to be widely distributed throughout the mammalian CNS (29, 37–39) and to be associated with the different types of neurons and glia. EGFRs have also been shown to bind to and be activated by the EGF-related polypeptides (now given the consensus name neuregulins) and by other polypeptide growth factors (39–43).

In conclusion, we can speculate that equivalent animal models of human neurodegenerative diseases may allow the development and evaluation of new therapeutic strategies. In this study we used the TGX rat as the most accurate model of human Cbl-D central neuropathy (18). Since reduced EGF synthesis in the CNS of TGX rats has been shown to be specifically linked with their permanent Cbl-D status (4), it follows that exogenous EGF (in this study administered to TGX rats) becomes an etiologically-based therapy of the neuropathological lesions (in this study administered to TGX rats) becomes an etiologically-based therapy of the neuropathological lesions characteristic of experimental SCD. Similarly, we have recently demonstrated that the level of the biologically active form of TNF-α protein is higher in the SC of TGX rats, and that the ICV administration of specific antibodies to TNF-α in TGX rats substantially prevents the myelinolytic lesions in their SC white matter (3).

Finally, although experimental neuroprotective therapy is still in its early stages (44), the TGX rat represents one of the few examples in which a deficiency in the synthesis of a neurotrophic factor (EGF) causes or, at least, contributes to a neurological disorder, which is likely to be cured by the administration of the lacking factor. A neuregulin has been shown to prevent clinical disability in animal models of multiple sclerosis (41). Reduced EGF CSF levels have been found in amyotrophic lateral sclerosis (45) but not in Parkinson disease (46), thus suggesting that a decrease in EGF synthesis in the human CNS might be involved in the pathogenesis of neurodegenerative diseases other than SCD. Among the fundamental questions yet to be answered in understanding the pathophysiology of SCD are 1) whether permanent Cbl deficiency may also cause a decrease in the number of and/or an alteration in EGFR in the rat CNS; and 2) whether the increase in TNF-α synthesis (3) and the decrease in EGF synthesis (4) in the SC of TGX rats are disparate and unrelated, or somehow linked with each other. Notwithstanding the conventionally distinct roles of neuroprotection by EGF (29–32) and neurotoxicity by TNF-α (6, 47, 48), there are many indications that there may be a potential antagonistic interaction between them, as has been demonstrated in some cultured neoplastic cell lines (49–52) and in primary human trophoblasts (53, 54). If such an antagonism were responsible for SCD-like lesions in Cbl-D rats, our model of Cbl-D central neuropathy would resemble the new model of neurodegeneration recently provided by Vinters et al (55), who suggest that neurodegeneration in the CNS may involve significant interplay between neurotoxic cytokines and neuronal survival factors. In the developing mature and degenerating CNS, neurons and glia are situated in a milieu that contains both neurotrophic and neurotoxic molecules, thus making any study of the shifting of the ratio between them of paramount importance to understanding the pathogenesis of human neurodegenerative diseases.

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