Identification of an insulin-like growth factor-binding protein in human cerebrospinal fluid with a selective affinity for IGF-II

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Human cerebrospinal fluid (CSF) has been found to contain several different molecular forms of IGF-specific binding proteins (BPs). Qualitatively, they are similar to those present in serum, although their relative proportions are very different, as well as to those present in the culture media of brain tissue from which these BPs presumably arise. One particular form of BP is predominant in CSF. It has an M_r of 34000, as estimated by SDS-polyacrylamide gel electrophoresis followed by transfer onto nitrocellulose, and an isoelectric point around 5.0 based on chromatofocusing. It has a selective affinity for IGF-II ($\sim 4 \times 10^{10}$ M^{-1}) as shown by competitive binding experiments in which biosynthetic IGF-I was about 40-times less potent than native IGF-II in displacing ^{125}I-labelled IGF-II. These findings are in agreement with the preponderance of IGF-II in nervous tissue and in CSF and suggest that this BP plays an important role in the interaction of IGF-II with its target cells.

(Cerebrospinal fluid) Insulin-like growth factor Insulin-like growth factor-binding protein Western blotting Chromatofocusing Competitive binding assay

1. INTRODUCTION

In [1,2] we found that insulin-like growth factors (IGFs) and their binding proteins (BPs) are produced by nervous tissue in culture and that the BPs are also present in cerebrospinal fluid (CSF) [3]. The biosynthesis of IGFs by nervous tissue has since been confirmed and IGF-II has been shown to be the predominant form in both nervous tissue and CSF [4–7]. The aim of this study was to characterize the BPs in CSF. The results indicate the preponderance of a BP with an M_r of 34000 and a selective affinity for IGF-II, from which an important physiological role in the interaction of IGF-II with its target cells can be inferred.

2. MATERIALS AND METHODS

2.1. *Biological samples*

CSF samples were collected during neuroradiological examination of otherwise healthy subjects with herniated intervertebral discs. A pool was made up from samples taken from 20 subjects, concentrated by ultrafiltration and then stored at −25°C. Pools of serum were made from samples from healthy subjects and hypopituitary patients. Samples of cerebral cortex were obtained from one adult (healthy tissue excised during surgery on a deep lesion) and two 26-week-old foetuses (therapeutic abortions) and maintained in organ culture as described [1]. CSF and serum samples were taken simultaneously from one of the foetuses.

2.2. *Peptides*

IGF-I (Prep. 1:4) and IGF-II (Prep. 9 SE IV) purified from human serum were generously provided by Dr R.E. Humbel (Zürich). Iodination of the IGFs and their subsequent purification were performed as in [8]. Biosynthetic IGF-I was kindly supplied by Genentech, Inc. (USA).

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2.3. *Analytical gel filtration*

Pooled CSF samples (1–15 ml initial volume) were incubated with ^{125}I-labelled IGF-II (~12 h, 4°C) and chromatographed at 4°C on a 1.5×40 cm column of Ultrogel AcA 44 (IBF, France) equilibrated with 0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% NaN_3, at a flow rate of 10 ml/h (1 ml/fraction). The column had previously been calibrated with different markers (see fig.1), some of which (^{3}H-labelled DNA, ^{14}C-labelled ovalbumin, ^{125}I) were used as internal markers.

2.4. *Chromatofocusing*

12 ml initial volume of the CSF pool was incubated with ^{125}I-labelled-IGF-II (~12 h, 4°C) and chromatographed at 4°C on a 0.9×60 cm column of anion exchanger (PBE 94, Pharmacia, Sweden), equilibrated with 0.025 M imidazole-HCl buffer, pH 7.4. The proteins were eluted in a gradient of pH 7.4–4.0 with Polybufffer 74-HCl, pH 4.0 (12.5% v/v), in 4 ml fractions at a flow rate of 20 ml/h.

2.5. *SDS-PAGE and Western blotting*

The techniques have been described elsewhere [9]. Briefly, the samples, either concentrated or not by ultrafiltration, were brought to the same final volume (150 μl) and the same ionic strength (~0.1 M NaCl) in the presence of 2.5% SDS, heated for 2 min at 100°C and subjected to SDS-PAGE (11% acrylamide slab gel, 15×15×0.15 cm) performed according to Laemmli [10], but without reducing agent (except in the case of the ^{14}C-labelled reference proteins) (Amersham, England). Thereafter, the proteins were electroblotted onto a nitrocellulose sheet which was then treated with Nonidet P40, BSA and Tween, and finally incubated with ^{125}I-labelled IGF-II (200 000 cpm/100 cm^2) so that the BPs could be detected by autoradiography.

2.6. *Competitive binding studies*

These were performed on BPs prepared from the CSF pool which had been gel filtered in acetic acid [11]. Titration curves were first established with ^{125}I-labelled IGF-I and -II in order to determine the concentration of BP yielding 20–25% binding of the tracer. The affinities of the BPs for the IGFs were then compared in experiments using ^{125}I-labelled IGF-I and -II (~2000 cpm/tube) and increasing concentrations of IGF-I and IGF-II. Incubation time was 24 h. Charcoal was used for the separation step.

2.7. BP and IGF assays [8,11]

These assays all comprised a prior gel filtration step in 1 M acetic acid, 0.15 M NaCl, which separates the IGFs from their BPs. Binding activity measurements were done at six concentrations in the presence of ^{125}I-labelled IGF-II and estimated by comparison with a preparation of BPs extracted from a pool of normal serum. IGF-I was determined by RIA using an anti-Sm-C antiserum prepared by Dr J.J. Van Wyk (Chapel Hill, NC) and kindly provided by the Hormone Distribution Program, NIDKK. IGF-II was measured by protein-binding assay using BPs extracted from CSF.

3. RESULTS

Neutral pH gel filtration of CSF incubated with ^{125}I-labelled IGF-II yielded two peaks (fig.1), one with a K_d of 0.74 corresponding to free IGF, the other with a K_d of 0.40 corresponding to material with an apparent M_r of ~40 000. The latter was

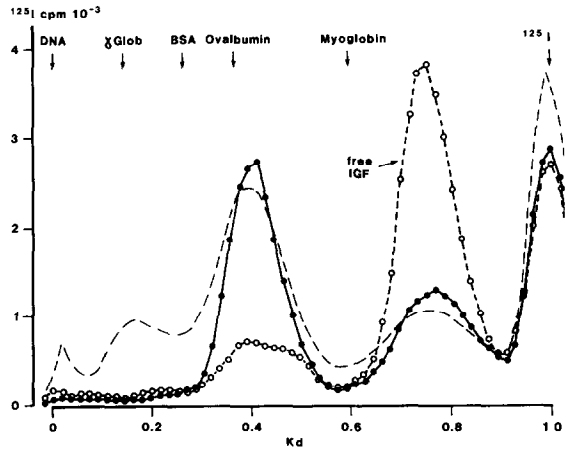

Fig.1. Gel filtration analysis of CSF IGF-binding proteins using Ultrogel AcA 44 at pH 7.4. 1 ml CSF was incubated with ^{125}I-labelled IGF-II (50 000 cpm) with (○---○) or without (●——●) excess IGF (crude preparation) and chromatographed in the presence of internal markers (see section 2). By way of comparison, the elution profiles of the serum IGF-BP complexes are included (0.5 ml normal serum incubated with ^{125}I-labelled IGF-II) (– – –).

displaced by excess IGF and therefore specifically bound to the tracer. The presence of BPs and IGFs in this material was demonstrated by assays carried out after acidic gel filtration (not shown). Neither BPs nor IGFs were however detected in the fractions with $K_d < 0.20$ which correspond to the elution zone of the ~150 kDa IGF-BP complex found in the serum (experiment done on a sample containing 15 ml CSF).

With chromatofocusing (fig.2), the CSF ^{125}I-labelled IGF-II-BP complexes eluted from the pH 7.4–4.0 gradient as a single broad peak at pH ~5.0. By comparison, the serum ~40 kDa IGF-BP complex yielded a major peak superimposable with that of the CSF BPs and a minor peak at pH ~6.0.

IGF and BP concentrations in CSF were determined in 10 subjects (table 1). Total protein concentration in these samples varied from 0.14 to 0.65 mg/ml (mean, 0.37 mg/ml). The total IGF concentration in CSF was 30-times lower than that determined for 12 samples of normal adult serum. IGF-II concentrations in CSF, however, were 15–20-times higher than those of IGF-I. Binding activity, determined using ^{125}I-labelled IGF-II, was approx. one-fifth of that of serum samples.

In the competitive binding studies (fig.3), the CSF BPs exhibited a strong preferential affinity for IGF II. With ^{125}I-labelled IGF-II as tracer, the

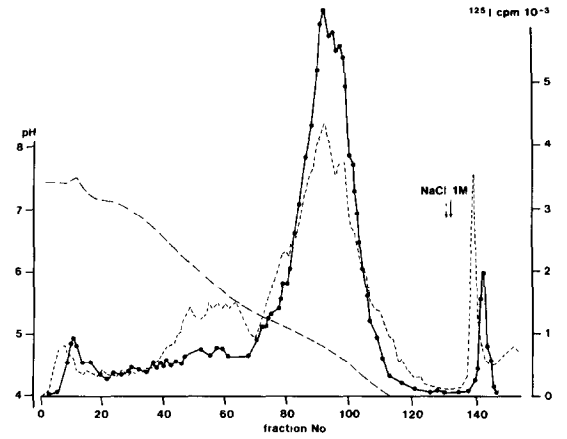

Fig.2. Chromatofocusing of CSF IGF-binding proteins. The elution profile was obtained after incubation of 12 ml CSF with ^{125}I-labelled IGF-II (●——●). By way of comparison, the elution profile is included of the serum ~40 kDa IGF-BP complex isolated by gel filtration at pH 7.4 (– – –).

Table 1

Levels of IGF-binding proteins and IGFs in the CSF and serum of normal adults (means ± SE)

	IGF BPs (mU/ml)	IGF-I (ng/ml)	IGF-II (ng/ml)	IGF-II/ IGF-I
CSF				
(n=10)	215±31	2.8± 0.2	48± 3.8	17 ±1.3
Serum				
(n=12)	967±43	279 ±11	1282±64	4.6±0.3

Samples were assayed after acidic gel filtration. IGF-I was measured by RIA and IGF-II by a specific protein-binding assay. IGF-BP levels were determined by comparing the binding to ^{125}I-labelled IGF-II of different concentrations of the CSF samples and a reference serum BP preparation assigned a value of 1 U IGF-BP per ml

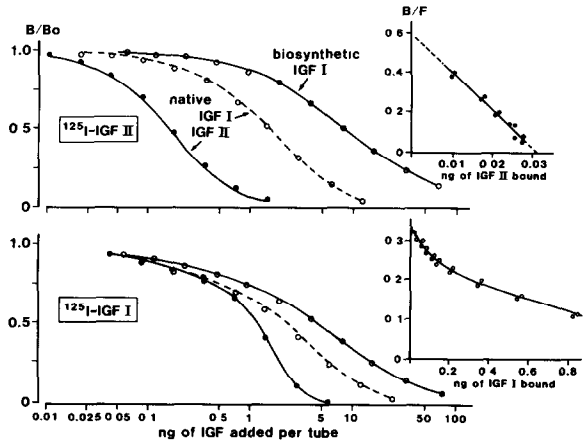

Fig.3. Competitive inhibition of the binding of ^{125}I-labelled IGF-II and ^{125}I-labelled IGF-I to CSF IGF-binding proteins by IGF-II and IGF-I. The quantities of BP were those yielding 25% binding of the tracer in the absence of cold IGF (B_0). These corresponded to 1 μl CSF for ^{125}I-labelled IGF-II and 20 μl for ^{125}I-labelled IGF-I. Insets: Scatchard plots of the data.

concentration of IGF-II required for half-maximal displacement was about 9-times lower than that of native IGF-I. At equal concentrations (determined by IGF-I RIA), the biosynthetic IGF-I was about 5-times less potent than native IGF-I in displacing ^{125}I-labelled IGF-II and 40-times less so than IGF-II. The specificity of binding was confirmed by the absence of cross-reaction with insulin (25 μg/ml). The affinity of the CSF BPs for IGF-II, calculated

from Scatchard analysis, was $4.2 \pm 1.4 \times 10^{10}$ M^{-1} and the binding capacity, $5.5 \pm 0.5 \times 10^{-9}$ M, i.e. 41 ng IGF-II bound per ml CSF (mean ± SD of 6 determinations).

With ^{125}I-labelled IGF-I as tracer, IGF-II remained more potent than IGF-I, but the margin was smaller than that with ^{125}I-labelled IGF-II. Scatchard plots of the data curved upwards, suggesting independent classes of binding sites.

Western blot analysis: in CSF, there was a predominant BP which corresponded to the 34 kDa BP of serum (fig.4). The other serum forms (41.5, 38.5, 3O and 24 kDa) were also present in CSF but they were far less abundant than the 34 kDa form. The 30 kDa BP appeared less distinct in CSF than in hypopituitary serum or certain other biological media (amniotic fluid, liver culture medium; unpublished). Very similar migration profiles were obtained from 10 different CSF samples, each showing the preponderance of the 34 kDa form (fig.5).

By way of comparison, fig.4 also shows the migration profiles of CSF BPs from a foetus and from the culture media of foetal and adult human nervous tissue. In the case of the CSF and the culture media from foetus no.1, the 34 kDa and 24

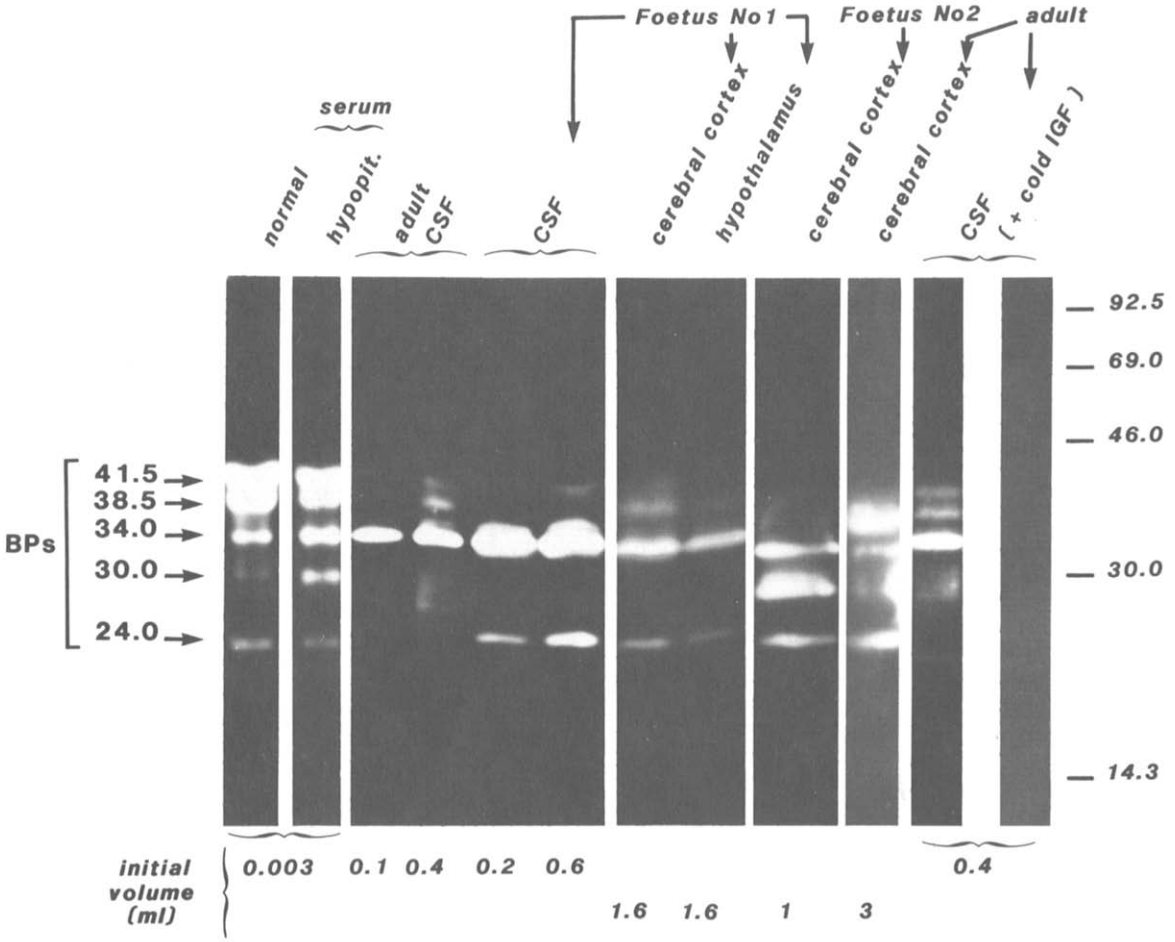

Fig.4. Western blot analysis of IGF-binding proteins present in CSF and serum and in one adult and two foetal brain culture media. The binding proteins were identified after incubation of the nitrocellulose with ^{125}I-labelled IGF-II. Binding specificity was demonstrated by incubation with excess IGF of part of the nitrocellulose containing the transferred BPs. The positions of the protein markers (run in the presence of β-mercaptoethanol) are shown on the right. The numbers indicate their $M_r \times 10^{-3}$ as well as those of the BPs (arrows).

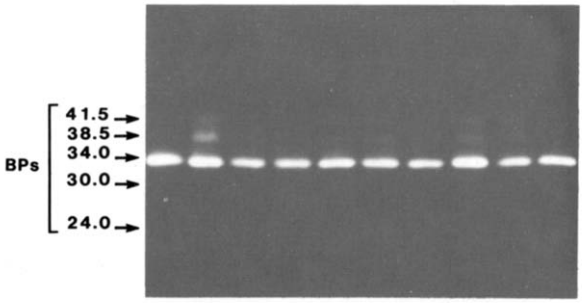

Fig.5. Western blot analysis of CSF IGF-binding proteins from 10 different subjects (see section 3 and table 1). 130 μl CSF was placed in each slot. At this concentration, the 30 and 24 kDa forms were barely detectable.

kDa forms were predominant, whereas in the culture media from foetus no.2, the 34, 30 and 24 kDa forms dominated. In the adult cerebral cortex, the 38.5, 34 and 24 kDa forms were predominant.

The chromatofocused CSF material was also analysed electrophoretically. The 34 kDa form was present in all fractions of the peak, whereas the 41.5, 38.5 and 30 kDa forms appeared in the second half of the peak at pH 4.5 (not shown).

4. DISCUSSION

It is well known that in biological fluids the IGFs are associated with high-affinity binding proteins [12–14]. Both IGFs and their BPs are present in CSF at concentrations considerably lower than those in serum. Nevertheless, the relative concentrations of IGFs and BPs as compared with the total protein concentration in CSF were high, being about 10- and 60-times higher, respectively, than those in serum. Moreover, the preponderance of IGF-II in CSF is expressed by an IGF-II/IGF-I ratio 4-times that in serum.

Serum IGF-BP complexes elute from neutral pH gel filtration as two peaks, one, which appears to be under the control of GH, with an apparent M_r of ~150000 ('large' complex) and the other with an apparent M_r of ~40000 ('small' complex) [12–14]. The CSF IGF-BP complexes eluted as a homogeneous peak with an apparent M_r similar to that of the small complex, but no material was found in the zone corresponding to the large complex. With chromatofocusing, the CSF complexes gave a profile which was partially superimposable with that of the serum small complex.

SDS-PAGE analysis of the CSF BPs followed by transfer onto nitrocellulose revealed the same molecular forms as those found in serum, but in very different proportions. The 41.5 and 38.5 kDa forms appeared in minute quantities as compared with normal serum where they are concentrated in the large complex [15]. The 34 kDa form, which is minor in normal serum, was predominant in CSF. The 30 kDa form was also more abundant in CSF than in serum. Interestingly, the 34 and 30 kDa forms are increased in hypopituitary serum, which shows that they are not dependent on GH. In nervous tissue culture media, the 34 kDa form was abundant, as was the 30 kDa form in one of the foetuses.

In the Western blot technique we adapted to the investigation of IGF-binding proteins, it is necessary that SDS-PAGE be performed in the absence of reducing agents which prevent binding between labelled IGF and BPs transferred onto nitrocellulose [9]. In a previous study involving cross-linking of the IGF-BP complexes and SDS-PAGE with or without reducing agent, we saw only a wide major band at 40 kDa and a minor band at 46 kDa [16]. We can therefore infer that the 34 kDa form, which is the major CSF BP revealed by Western blotting, is not a subunit of a higher molecular mass binding protein.

The particular affinity of the CSF BPs for IGF-II has been the basis of their use in a specific assay for IGF-II [11]. Although the ability of native IGF-I to displace ^{125}I-labelled IGF-II was 9-times lower than that of IGF-II, it was 5-times higher than that of biosynthetic IGF-I. This suggests either the presence of some contaminating IGF-II in the IGF-I prepared from human serum, or small structural differences between the native and biosynthetic forms of IGF-I. The selective affinity of the CSF BPs for IGF-II can be attributed essentially to the 34 kDa form in view of its abundance and since experiments done on the material eluting at the beginning of the chromatofocusing peak, which contains only the 34 kDa form, yield results similar to those obtained with native CSF BPs (not shown). The other forms of BP analysed in serum and in liver culture media also have distinct affinities for IGF-I and IGF-II [15].

The fact that brain explants release into the

culture medium IGF-binding proteins similar to those in CSF, with a large proportion of the 34 kDa form, strongly suggests that the CSF BPs arise from the nervous system.

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