PRODUCTION OF INSULIN-LIKE GROWTH FACTORS AND THEIR CARRIER BY RAT PITUITARY GLAND AND BRAIN EXPLANTS IN CULTURE

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1. Introduction

Cartilage growth-promoting substances are known either as somatomedins (SM), which reflects their dependance on GH [1], or as insulin-like growth factors (IGF), because of the structural homologies with insulin of IGF I and II which are, to date, the only two SM to have been totally identified [2,3]. SM or IGF have been shown to circulate in the blood transported by a specific carrier protein [4,5]. Following the pioneering work of McConaghey [6], the liver has been considered as the primary source of these growth factors. Using an organ culture technique, we have shown that both the IGF and their carrier(s) are produced by the liver [7–9] (reviews [9–11]).

In view of the increasing number of gastrointestinal and pancreatic peptides that are also found in the brain [12–15] and the fact that certain growth promoting factors (such as fibroblast growth factor) have been extracted from the pituitary gland and/or brain [16–19], we have considered it interesting to apply our organ culture technique to these tissues and determine whether they produce IGF. Here, we report the first evidence that in the rat the pituitary gland and various brain tissues (hypothalamus, cerebral cortex, cerebellum) maintained in organ culture, produce IGF and their carrier protein(s).

2. Materials and methods

2.1 *Organ culture*

The technique used was the same as that employed in [7] for liver culture. Male Sprague Dawley rats (100–120 g) were decapitated and the organs quickly excised, sliced and pooled before distribution into culture dishes containing 2 ml McCoy's 5a medium (Eurobio) without serum. The quantities of tissue/dish were the following: 4 hemi-anterior pituitary lobes; 2 neuro-intermediate lobes; 4 hemi-hypothalamus; 8 cerebral cortex or cerebellum explants (15–25 mg each). For each tissue, 6 dishes were routinely prepared/experimental condition. After 24 h culture, the medium was discarded and replaced, then the culture continued a further 3 days. The media from each set of experimental conditions were then pooled and centrifuged (10 min, 3000 × *g*). An aliquot of the supernatant was put aside for the total protein assay [20]. The remainder was filtered on disposable Sephadex G-25 columns (Pharmacia) in 0.03 M NH_4HCO_3, then the void volume material was lyophilized as in [7] and used for subsequent studies (sections 2.2 and 2.5).

2.2. *Extraction of IGF and their carrier from the culture media*

Lyophilized material corresponding to 5–10 ml culture medium was gel filtered on 1.5 × 30 cm Ultrogel AcA54 (IBF) columns in 1 M CH_3COOH, 0.15 M NaCl. The fractions containing the IGF and the carrier were pooled separately, lyophilized and, before assaying, filtered on Sephadex G-25 disposable columns equilibrated with 0.1 M phosphate buffer. The material used for the assays was that eluted in the void volume (sections 2.3, 2.4).

2.3. *Competitive protein-binding assay for IGF*

Here the carrier protein extracted from rat liver culture media was used, which has an elective affinity for SM or IGF (no cross-reaction with other polypeptides including insulin and proinsulin) [9,21]. The ligand was NSILA-S (non-suppressible insulin-like

activity soluble, a gift from Dr Zapf, Zurich). The partially purified preparation used as standard (4.5 mU/mg insulin-like activity) was an approximately equimolar mixture of the 2 IGF. The preparation used as tracer contained mainly IGF I.

The total incubation volume was 0.4 ml 0.1 M phosphate buffer, each assay tube containing ~3000 cpm ^{125}I-labelled NSILA-S and sufficient carrier (corresponding to 0.1–0.2 ml original liver culture medium) to obtain ~25% binding in the absence of unlabelled NSILA-S (B_0 samples). Each sample was assayed in triplicate + 1 control (no carrier). After 24 h incubation, 'free' and 'bound' IGF were separated with charcoal. Results are expressed in serum units of IGF compared with a pool of normal adult male rat serum arbitrarily assigned a value of 1 serum unit IGF/ml.

2.4. *Radioligand assay for the IGF carrier protein*

With this assay, the relative IGF carrier concentration in an unknown sample was assessed in terms of its binding at different concentrations to ^{125}I-labelled NSILA-S [22]. The reference curve was obtained with a carrier preparation extracted from a pool of normal adult male rat serum arbitrarily assigned a value of 1 serum unit carrier/ml. The incubation and separation were identical to those used for the competitive protein-binding assay (section 2.3). Results are expressed in serum units of carrier.

2.5. *Gel filtration studies of the [IGF–carrier] complex*

Lyophilized samples (section 2.1) were incubated with ^{125}I-labelled NSILA-S in TNE buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF (phenylmethylsulphonylfluoride, Serva)) then chromatographed at 4°C on Ultrogel AcA44 equilibrated with the same buffer, using a 1.5 × 90 cm column pumped at a flow rate of 11 ml/h. The calibration curve was established with various proteins (Sigma) (see fig.4). For each run, SV 40 virus [^{3}H]DNA (100 μCi/mg, gift from J. L. Mandel and K. Dott, Strasbourg) and ^{125}I were used to determine V_0 (void volume) and V_t (total volume accessible to the solvent), respectively.

3. Results and discussion

IGF-like substances were found to be present in the culture media of all the rat brain tissues studied,

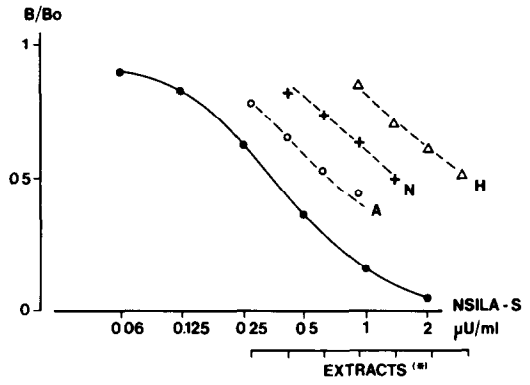

Fig.1. Competition curves obtained with IGF-like substances extracted from rat pituitary gland and hypothalamus culture media in the competitive protein-binding assay for IGF (A, anterior lobe; N, neuro-intermediate lobe; H, hypothalamus). The binding protein employed for the assay was the specific IGF-carrier protein produced by rat liver in culture [9,23]. The ligand used as tracer and standard was a purified NSILA-S preparation (see section 2). The values along the abscissa represent units of insulin-like activity.

*The curves for the IGF-like substances extracted from the culture media have been drawn in such as way as to facilitate comparison with the slope of the reference curve. They are therefore to be read along the abscissa on a scale with intervals corresponding to a dilution factor of 1.5. The first point on the curve corresponds to 0.12 ml original culture medium for A; 0.10 ml for N; and 0.14 ml for H

i.e., the anterior and neurointermediate lobes of the pituitary gland, the hypothalamus, cerebral cortex and cerebellum (fig.1, table 1). Gel filtration of these media in CH_3COOH under the conditions used to extract IGF from serum [22] yielded, in the fractions in which NSILA-S elutes, substances competing with ^{125}I-labelled NSILA-S in its binding to the IGF-specific liver carrier protein. These substances have displacement curves parallel to that of NSILA-S (fig.1).

IGF carriers were also present in these culture media:

(i) Treatment of these media under the identical conditions used to extract the carrier from liver culture media (fig.2 legend and [22]) yielded a substance with a strong affinity for NSILA-S, similar to that of the liver carrier protein, and with binding sites saturable by quantities as small as ~2.0 μU/ml NSILA-S (fig.2). In addition, in these competitive binding experiments, neither insulin nor proinsulin displaced the ^{125}I-labelled NSILA-S despite the fact that they have nearly

Table 1
IGF, IGF carrier and protein concentrations in the culture media of rat pituitary gland and various brain tissues

Tissue (mg/ml culture medium)	IGF (mU/ml) (serum units)	Carrier (mU/ml) (serum units)	Proteins (mg/ml)	IGF/ proteins (mU/mg)	Carrier/ proteins (mU/mg)	release/tissue weight	
						IGF (mU/mg)	Carrier (mU/mg)
Anterior lobe of pituitary	*0.7, 1.7, 5.2, 3.6, 4.6 2.8, –, 5.8, –, 2.2*	*13, 20, 18, 20, 11, 33, 10, 22, 17, 21*					
4.29	3.20 ± 0.63	18.5 ± 2.1	0.151 ± 0.008	21 ± 3.8	128 ± 14	0.77 ± 0.15	4.54 ± 0.44
Neuro-intermediate lobe	*1.0, 1.2, 5.6, –, 3.2 1.1, –, –, –, 2.2,*	*2.0, 4.0, 8.0, 6.6, 6.0 5.0, 6.0, 2.7, 4.4, 5.2*					
1.08	2.38 ± 0.73	4.99 ± 0.57	0.013 ± 0.002	162 ± 33	378 ± 55	2.21 ± 0.68	4.62 ± 0.53
Hypothalamus	*1.0, 1.9, 2.9, 3.0*	*1.3, 4.4, 4.5, 5.0*					
18	*4.7, 1.0, 1.6*	*2.5, 1.3, 3.0*					
(16–20)	2.30 ± 0.50	3.14 ± 0.58	0.070 ± 0.006	37 ± 9	53 ± 14	0.15 ± 0.04	0.21 ± 0.06
Cerebral cortex							
90							
(80–100)	*1.3, 0.4*	*3.5, 0.7*	0.203 ± 0.058	3.70	8.60	0.01	0.02
Cerebellum							
70	*0.8, 2.9, 1.3, –,*	*1.7, 7.7, 3.5, 2.0*					
(60–80)	1.67	3.73	0.312 ± 0.050	6.90	14	0.02	0.05
Serum	1000 (arbitrary reference values)	1000	~70	~14	~14		

The results are those for 10 expt in which the pituitary gland anterior and neuro-intermediate lobes were cultured simultaneously, with or without the brain tissues. They represent the cumulative concentrations of the substances released over the final 3 days of culture (see section 2). All three parameters were measured in all samples, although in some cases the IGF were non-detectable (–) owing to insufficient volumes of original culture medium. Results for IGF and carrier are expressed in serum units (see section 2). The values in italics are individual results, the others the mean ± 1 SEM for all expt

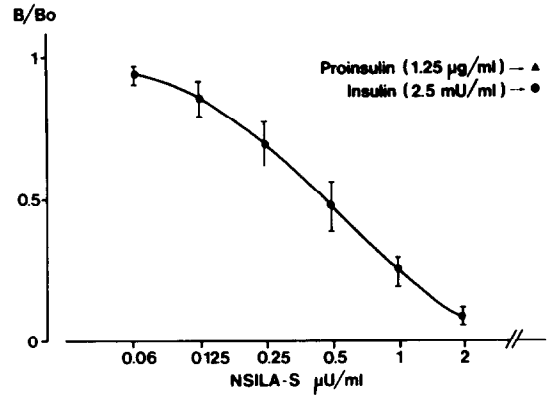

Fig.2. Competitive binding of ^{125}I-labelled NSILA-S to IGF carrier proteins extracted from rat adenohypophysis culture media. The IGF carrier preparations were obtained by Sephadex G-50 gel filtration in 1 M CH_3COOH of culture media desalted on Sephadex G-25 and lyophilized. The void volume material was collected and stored lyophilized. The volume for each test tube corresponded to 0.2 ml original culture medium and a total protein concentration of ~10 µg/ml. Incubation with NSILA-S and separation were done as for the IGF competitive binding assay (see section 2). The results are the mean ±1 SEM of 3 expt done with 3 different preparations of carrier in which the proportions of bound ^{125}I-labelled NSILA-S in each B_0 sample were 23, 38 and 29%, respectively. The values on the abscissa are expressed in units of insulin-like activity.

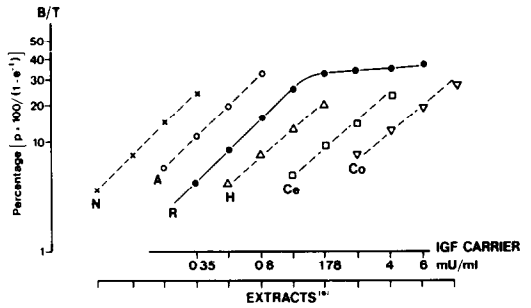

Fig.3. Titration curves of IGF carrier proteins extracted from rat pituitary gland and various brain tissue culture media (A, anterior lobe; N, neuro-intermediate lobe; H, hypothalamus; CO, cerebral cortex; CE, cerebellum). Increasing concentrations of the various carriers were studied in the presence of ^{125}I-labelled NSILA-S under the same conditions as those for the radioligand assay for the IGF carrier proteins (see section 2). The reference curve (R) was obtained with a preparation of carriers extracted from a pool of rat serum. The abscissa carrier values are expressed in serum units of carrier (see section 2).

*The curves for the carriers extracted from the culture media (of which only the linear portion is shown) have been drawn in such as way as to facilitate comparison with the slope of the reference curve. They are to be read along the abscissa on a scale with intervals corresponding to a dilution factor of 1.5. The first point on the curve corresponds to 0.04 ml original culture medium for A; 0.19 ml for N; 0.42 ml for H; 0.23 ml for CO; and 0.28 ml for CE.

50% homologies with IGF [3]. This suggests that the binding material was specific for the IGF.

(ii) Fig.3 shows the titration curves of the IGF carrier produced by the pituitary gland and other brain tissues, compared with the reference curve obtained with the serum carrier. It should be noted that for any given experiment these titration curves were not always parallel to the reference curve (not shown). This is in sharp contrast with what we observed for the liver carrier protein (in preparation) and suggests possible structural differences of molecular heterogeneity.

(iii) Ultrogel AcA44 chromatography at neutral pH of the material concentrated from the culture media and incubated with ^{125}I-labelled NSILA-S gave two peaks (fig.4). The first, which corresponds to the [IGF–carrier] complex, as demonstrated by experiments using unlabelled NSILA-S (in preparation), was asymmetrical with K_d ~0.21–0.52 and a mean K_d extrapolated to 0.39 ± 0.03 (SD) (n = 10). The second peak with K_d ~ 0.70 corresponds to free IGF. Prior treatment of the samples with 1 M CH_3COOH had no effect on the elution profile (not shown). The heterogeneity of the [IGF–carrier] complex, as suggested by the chromatographic profile, was confirmed by further experiments involving rechromatography and sedimentation of the complex (in preparation).

By way of comparison, fig.4 also shows the elution pattern of a liver culture medium chromatographed under the same conditions after incubation with ^{125}I-labelled NSILA-S. Here the [IGF–carrier] complex also eluted with a mean K_d 0.39 ± 0.01 (n = 9). The peak was however symmetrical and narrow: K_d ~0.32–0.52 (here the minor peak at K_d 0.26 corresponds to molecules non-specifically bound to ^{125}I-labelled NSILA-S) (in preparation). The app. M_r of ~40 000 and r_S of ~28 Å deduced from the K_d of 0.39 and attributed to the liver [IGF–carrier] complex ([9], in preparation) are merely given here as an indication for the pituitary gland and brain [IGF–

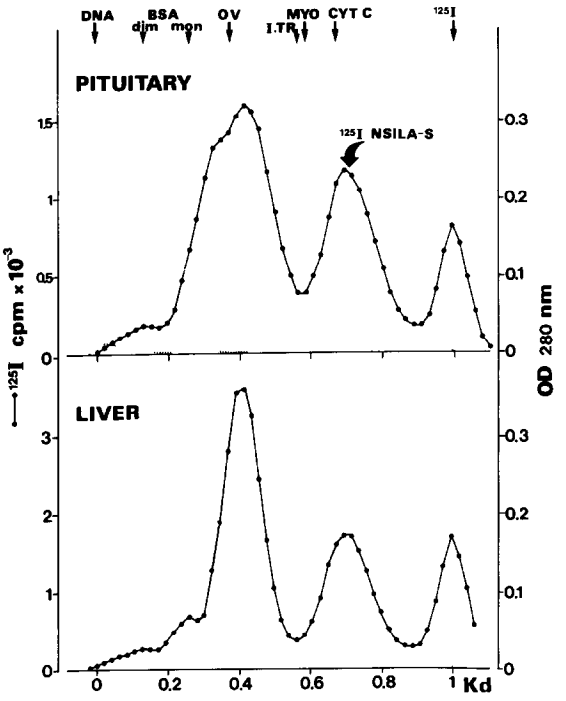

Fig.4. Gel filtration study of pituitary gland and liver IGF carriers. Lyophilized samples corresponding to either 10 ml rat pituitary gland culture medium (0.55 mg protein) or 10 ml rat liver culture medium (1.2 mg protein) were dissolved in TNE buffer and incubated overnight with ^{125}I-labelled NSILA-S at 4°C, mixed with SV 40 [^{3}H]DNA and [^{14}C]BSA as internal markers and chromatographed at pH 7.4 on AcA44 Ultrogel (see section 2). Proteins were monitored by A_{280} and radioactivity measured in a γ and a β counter. Arrows indicate the elution position of the markers: SV 40 [^{3}H]DNA (3.3×10^{6}); unlabelled or [^{14}C]BSA dimer (134 000); and monomer (67 000); ovalbumin (43 000); soybean trypsin inhibitor (20 100); horse myoglobin (17 200); and cytochrome *c* (11 700). Profiles similar to those for the adenohypophysis where obtained with the culture media of the neuro-intermediate lobe, the hypothalamus, cerebral cortex and cerebellum.

carrier] complexes in view of their molecular heterogeneity.

Table 1 shows the different concentrations of IGF and of their carrier in the various culture media.

Whether these are expressed in terms of the total protein concentration or the amount of tissue cultured, the anterior and neuro-intermediate pituitary lobes were found to have released the largest quantities of both IGF and carrier and the cerebral cortex and cerebellum, the least. Under the same experimental conditions, the liver production/mg tissue was similar to that of the two pituitary lobes (in preparation). If:

(i) The variations in production from one tissue to another;

Table 2
Effects of cycloheximide

Culture medium	Cyclo-heximide (μg/ml)	^{3}H-Labelled trichloroacetic acid-precipitable material (dpm/ml)	(% Inhib.)	IGF mU/ml (serum units)	% Inhibition	Carrier mU/ml (serum units)	% Inhibition
Anterior lobe	0	815 740		2.77		33	
of pituitary	100	3140	>99	1.17	58	1.19	94
Neurointer-	0	36 340		1.14		5	
mediate lobe	100	300	>99	1.05	8	1.85	63
Hypothalamus	0	5280		0.99		2.5	
	100	115	98	<0.44	>56	2.15	14
Cerebral cortex	0	8320		1.29		3.5	
	100	835	90	0.64	50	2.42	31
Cerebellum	0	4690		1.32		3.5	
	100	990	80	0.87	34	2.8	20

Rat pituitary gland and brain explants were cultured in 12 dishes for each organ, half with and half without cycloheximide (Sigma). After 24 h the culture medium was replaced by fresh medium with 4 μCi/ml [^{3}H]leucine (110 Ci/mmol NEN) with or without cycloheximide. The culture was continued a further 3 days and the media for each set of experimental conditions then collected and pooled separately. [^{3}H]Leucine incorporation into trichloroacetic acid-precipitable material [7] was determined in 100 μl aliquots of culture medium. Values are the mean of 5 measurements corrected by subtraction of the control value (culture medium with [^{3}H]leucine, without explants) of 450 dpm/ml. IGF and carrier were assayed after extraction and their concentrations expressed in serum units (see section 2). Similar results were obtained in a second experiment

(ii) The predominant production of carrier in all tissues; and
(iii) The relative concentrations of IGF or carrier/total amount of protein as compared with those in serum, are taken into account, any notion of a simple mechanical release of material formerly bound to the tissues into the culture medium would seem highly unlikely. Moreover, this possibility would be minimized by the change of medium after 24 h culture.

Table 2 shows the effects of cycloheximide added to the culture medium. It appears that, under the influence of the drug:

(i) The incorporation of [^{3}H]leucine into the proteins released in the media was strongly inhibited in all the tissues;
(ii) The IGF and/or carrier concentrations in the culture media decreased in every tissue cultured (the strongest inhibition occuring in the adenohypophysis culture).

The results suggest a synthesis of IGF and carrier in the pituitary gland and the brain.

4. Concluding remarks

The anterior and neuro-intermediate lobes of the pituitary gland, the hypothalamus, cerebral cortex and cerebellum of the rat, maintained in organ culture, release both IGF and their carrier protein. Production is highest in the pituitary lobes and lowest in the cerebral cortex and cerebellum, although it is possible that the latter was due to inadequate culture conditions for these tissues.

The nature of the brain IGF remains unknown as the rat liver carrier protein used to identify them recognizes IGF I, IGF II, SM-A and MSA [21]. The brain tissue IGF carriers appear to be different from that produced by the liver and the relationships between these various carriers and that present in the serum remain to be clarified.

The control of the synthesis of the brain IGF and their function have yet to be determined. It is unlikely that they play a physiological role in somatic growth (computations from our results suggest that the total production by the brain would correspond to <5% of that by the liver 'in vitro'). Nevertheless, it can be imagined that the IGF synthesized by the brain and pituitary gland have a local role, as suggested by the detection of somatomedin receptors in the brain indicating a possible action for these peptides as neuroregulators [23] and brain growth factors [24,25].

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