Isolation of heparin-binding growth factors from dogfish (*Mustela canis*) brain and retina

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We have purified two growth factors from dogfish brain and retina by using their binding affinity for heparin-Sepharose. These growth factors were eluted at 1 and 2 M NaCl similarly to those purified from bovine brain or retina. Their mitogenic activity was assayed in vitro on the same mammalian cells: bovine lens epithelial cells or human fibroblasts. All these data seem to indicate that these growth factors belong to the families of other well defined mammalian growth factors: EDGF I, brain basic FGF, AGF II, on the one hand and EDGF II, brain acidic FGF, AGF I, ECGF, on the other. Thus, these growth factors have been widely distributed during evolution and retain at least a conservative sequence to stimulate cell proliferation, in mammalian cells.

(Brain, Retina) *Growth factor* *Heparin binding* *Evolution*

1. INTRODUCTION

Our investigations on the role of retina in the control of lens growth and development have demonstrated that adult bovine retina contains a growth factor which can stimulate the proliferation of lens epithelial cells in culture [1]. This growth factor was further purified and its presence in various ocular tissues as well as its activity in vitro and in vivo [2–5] on many cell types prompted us to name it EDGF (eye derived growth factor) and to compare it to other growth factors extracted from the brain or retina [6,7]. Recently a major technological development, the use of heparin-Sepharose chromatography, has allowed several groups including our own to purify two families of growth factors to homogeneity [8–13] from bovine or human brain and also from the retina [14,15]. The strong similarity between brain and retina growth factors can be deduced from these works and from recent data by either direct microsequencing [6] or immunological techniques [16]. The complete sequence of bovine acidic FGF has just been published [17]. While most of these studies have been performed on human or bovine tissue, we [2], as well as others, have reported that these growth-stimulating activities may be observed with extracts from chick, mouse or rat tissues. These growth factors also seem to be present in other tissues [18]. According to their affinity for heparin we have named them EDGF I and II corresponding respectively to basic and acidic FGF (the latter being also termed ECGF). The interesting observation is that these growth factors have a very broad spectrum of activity easi-

Part of this work has been performed at the Woods Hole Marine Biological Laboratory in Dr Max Burger's Laboratory and has been reported in abstract form in the Biological Bulletin, 165 (1985)

Abbreviations: EDGF, eye derived growth factor; BDGF, brain derived growth factor; ECGF, endothelial cell growth factor; FGF, fibroblast growth factor; BEL cells, bovine epithelial lens cells; AGF I and II, astroglial growth factor; PBS, phosphate-buffered saline

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ly crossing the barrier species [2]. For instance, EDGF from bovine retina stimulates the proliferation of chick myoblast, rabbit chondrocytes, rat neuroblastoma cells or human endothelial vascular cells.

The origin of these growth factors has not yet been clearly defined; however they are found in adult retina which is mostly post-mitotic after birth with the exception of the capillary cells of the microvessels. It was thus of interest to investigate the possibility that these growth factors are also present in the retina and brain of dogfish which are distant, in evolutionary terms, from mammals or birds. In addition, fish neuronal cells of the retina divide constantly during their life span [19,20]. We report here that dogfish brain and retina contain factors which exhibit EDGF-like activities.

2. MATERIALS AND METHODS

Dogfish (*Mustela canis*) brain and retina were purchased at the Woods Hole Marine Biological Laboratories supply department. Preliminary experiments were also performed on dogfish (*Scyliorhinus caniculus*) provided at the CNRS Roscoff Marine Station (France). The animals (50–100 cm long) were killed by decapitation and their eyes or brain removed and kept on ice until treated for growth factor extraction, within 1 h after death. Twenty fresh retina or retinal pigmented epithelium were pooled for each preparation.

The tissues were then homogenized in PBS and treated by the same techniques used to prepare EDGF or BDGF and described extensively in [21]. After centrifugation at $10000 \times g$ and $100000 \times g$, the crude extracts were dialysed against 0.1 M acetic acid, a first important step in the purification procedure. This acid extraction was named the 0.1 M purification step. These fractions were then loaded on heparin-Sepharose CL 6B (0.5 g per 10 retinas) at 0.6 M NaCl in 0.01 M PBS, pH 7.2, and eluted in 1 and 2 M NaCl. The activity of the different fractions was determined by measuring [^{3}H]thymidine incorporation into BEL cells (10th passage) or in human fibroblast AG 1523 NIA cells (8th passage) as described [21].

Bovine FGF was prepared in our laboratory according to [14].

Protein concentration was determined as described by Bradford [22] using bovine serum albumin as a standard. 20% polyacrylamide-SDS gel electrophoresis was performed according to Laemmli [23], staining being performed with silver nitrate.

3. RESULTS

Dogfish retinal crude extracts were assayed for their activity on BEL cells and human fibroblasts.

As can be seen in table 1 both cell types were stimulated to incorporate [^{3}H]thymidine in a dose-dependent fashion. The maximum incorporation was lower with the highest amount of extracts than with bovine EDGF 0.1 M extract probably due to the presence of an inhibitor. The retinal pigmented epithelium layer did not seem to contain any significant activity (not shown). We then compared [^{3}H]thymidine incorporation into BEL cells induced by the acidic extracts of brain and retina. As expected, both preparations were active in a dose-dependent fashion and the maximum activity was obtained with 6 μg per ml culture medium. Heat denaturation was sufficient to destroy most of the activity (table 2).

We thus loaded both preparations on heparin-Sepharose column at 0.6 M NaCl in PBS. After extensive washing, two fractions were eluted by 1 and 2 M NaCl respectively, and the activity assayed for

Table 1

Effects of various concentrations of retinal extracts on [^{3}H]thymidine incorporation into DNA

Dogfish retina	Protein concentration ($\mu g \cdot ml^{-1}$)	cpm in	
		BEL cells	Fibroblasts
Crude extract	180	8200	5800
	18	3000	1700
	1.8	300	200
Bovine EDGF, 0.1 M acetic acid extract	5	13200	13500

Means of triplicate experiments. The background incorporation (1300 cpm for BEL cells and 500 cpm for fibroblasts) has been subtracted

Table 2

Comparison of various growth factors extracted from dogfish brain and retina on [^{3}H]thymidine incorporation – acetic acid purification step

Dogfish retina	$\mu g \cdot ml^{-1}$	cpm
0.1 M	6	145000
	3	105000
	0.3	35000
0.1 M, treated at 100°C for 3 min	6	39000
Dogfish brain, 0.1 M	6	135000
Bovine EDGF, 0.1 M	0.5	65000

Means of triplicate experiments. The background incorporation (35000 cpm) has been subtracted

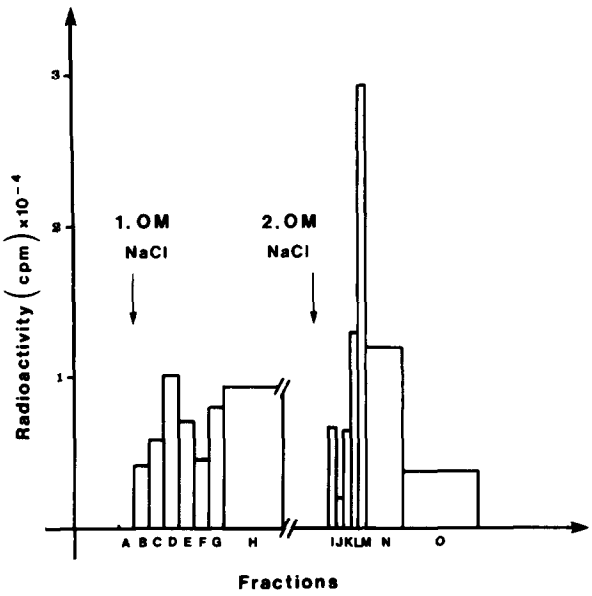

Fig.1. Purification of dogfish brain growth factor on heparin-Sepharose. Heparin-Sepharose chromatography of dogfish brain homogenate after acetic acid extraction. After loading the extract at 0.6 M NaCl at room temperature in PBS, 4 ml fractions were collected at 20 ml/min, at 1 and 2 M NaCl successively. 10 μl of each fraction was assayed for growth factor activity by [^{3}H]thymidine incorporation into BEL cells.

Fig.2. SDS-polyacrylamide gel electrophoresis of heparin-binding growth factors. Molecular mass markers (a,f), dogfish total retinal extract (g). Heparin-binding growth factors extracted from brain and eluted at 2 M NaCl (b), extracted from retina and eluted at 2 M NaCl (c), at 1 M NaCl (d), acidic bovine FGF (e).

each fraction. Two peaks of activity were detected. None was eluted at 3 M NaCl. The same results were obtained whether brain or retina extracts were used. The dose which gave rise to half of the maximum activity of these fractions was in the range 10 ng/ml, similar to what has been described for bovine retina [14]. When the crude extracts were used instead of the 0.1 M acidic extracts, some activity was not retained at 0.6 M NaCl. The different fractions were dialysed against ammonium formate and lyophilised. They were then separated by electrophoresis on SDS gel under reducing conditions. As shown in fig.2, dogfish brain and retina heparin-binding proteins stained by silver nitrate migrated in the same region as the bovine acidic FGF which has a molecular mass of 17.5 kDa. In the 2 M NaCl fraction eluted from the brain, the main band seems to migrate at a higher molecular weight mass, above 20 kDa, than the same preparation extracted from the retina. This latter fraction contains 3 bands. They may be either degradative products of native molecules as this has been shown for bovine bFGF [6] or other proteins with binding affinity for heparin but with no direct mitogenic activity. There may also be an overspill of the 2 M fraction on the 1 M fraction during the heparin chromatography purification step. These questions will be solved in further studies using HPLC chromatography and amino acid analysis but comparison of the activity of the various preparations described here shows that they have a similar specific activity to homogeneous preparations of bovine brain or retina FGFs.

4. DISCUSSION

Our results clearly indicate that two growth factor activities can be separated on heparin-Sepharose from dogfish brain and retina extracts. While only their sequences will enable us to compare their structures with those of the bovine or human brain and retina growth factors purified by the same technique, it is clear that there must have been great conservation in these families of growth factors during evolution since they can stimulate the same mammalian cells. These results extend our previous data on the relatively low species specificity of bovine EDGF, BDGF or FGF [2]. Recently we have shown that bovine EDGF II and III can induce in vitro newt iris transdifferentiation into lens cells (Cuny, R. and Courtois, Y., unpublished). Thus there is a good possibility that the same receptor site on the cells has been conserved. Recently FGF receptors have been isolated [24]. It will be of interest to look for the conservation of these receptors in various species.

Since the fish retina has a great capacity for continuous growth as well as regeneration [18,19], the presence of these growth factors in the fish may be extremely useful to understand their role in eye or brain development and physiology.

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