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Brain basic fibroblast growth factor stimulates the proliferation of rat neuronal precursor cells in vitro

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Neuronal cells from cerebral hemispheres of 13-day-old rat embryos were grown in a serum-free culture medium for 48 h, 4 or 8 days. The neuronal precursor cells proliferate for 5 days. The addition of bovine brain basic fibroblast growth factor stimulates their proliferation as determined by measurement of [^{125}I]-iododeoxyuridine incorporation and by autoradiographic analysis after [^{3}H]thymidine incorporation. The proliferating responsive cells were characterized as neurons by immunostaining against neurofilament proteins. Five other growth factors tested were without effect on the proliferative activity of these neuronal cells. The present results show that bFGF is mitogenic in vitro for rat neuronal precursor cells of the central nervous system.

Fibroblast growth factor; Proliferation; Stimulatory effect; (Rat neuronal cell)

1. INTRODUCTION

Little is known about factors which control the proliferation of neuronal precursor cells during brain development. We have previously shown that embryonic chick and rat brain neuroblasts in primary culture can proliferate [1–4]. A few other studies have also reported that neuroblasts from fetal rat brain show division in culture [5,6]. We then demonstrated that meningeal cells as well as meningeal and brain extracts stimulate the multiplication of these neuroblasts [1–4]. In addition, brain extracts also enhance the development of glial cells [7,8]. Recently, we purified to homogeneity from bovine brain two astroglial growth factors (AGF1, AGF2), which influence the proliferation and maturation of rat astroblasts in culture [9]. AGF1 and AGF2 are identical to the acidic and basic fibroblast growth factor (FGF) [9], respectively. Basic FGF (bFGF) also stimulates the proliferation of oligodendrocytes [10]. We now report that bFGF enhances the proliferation of neuronal precursors from embryonic rat brain. Since in rat brain this factor has been found to be localized essentially in neuronal cells [11], it may function as an autocrine growth factor for these cells during ontogenesis.

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2. MATERIALS AND METHODS

Primary cultures of neuronal cells were established from cerebral hemispheres of 13-day-old rat embryos as in [4]. Meningeal membranes were removed prior to culture. Cells were plated in poly(L-lysine)-precoated Falcon petri dishes (5 ± 10^5 cells per 35 mm dish) in a serum-containing medium, consisting of Dulbecco's modified Eagle's H16 medium (DMEM, Flow Laboratories) containing 20% fetal calf serum (FCS, Flow Laboratories). After 2 h, cultures were switched to a serum-free chemically defined medium: DMEM plus 5 μg/ml insulin (Sigma), 50 μg/ml transferrin (Sigma), 20 nM progesterone (Sigma), 100 μM putrescine (Sigma) and 30 nM

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sodium selenite (Merck). 4 h after culture initiation experimental cultures were treated for 24 h with bFGF, also called astroglial growth factor 2 (AGF2) or with other growth factors (EGF, epidermal growth factor from Sigma; PDGF, platelet-derived growth factor from Bioprocessing, UK; IGF-I, recombinant insulin-like growth factor I from Amgen, USA; IL-2, recombinant interleukin-2 from Transgene, Strasbourg; and thrombin provided by the Centre de Transfusion Sanguine, Strasbourg). Pure bFGF was prepared from bovine brain in our laboratory according to our second purification procedure [9].

Neurons were identified by immunostaining against neurofilament (NF) proteins [4,12]. Rabbit anti-NF antiserum was kindly made available by Dr J.F. Leterrier, Centre de Neurochimie, Strasbourg.

Cell proliferation was measured by 5-[^{125}I]iodo-2'-deoxyuridine (^{125}I-dUrd) incorporation. At 24 h, 0.25 μCi ^{125}I-dUrd (5 Ci/mg, Amersham) was added to the culture medium (2 ml) and cells were harvested 24 h later. Cultures were rinsed four times with 0.9% NaCl. The cells were scraped off with a rubber policeman and sedimented at 2000 × g for 10 min; the cell pellets were counted in an LKB 1260 gamma counter.

For autoradiographic analysis of neuronal cell proliferation cultures were exposed for 24 h to 2 ml nutrient medium containing 0.1 μCi [6-^{3}H]-thymidine ([^{3}H]dThd, 20–30 Ci/mmol; CEA, France) per ml starting 4 h after culture initiation unless otherwise indicated. The radioactive medium was then removed, and cultures were rinsed 5 times with DMEM and supplied with fresh nutrient medium. After 8 days cultures were immunostained with NF antiserum (1:100) [12], rinsed with distilled water, air-dried and submitted to autoradiography [13]. Ilford K5 photographic emulsion was used. The autoradiograms were exposed for 15 days in the dark at 4°C.

3. RESULTS AND DISCUSSION

The addition of bFGF to cultures derived from cerebral hemispheres of 13-day-old rat embryos stimulates the incorporation of ^{125}I-dUrd into the cultured cells (table 1). Results are similar for cultures started at various cell concentrations ranging from 3 to 7.5 × 10^5 cells per dish. The level of incorporation is 2-times higher in the treated cultures compared to controls. The dose-response curve for the stimulation by bFGF of the brain cell proliferation (fig.1) shows that maximal responses (about 95–98% stimulation) are observed at concentrations of 2–8 ng/ml culture medium. Among several purified growth factors only bFGF affects the proliferative activity of brain cells (table 1).

Table 1

Effect of various growth factors on neuronal cell proliferation

Treatment	Concentration	^{125}I-dUrd incorporation (% of control)
Control		100 ± 6
bFGF	5 ng/ml	200 ± 25
EGF	10 ng/ml	111 ± 11
PDGF	2 U/ml	101 ± 13
IGF-I	60 ng/ml	104 ± 7
IL-2	25 U/ml	113 ± 6
Thrombin	0.05 U/ml	107 ± 15

4 h after culture initiation cultures were treated with the various growth factors for 24 h. Values are means ± SD from 4 independent experiments

A combination of tritiated thymidine ([^{3}H]-dThd) autoradiography and immunoperoxidase technique for neurofilament (NF) proteins shows that neuronal cells are radioactive in control as well as in treated cultures (fig.2), indicating that neuronal precursor cells proliferate under our

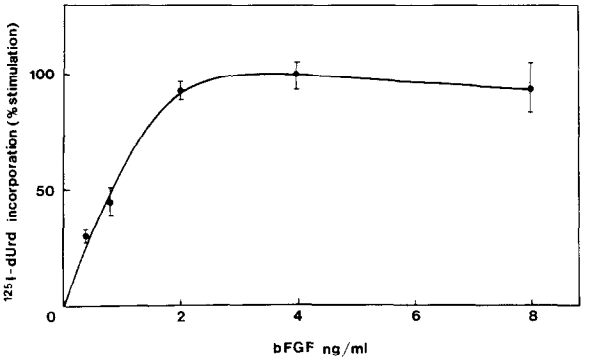

Fig.1. Stimulation of cell proliferation by varying concentrations of bFGF. 4 h after culture initiation cultures were treated for 24 h with the various amount of bFGF. Values are means ± SD from triplicate cultures.

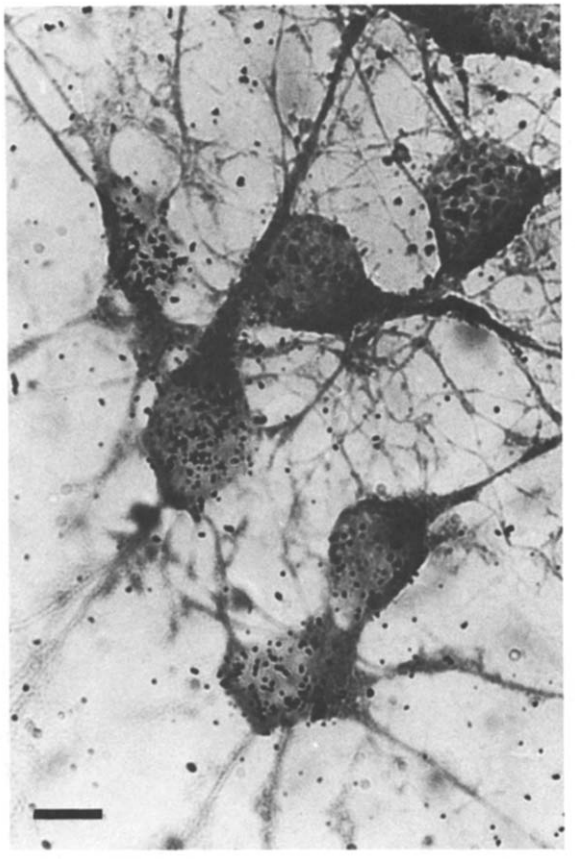

Fig.2. Combination of [^{3}H]thymidine autoradiography and immunoperoxidase staining with rabbit anti-NF antibody of neuronal cell cultures treated with bFGF (5 ng/ml). Bar: 10 μm.

culture conditions. During the first days of culture, with and without bFGF, only a few cells exhibiting astroglial morphologies and immunostained against glial fibrillary acidic protein (GFAP) were seen. Moreover, only some of these cells were radiolabelled when [^{3}H]dThd was added between 4 and 24 h or 24 and 48 h.

Between 3 and 6 days, under normal culture conditions, many round cells are associated into small clumps. Other cells are fusiform or of irregular shape bearing two or several processes and are dispersed between the aggregates (fig.3a). Most of these cells present a neuronal morphology and are positively immunostained for NF proteins (not shown). In the presence of bFGF the cellular clumps have increased in size compared to control cultures (fig.3b). Most cells and the dense fiber network contain NF proteins (not shown). The astroglial GFAP positive cells remain rare under this treatment, up to 4 days. At day 6 the number of GFAP positive cells represent less than 10% of the total population.

We found an amount of 8.5, 27 and 16.5% radiolabelled NF$^+$ neuronal cells to total NF$^+$ cells for control cultures exposed to [^{3}H]dThd from 4 to 24 h, 24 to 48 h and 4 to 5 days, respectively. In the preparations treated with radioactive thymidine between 7 and 8 days radioactivity could no longer be detected in neurons. In the presence of bFGF and [^{3}H]dThd from 4 to 24 h about 4-times more radioactive neuronal cells were observed (table 2). When cells were treated with bFGF and with the radioactive precursor after 24 h too many cells were present in large aggregates and it was impossible to determine the number of radiolabelled neurons.

The above results show that neuronal precursor cells from brain of 13-day-old rat embryos can proliferate for 5 days in our culture conditions and stop multiplying after 7 days. The highest proliferative activity was observed between 24 and 48 h. Moreover, the present report is the first evidence that bFGF, which is mitogenic for glial cells and for a great variety of other cell types, is also a mitogen for neuronal precursor cells of the central nervous system. The selectivity of these cells for bFGF should be pointed out since they do not respond to five other well characterized growth factors which are active on the proliferation of glial cells.

Since bFGF has been found to stimulate the development of astroglial [9] and oligodendroglial [10] cells it can be considered possible that the trophic effect observed in the present study may be mediated via glial cells. However, in our culture system most of the cells were found to express NF protein and few cells show GFAP, at least up to 4 days and even in the presence of bFGF. Moreover, determinations of 2′,3′-cyclic nucleotide 3′-phosphodiesterase activity performed on the cultures to check the eventual presence of oligodendroglial cells indicated that no activity was detectable before 2 weeks (not shown). Thus, the cultures appear virtually devoid of glial cells, especially during the first 48 h after culture initiation when most of the experiments have been performed. There-

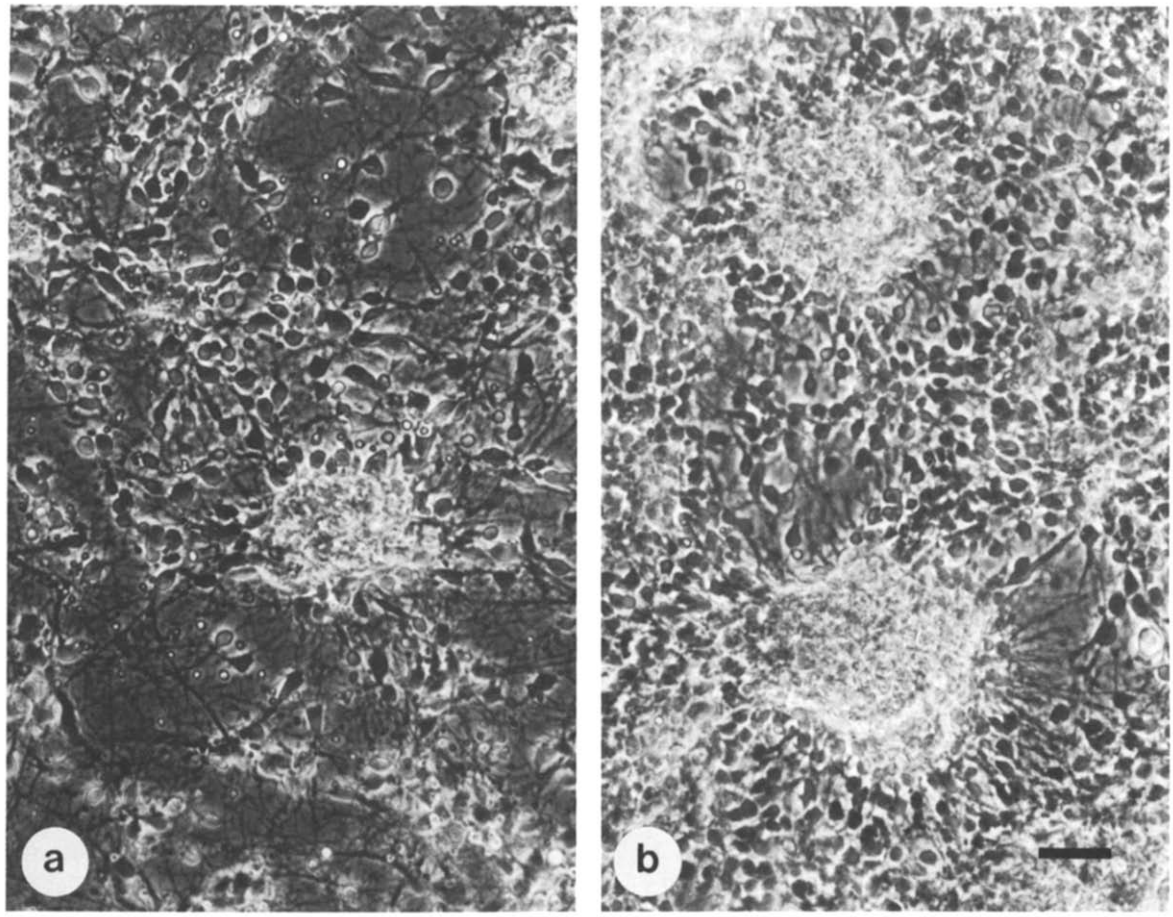

Fig.3. Phase-contrast micrographs of brain cells from 13-day-old rat embryos cultured in the absence (a) and presence (b) of bFGF (5 ng/ml). Cells were grown for 4 days. Bar: 50μm.

fore, the effect of bFGF on neuroblast proliferation is probably the result of a direct action.

Recently, it has been shown by us [14] and others [15,16] that bFGF is able to support neuronal survival and to promote neurite outgrowth. Since this factor has been found in rat brain to be localized essentially in neuronal cells [11], neurons may both secrete and respond to bFGF. Thus, bFGF may function as an autocrine factor for these cells.

Table 2

Stimulation of neuronal cell proliferation by bFGF

	% ^{3}H-labelled NF$^+$ neurons to total NF$^+$ neurons
Control	8.5 ± 2.5
bFGF (5 ng/ml)	31.5 ± 3.5

Approx. 1000 NF positive cells were counted in each case on autoradiograms. Results are means ± SE of several counts from 2 independent experiments

Further studies will be required to investigate whether neuronal cells release bFGF to which they can respond and whether this factor plays an important role in vivo on neuronal development during brain ontogenesis.

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