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Concentration-dependent modulation of basic fibroblast growth factor action on multiplication and locomotion of human teratocarcinoma cells

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A human teratoma cell line (Tera 2) was grown in serum-free medium, and the population multiplication was stimulated by the addition of 1–10 ng basic fibroblast growth factor (bFGF)/ml. The bFGF-effect was abrogated by the addition of protamin sulphate. When high concentrations of bFGF were added, a preferential effect on cell locomotion was observed. 100 ng bFGF/ml stimulated cell movement but only exerted a marginal effect on cell multiplication. These observed exogenous requirements for multiplication and locomotion were complemented by the expression of bFGF receptors. Scatchard analysis of binding data suggests the existence of a high-affinity and a low-affinity class of receptors.

Fibroblast growth factor; Cell multiplication; Cell motility

1. INTRODUCTION

The acidic and basic fibroblast growth factors were the first members of a class of highly mitogenic peptides with a strong affinity for heparin to be isolated. Since their discovery, six members of the larger family have been isolated and characterised from a wide variety of sources and exhibit pleiotropic effects on a broad range of mainly mesoderm- and neurectoderm-derived cell types [1,2]. They stimulate cell proliferation, migration and differentiation at a range of concentrations with maximal effects between 0.1 and 1 ng/ml, and almost uniquely show qualitative modulation of their biological functions with changes in concentration. For example in the eye, bFGF stimulates proliferation of lens epithelial cells at low concentrations but induces cell migration at higher doses [3].

The range of properties shown by the FGF family suggests that these might be important molecules in the processes of embryogenesis. The factors may modulate the rate of expansion of stem cell populations by affecting both differentiation (for example induction of collagen synthesis in chondrocytes [4], and differentiation of preadipocyte fibroblasts in to adipocytes [5]) and proliferation, e.g. by repressing the terminal differentiation of myoblasts into myotubes [6]. Additionally, they have been implicated as potential mediators of morphogenetic information in amphibian development [7,8] and as important factors in the modelling of tissues during organogenesis [9]. The embryonic distribution of mRNA for these species has been described recently [10].

Heparin-binding growth factors are reported to be synthesized in the ovary and testis [11,12], messenger RNAs are expressed in and proteins have been isolated from proliferating and differentiated populations of mouse and human teratocarcinoma cells (reviewed in [13]). In one instance it is known that the FGF-related molecules (ECDGF) are able to participate in a paracrine growth loop by stimulating the proliferation of the differentiated derivatives of the EC population [14], and it has been speculated that parietal endoderm of the mouse embryo may have a similar role. As these processes may reflect a normal interaction during embryogenesis, it is intriguing that such loops may continue to operate in tumour tissue. This suggests that continued inappropriate paracrine growth stimulation may contribute to the proliferation of the tumour tissue, influence the various differentiation pathways seen in teratocarcinoma [15], or due to the potent angiogenic properties of the FGFs, stimulate tumour invasion by capillaries.

We have previously shown that undifferentiated populations of the cloned human teratocarcinoma cell line Tera-2 synthesise messenger RNA for basic fibroblast growth factor and K-FGF [15], but it has not been possible to purify these peptides from conditioned medium. This problem was also reported for several mouse teratocarcinoma cell lines [16]. We report here that Tera-2 will proliferate in serum-free medium in the sole presence of recombinant basic FGF. This effect is accompanied by changes in cell shape and motility. The effects are mediated through classical FGF receptors,

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are sensitive to protamine sulphate, and demonstrate a qualitative dependance on concentration.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Tera-2 cells were routinely passaged as described by Thompson et al. [17] in alpha modified Eagles' medium and 10% heat-inactivated fetal bovine serum (SeraLab). Serum-free medium was essentially as described by Biddle et al. [18] being composed of a 1:1 mixture of alpha modified Eagles medium (lacking nucleosides and deoxyribonucleosides) and Hams' F12 medium (Gibco, UK), supplemented with 10 μg/ml human transferrin preloaded with iron in accordance with the manufacturers instructions.

Two days prior to an experiment, cultures were weaned onto alpha/Ham supplemented with 10% fetal calf serum, to allow the cells to adapt to the basal medium used in the serum-free culture experiments. Cells were harvested by rinsing in PBS (solution A [19]) and then briefly exposed to trypsin versene, and chick plasma. Cells were taken up in 10% FCS, collected by centrifugation and counted in a haemocytometer.

All subsequent procedures were carried out as described in Biddle et al. [18]. Cells for growth curves were plated out at a density of 1.4–3 × 105 cells in each 6-cm diameter Primaria dish (Beckton-Dickinson, UK) and allowed to attach overnight in 10% FCS/alpha/Ham. Cultures were then extensively washed and medium with FCS replaced with serum-free medium. The following day cells were counted, and this was taken as the starting point of the experiment. Counting of cells exposed to experimental conditions was carried out after exposure of washed monolayers to trypsin/EDTA for 20 min at room temperature to ensure complete harvesting. The range of cell counts in the duplicate and triplicate dishes rarely exceeded 10% of the mean cell count.

The results of several of these studies were expressed as the number of population doublings that had occurred since the start of the experiment. This method eliminates day to day variation in the rate of population increase and was a single point assay, detailed in Biddle et al. [18].

2.2. *Growth factors*

The additives used included human basic fibroblast growth factor (R&D via British Biotechnology, Oxford) and insulin-like growth factor-I (Amersham, UK). Protamine sulphate was purchased from Sigma, Poole, UK.

2.3. *Clonal analysis of cell multiplication and cell locomotion*

The cells were plated at different densities ranging from 10–100 cells per cm^2 into equilibrated medium in the 35-mm wells of six-cluster dishes. After 5 days, each culture was fixed in 3:1 methanol/acetic acid for at least 24 h, hydrated in Analar water, and finally stained with Crystal violet. Each dish was examined under a Leitz inverted microscope equipped with an eyepiece graticule inscribed with 10 concentric circles. The distance between two circles was taken as one relative unit. Image analysis using a C-Scan (Cambridge) analyser allowed for quantitation of colony size in parallel and gave identical results to the manual method.

Each dish was scanned in the microscope and the location of each colony was marked. The diameter of each colony was determined by use of the graticule, whereafter the number of cells in each colony was counted. In several instances the colonies were photographed under a Leitz inverted microscope with an attached camera system.

2.4. *FGF binding assays*

Radioiodinated FGF was prepared according to Neufeld and Gospodarowicz [20] and purified by heparin Sepharose affinity chromatography. Specific activities of 4–8 × 10^4 cpm/ng were obtained in this way.

Tera-2 cells were plated out on gelatinised 24-well cluster plates at 2 × 10^5 cells per well in alpha MEM/10% FCS and allowed to attach and grow overnight. Plates were transferred to ice, medium aspirated and cells washed 5× with 500 μl phosphate-buffered saline. Subsequently 200 μl of binding buffer (alpha MEM + 25 mM HEPES + 0.2% gelatin) were added to each well together with increasing concentrations of labelled FGF (0.01–10 ng/ml). Non-specific binding was estimated by including 2 μg/ml unlabelled FGF with duplicate samples to which had been added 0.5 ng of iodinated bFGF/ml and binding to dishes by adding 5 ng of iodinated bFGF/ml to dishes without cells in 200 μl of binding buffer. Plates were incubated at 4°C for 4 h, medium aspirated and cells washed with 0.5 ml PBS/0.1% BSA three times. Membranes were then solubilised with 400 μl of Triton X-100 + 0.1% BSA and counted in an LKB rack gamma counter. Greater than 95% of counts bound to cells on incubation with 0.5 ng/ml of iodinated FGF could be displaced by 2 μg/ml of unlabelled competitor in repeat data sets. This was taken as a measure of non-specific binding and subtracted from the binding data. This data was subsequently converted to the Scatchard [21] form and analyses using the programme LIGAND. All figures were corrected for non-specific binding as described above.

2.5. *FGF receptor cross-linking*

Receptor cross-linking was carried out essentially according to Neufeld and Gospodarowicz [22]. 1 × 10^6 Tera-2 cells were plated out onto gelatinised 600-mm dishes and allowed to attach overnight. Binding was carried out at 4°C in 1.2 ml of the binding buffer of Neufeld and Gospodarowicz [20]. 100 ng/ml of iodinated FGF was added to each dish. Competitor was added at 2 μg/ml to one set of dishes and increasing concentrations of protamine sulphate (1,5 and 10 μg/ml) to the remaining dishes. Dishes were incubated for 2.5 h on a rotating platform at 2 cycles per second, washed in 10 ml of PBS and cross-linked at room temperature with 20 mM disuccinimidyl suberate (Aldrich) in 2 ml of PBS. Harvesting of membranes was carried out as described in [22] and analysed on 5% Laemmli SDS-polyacrylamide denaturing gels using the Amersham (Amersham, UK). Rainbow markers for calibration.

3. RESULTS

3.1. *Growth effects*

Daily addition of recombinant FGF to Tera-2 cells plated out in serum-free medium over a 4-day period showed that proliferation of the cells could be supported by 20 ng/ml FGF as the sole macromolecular additive, resulting in a 1.3-fold doubling in population over the time course of the experiment as compared with controls which showed essentially no growth. The proliferative response increases with increasing concentrations of bFGF and reaches a maximum at 20 ng/ml (Fig. 1). No further stimulatory effect could be achieved by adding higher doses of the growth factor. The effects of bFGF are inhibitable by low concentrations (10 μg/ml) of protamine sulphate (data not shown) as previously reported by Gospodarowicz et al. [23] and inclusion of this agent in growth experiments substantially reduced the biological effects of the bFGF suggesting that the molecule is acting through similar receptors to those previously characterised.

3.2. *Cell migration*

To study the relationship between cell multiplication and cell locomotion we seeded Tera-2 cells at clonal density and grew them with the addition of 10 ng/ml of basic FGF. In this set of experiments the number of cells

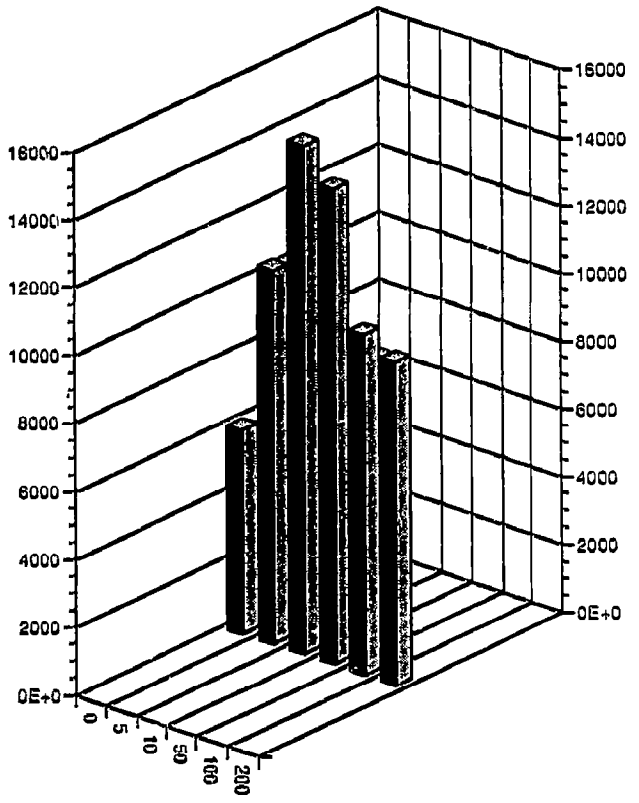

Fig. 1. Dose-dependent stimulation of Tera-2 cell proliferation by basic FGF. Cells were plated out at a nominal density of 1×10^5 cells per 600-mm dish, washed after 24 h and medium replaced with alpha/Ham alone or supplemented with basic FGF at 5–200 ng/ml. After 4 days replicate dishes were counted in triplicate and figures related to appropriate controls.

as well as the diameter was scored for each colony. In order to exclude the possibility that any observed effects on cell migration were a consequence of an increased rate of cell proliferation, the diameters of colonies that consisted of equal numbers of cells were examined in the presence and absence of bFGF.

It was shown that bFGF stimulates clonal cell proliferation as determined by cells/colony in a concentration-dependent manner. The maximum increase in cell numbers was observed at 10 ng/ml. Increasing the concentration over and above this slightly decreased cell numbers. Fig. 2A–C demonstrates that irrespective of colony size, as defined by numbers of cells per colony, only high doses of bFGF (100 ng/ml) significantly increased the colony diameter. In contrast, lower concentrations of bFGF (1–10 ng/ml) did not significantly increase the colony diameter. The stimulatory effect on cell locomotion was ascribable to bFGF since it could be completely abrogated by simultaneous addition of protamine sulphate (data not shown).

3.3. *FGF receptors*

It is important in this type of analysis to establish that the observed requirements are complemented by expression of the appropriate growth factor receptors. Fig. 3 shows the results of FGF binding experiments on Tera-2 cells. Scatchard analysis of the binding data yields a total number of 22,500 and 200,000 sites per cell made up of two populations of sites with high- and low-affinity dissociation constants of 16.7 pM and 51 nM, the former accounting for approximately 22,500 sites per

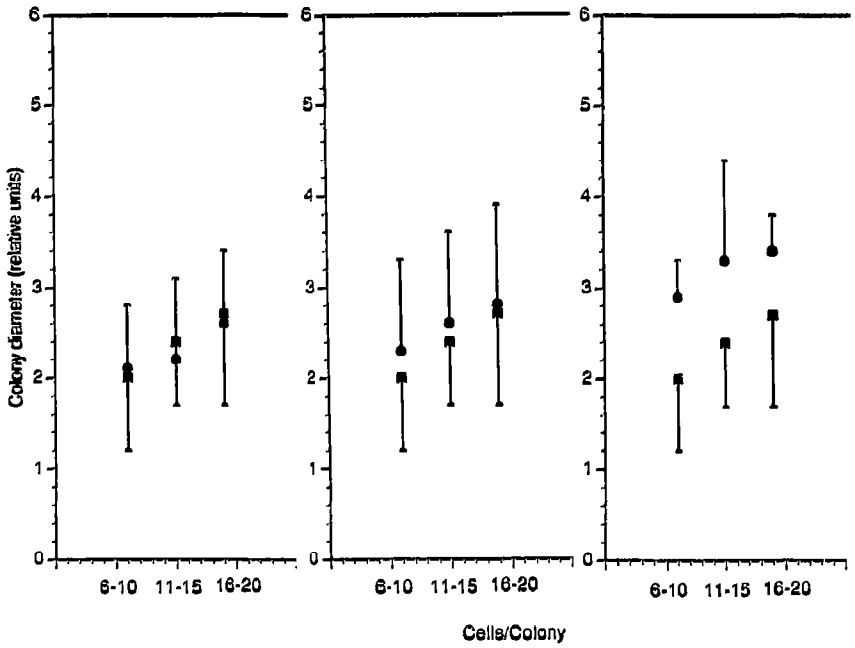

Fig. 2. The effect of bFGF on clonal locomotion of Tera-2 cells. Cells were seeded onto 35-mm dishes at a concentration of 20 cells/cm^2 in 1% serum. The media were either supplemented with 1 (A), 10 (B), or 100 (C) ng bFGF per ml (●), or nothing (■). After 5 days the dishes were fixed and the diameter and the cell number of each colony determined under a light microscope. The mean diameters were calculated on colonies with comparable cell numbers. For this purpose the colonies were pooled into clusters that ranged over 5 cell numbers (abscissa), e.g. 6–10, 11–15, 16–20 etc. as described in [30]. The curves represent means ± S.D. from three different experiments.

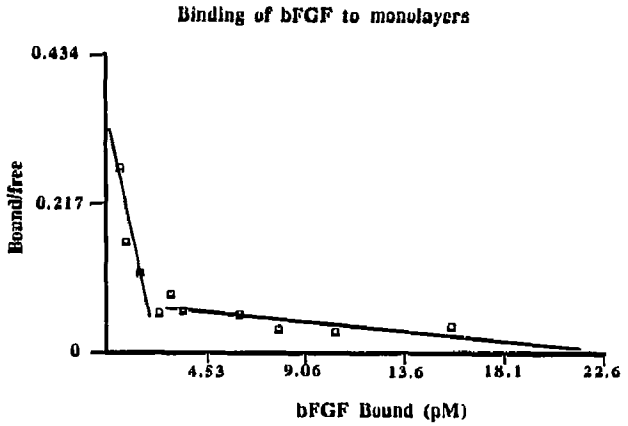

Fig. 3. Binding of [^{125}I]bFGF to monolayers of Tera-2 cells was carried out as described in section 2. In this experiment the specific activity of bFGF used was 1×10^5 dpm/pmol. Saturation binding data collected in triplicate were analysed using the programme EBDA (BioSoft) and the resultant estimates of K_d and saturation binding analysed using the iterative curve fitting programme LIGAND. The data were best fitted to a model with two non-cooperative classes of binding site ($P < 0.05$). The highest affinity sites having a K_d of 16.7 pM (22,500 receptors per cell, and the lowest affinity 51 nM (200,000 receptors per cell).

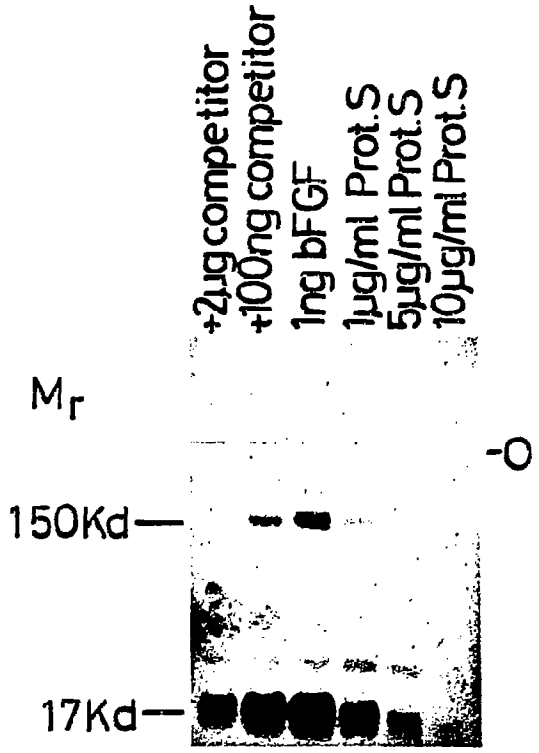

Fig. 4. Cross-linking of [^{125}I]bFGF to Tera-2 cell membranes. 100 ng/ml [^{125}I]bFGF was incubated with monolayers of Tera-2 cells, at 4°C as described in section 2, competitor (unlabelled bFGF) was added at 1 ng/ml, 100 ng/ml and 2 μg/ml to one set of dishes and to a set of dishes containing radiolabelled ligand alone, increasing concentrations of protamine sulphate (Prot. S) (1, 5 and 10 μg/ml). Ligand and receptors cross-linked at room temperature with 20 mM disuccinimidyl suberate (Aldrich) in 2 ml of PBS. Harvesting of membranes was carried out as described in section 2 and analysed on 5% Laemmli polyacrylamide-SDS denaturing gels using the Amersham (Amersham PLC, UK) Rainbow markers for calibration.

cell and the latter 200,000. Cross-linking of radioiodinated ligand to monolayers indicated a single species of receptor of M_r 150 kDa to which ligand binding could be competed with an excess of unlabelled ligand or increasing concentrations of protamine sulphate (Fig. 4). It is particularly important to note that the degree of protamine sulphate induced inhibition of binding of FGF to its receptor mirrors the dose–response relationship for inhibition of FGF stimulated growth thus providing good circumstantial evidence that the growth effects are mediated through the 150 kDa receptor.

4. DISCUSSION

Synthesis of heparin binding growth factors has been reported by several authors previously, for example NTera-2 a derivative of Tera-2 is known to express mRNA for bFGF and to synthesise material immunologically related to FGF [24], but the response of human teratocarcinoma cells to such factors has not previously been characterised. Although mRNA for bFGF has been detected in Tera-2 cells attempts to purify it from conditioned media have so far failed; a similar problem exists with the purification from mouse teratocarcinoma cells which also contain transcripts. This results in the potential autocrine or paracrine loop available for Tera-2 cell growth not being completed for basic FGF. This paradoxical finding that cells expressing bFGF also require it for their growth is frequent for FGF dependent cells, such as capillary endothelial cells and emphasises the problems associated with lack of secretion of FGF. However, it must be considered that bFGF is not the ligand to which cells are reacting in vivo. Just as the EGF receptor reacts equally well with TGFa, which is probably the most abundant physiological ligand, it is plausible that the different FGF receptors may also interact with different members of the FGF family [20,25–27].

Tera-2 possesses one size class of single chain high-affinity cross linkable receptor with an M_r of approximately 150 kDa. Scatchard analysis of binding data indicates two types of binding site, one high-affinity site with a K_D of 16.7 pM and 22,500 receptors per cell and a low-affinity site. A wide range of receptor numbers and affinities has been reported for different cells with K_d values ranging from 11 pM to 270 pM and numbers of receptors per cell from 0.2×10^5 to 1×10^5. Moreover, four distinct FGF-receptor genes have hitherto been cloned and sequenced [25–27].

In this Tera-2 shows a typical number of receptors with high affinity. Molecular weights have been reported between 110 and 165 kDa with most cells having at least two cell surface species resolvable by electrophoresis of ligand cross-linked receptor. The class of

low-affinity binding sites with a K of 51 nM is rather higher than the estimated binding of bFGF to extracellular matrix but it is not unrealistic to suggest that this class of binding site is indeed on the matrix as a large number of sites are present, equivalent to 200,000 per cell. Both the biological effects of bFGF are inhibited by protamine sulphate, and the concordance between dose/inhibition data for biological effect and binding to the receptor in cross-linking experiments supports the previous findings of Gospodarowicz et al. [9] and allows discrimination between two potential mechanisms of action of insulin-like growth factors, direct or via bFGF. Moreover, others have reported effects of FGF on migration of several cell types including fibroblasts and endothelial cells [28–30]. The concentration-dependent difference in biological effect is clearly demonstrated in studies with lens epithelial cells, and reflects the finding here that high concentrations of bFGF affect cell migration. It is tempting to speculate that the low-affinity sites either on the cells or more probably in the ECM are responsible for inducing this response (e.g. [23]).

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