Effects of Fibroblast Growth Factors 19 and 20 on Cell Multiplication and Locomotion in a Human Embryonal Carcinoma Cell Line (Tera-2) *In Vitro*

WILHELM ENGSTRÖM and MARIKA GRANERUS

Department of Biosciences and Veterinary Public Health, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden

Abstract. *Background: The effects of two recently discovered heparin binding growth factors, FGF-19 and FGF-20, on the human embryonal carcinoma derived cell line Tera 2 were examined. Materials and Methods: Cell numbers, as well as cell migration were examined at the clonal level by light microscopy. Results: FGF-19, as well as FGF-20 promoted Tera 2 cell multiplication. Whereas FGF-20 promoted cell multiplication at low doses, FGF-19 was required at high doses to achieve a comparable effect. Moreover, FGF-19 did not significantly stimulate cell locomotion, while FGF-20 promoted cell motility at high doses. Conclusion: FGF-19 and FGF-20 qualitatively exert different effects on cell survival and cell locomotion.*

The fibroblast growth factors (FGF) play an important, but not yet fully understood, role in the regulation of important cellular processes. The FGF family consists of 23 members ranging in size from 17 to 34 kDa and the last member was discovered more than five years ago (1). These polypeptides share a common core of approximatelly 140 amino acids (2). Whereas the first two members of the FGF family, acidic and basic FGF lack a signal peptide required for secretion, most other members of the FGF family have retained this leader sequence (2).

FGFs bind chiefly to two types of structures. FGF molecules form a low affinity interaction with heparan sulphate, an important structural component of the extracellular matrix. FGFs also bind to FGF-receptors (FGF-R 1-5 in mammals). Many of the diverse effects of the FGFs have been explained by their different affinities to different receptors (3).

This study was aimed at examining the effects of two recently characterised members of the heparin binding growth factor family (FGF-19 (4) and FGF-20 (5)) , that have both been assigned important regulatory roles in development. A well characterised human embryonal carcinoma cell line, Tera 2, frequently used for simultaneous studies of proliferation and locomotion on a clonal level (6-9), was employed. It is demonstrated here that while FGF-19 exerts an effect on proliferation at low concentrations but no effect on motility, FGF-20 exerts effects on proliferation at low concentrations and on motility at higher concentrations.

Correspondence to: Wilhelm Engström, Department of Biosciences and Veterinary Public Health, Swedish University of Agricultural Sciences, P.O. Box 7028, S-750 07 Uppsala, Sweden. e-mail: wilhelm.engstrom@bvf.slu.se

Key Words: Heparin binding growth factors, cell multiplication, cell locomotion.

Materials and Methods

Cell culture. Human embryonal carcinoma-derived Tera 2 cells (Clone 13) were routinely passaged as described by Thompson *et al.* (10) in alpha medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO). Serum-free medium was essentially as described by Biddle *et al.* (11). Briefly, it consisted of a 1:1 (v/v) mixture of alpha medium (lacking nucleosides and deoxyribonucleosides) and Hams F12 medium (GIBCO) supplemented with 10 μg/ml of human transferrin preloaded with iron in accordance with the manufacturer's instructions.

The cells used for the growth experiments were plated out at a density of 1.5-3.5x10^5 cells in each 6-cm diameter Primaria dish (Becton-Dickinson) and were allowed to attach overnight in 10% FCS. The cultures were then extensively washed and the FCS-containing medium replaced by serum-free medium. The following day, the cells were counted and this was taken as the starting point of the experiment. The counting of cells exposed to different experimental media was carried out after exposure of washed monolayers to 0.125% (w/v) trypsin, 0.0125% (w/v) EDTA for 10 min at room temperature to ensure complete harvesting. The range of cell counts in the duplicate and triplicate dishes only rarely exceeded 8% of the total cell count. The results of these counts were normalised by relating the number of population doublings since the start of the experiment. This procedure eliminated day-to-day variation during the population increase, as described in detail in Biddle *et al.* (11).

Growth factors. Recombinant FGF-19 and FGF-20 were purchased from Research Diagnostics Inc, (Concord, Mass, USA). Protamine sulphate was purchased from Sigma, Sweden.

Clonal analysis. Tera 2 cells were plated at different densities ranging from 10 to 100 cells per square centimetre in equilibrated medium in 35 mm wells of six cluster dishes. After a five-day exposure to the experimental media, each culture was fixed in 3:1 (v/v) methanol: acetic acid for at least 24 h, hydrated in Analar water and finally stained with 1% (w/v) aqueous crystal violet. Each dish was examined under a Leitz inverted microscope equipped with an eyepiece graticule inscribed with 24 concentric circles. The distance between two neighbouring circles was taken as one relative unit. Image analysis using a C-scan (Cambridge) gave identical results to the manual method. Each dish was subsequently scanned in the microscope and the location of each visible colony marked. The diamater of each colony was determined by the 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. This procedure was described in detail elsewhere (7).

Results

Growth effects. Daily additions of FGF-19 or FGF-20 to Tera 2 cells plated out in serum-free medium over a five-day period showed that multiplication of these cells could be supported by 10 μg per ml of any one of these growth factors, as the sole macromolecular additive. This resulted in at least one-half population doubling over the time course of the experiment and compared with controls that showed essentially no increase in cell number (Figure 1). However, the magnitude and dose response differed between the two factors. Whereas FGF-20 reached its dose optimum in the interval 10-100 μg/ml and then tailed off, FGF-19 increased its multiplication stimulating activity with increasing growth factor concentration and reached its maximum at 100 μg/ml; thereafter, the effect tailed off. In neither case could any further significant effect be achieved by adding a higher concentration of the growth factor.

The effects of FGF-19 and FGF-20 were both abrogated, if 10 μg protamine sulphate were concomitantly added in a manner previously described for bFGF (6) (data not shown) and inclusion of this agent in control experiments was hence used as a control of the specific effect of each of the two heparin binding growth factors.

Cell locomotion. To study the relationship between cell multiplication and migration, Tera 2 cells were seeded at clonal density and grown with different concentrations of FGF-19 or FGF-20. In this set of experiments, the cell number, as well as the relative diameter was scored for each colony. In order to exclude the possibility that any of the observed effects on cell migration were a consequence of an enhanced cell multiplication, the diameter of colonies that contained comparable cell numbers were examined in the presence or absence of the two growth factors under study. It was found that FGF-19, as well as FGF-20 stimulated clonal proliferation as determined by the number of cells per colony. Moreover, as shown in Table I, irrespective of colony size as defined by number of cells per colony, the effects on cell motility (as compared to the controls) were best achieved when high doses (50-100 μg/ml) were added. There was a conspicuous difference between the two factors in that FGF-19 did not result in any stastistically significant effect on cell locomotion. In contrast, FGF-20 significantly stimulated cell locomotion only when added at high concentrations (100 μg/ml).

In the case of FGF-19, there was no significant difference in colony size as compared to the controls in the range 5-100 μg/ml. However, for FGF-20 this effect was only significant for the highest concentration tested (100 μg/ml). The stimulatory effects on cell migration were ascribable to the addition of FGF-20 itself, since its effects were abrogated by simultaneous addition of protamine sulphate (data not shown).

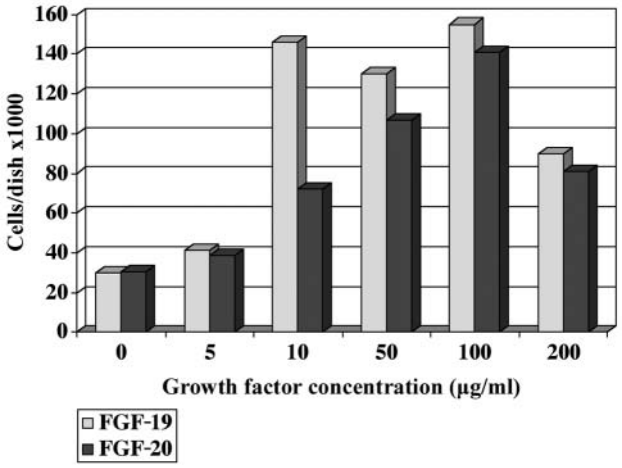

Figure 1. *Dose-dependent stimulation of Tera 2 cell multiplication by addition of two different heparin binding growth factors. The cells were plated out at a nominal density of 10^5 cells per 60 mm dish and exposed to serum-free medium supplemented with different concentrations of FGF-19 (dark grey) or FGF-20 (light grey). Daily addition of the assigned dose of growth factor was carried out through the experiment. After five days, replicate dishes were counted in triplicate and the figures related to appropriate controls.*

Discussion

In their classic study, McAvoy and Chamberlain showed that the two archetypical fibroblast growth factors, aFGF and bFGF, were capable of inducing different biological responses when supplied at different doses. When the growth factors were added to chick lens epithelial cells, preferential effects

Table I. *Colony diameter.*

Cells/Colony	6-10	11-15	16-20	21-25
Additives to serum-free medium				
None (Control 1)	10.5	12.8	14.5	15.6
10% Serum	10.7	13.5	17.6	17.1
5 μg FGF-19/ml	10.5	14.3	16.7	18.1
10 μg FGF-19/ml	12.5	13.9	15.5	15.4
50 μg FGF-19/ml	11.4	12.7	15-0	18.8
100 μg FGF-19/ml	13.0	15.0	14.4	16.9
5 μg FGF-20/ml	09.9	13.7	16.9	17.0
10 μg FGF-20/ml	11.0	14.5	17.6	17.6
50 μg FGF-20/ml	12.1	15.6	19.9	22.0*
100 μg FGF-20/ml	15.8*	18.2*	21.6*	21.7*

*$p<0.05$.

The effect of two heparin binding growth factors on cell locomotion in Tera 2 cells. Cells were seeded onto 35 mm dishes. The serum-free media were supplemented with daily additives, as specified in the Table. After five days, the dishes were fixed and stained and the diameter and cell number of each colony was determined by light microscopy. 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 as described by Schofield *et al.* (6), *e.g.,* 6-10, 11-15, 16-20 and 21-25. The figures represent means of four different experiments. The statistical significance between the means was determined by Student's *t*-test.

were observed on proliferation, locomotion of differentiation (as determined by activation of the beta-crystalline gene) depending on the growth factor concentration (12-14). This plethora of effects executed by a single growth factor fits rather well into the pattern of multiple effects of FGFs observed during normal and tumour angiogenesis.

We previously observed that the addition of nine different members of the FGF family gave rise to an increase in human teratocarcinoma cell numbers at very modest concentrations (6, 8, 9, 15). When the growth factor concentration was increased, a preferential effect on cell migration was observed. It was firmly concluded that the effect on cell numbers was chiefly due to an enhanced cell survival rather than recruitment of more cells into the cell cycle or an increased rate of cell cycle traverse (13).

In this study, we examined the effects of the two recently discovered growth factors, FGF-19 and FGF-20, on multiplication and motility at the clonal level. It was found that FGF-20 behaved in a fashion similar to all other examined members of the FGF family. FGF-20 exerted its maximum effect on clonal multiplication at low concentrations, whereas its effect on cell locomotion became most obvious at higher concentrations. In contrast, FGF-19 did not support Tera 2 cell multiplication at low concentrations, but a significant effect was achieved at high concentrations. Like other FGFs, FGF-20 stimulated cell motility at high concentrations, but this effect was conspicuously absent when FGF-19 was added.

It is reasonable to assume that the difference between FGF-19 and all other examined members of the FGF-family may relate to its receptor binding properties. FGFs bind to a class of FGF-receptors, where new isoforms with different ligand-binding properties are generated by alternative splicing mechanisms. There is an interesting difference between FGF-R1-3 and FGF-R4 in that the latter seems to lack alternative splice sites (9). It was recently shown that FGF-19 is unique in the sense that it only binds to one sole receptor - the FGF-R4. This exclusivity was explained by the unique structural properties of FGF-19 and FGF-R4. (16). This unusual affinity may well explain the absence of FGF-19 effects on cell motility since this effect is generally believed to be exerted *via* FGF-R2 (17). Since FGF-R2 is expressed in Tera 2 cells, it was assumed that this receptor is mainly responsible for its motility. However, older data suggest that membrane ruffling activities in malignant cells can be mediated *via* the FGF-R4 (18). Thus, the exact biological effects mediated by each receptor need to be further elucidated.

Acknowledgements

This study was supported by grants from Cancerfonden and Barncancerfonden, Sweden.

References

1 Jeffers M, Shimkets R, Prayaga S, Boldog F, Yang M, Burgess C, Fernandes E, Rittman B, Shimkets J, LaRochelle WJ and Lichtenstein HS: Identification of a novel human fibroblast growth factor and characterisation of its role in carcinogenesis. Cancer Res *61*: 3131-3138, 2001.

2 Powers A, MacLeskey S and Wellstein A: Fibroblast growth factors, their receptors and signalling. Endocrin Relat Cancer *7*: 165-197, 2000.

3 Granerus M: The Effects of Growth Factors on proliferation, survival and motility in a human embryonal carcinoma derived cell line. PhD-thesis, Swedish University of Agricultural Sciences, 1997.

4 Xie MH, Holcomb I, Deuel B, Dowd P, Huang A, Vagts A, Foster J, Liang J, Brush J, Gu Q, Hillan K, Goddard A and Gurney AL: FGF-19, a novel fibroblast growth factor with unique specificity for FGFR4. Cytokine *11*: 729-735, 1999.

5 Kirikoshi H, Sagara N, Saitoh T, Tanaka K, Sekihara H, Shiokawa K and Katoh M: Molecular cloning and characterisation of human FGF-20 on chromosome 8p21.3-p22. Biochen Biophys Res Commun *274(2)*: 337-343, 2000.
6 Schofield PN, Granerus M, Lee A, Ekström TJ and Engström W: Concentration dependent modulation of bFGF on multiplication and locomotion of human teratocarcinoma cells. FEBS letters *298*: 154-158, 1992.
7 Engström W: Different effects of EGF on locomotion and cell proliferation in a cloned human embryonal carcinoma cell line *in vitro*. J Cell Sci *86*: 47-55, 1986.
8 Granerus M and Engström W: Dual effects of four members of the fibroblast growth factor family on multiplication and motility in human teratocarcinoma cells *in vitro*. Anticancer Res *20*: 3527-3532, 2000.
9 Granerus M and Engström W: Effects of fibroblast growth factor 8, fibroblast growth factor 9 and keratinocyte growth factor on multiplication and locomotion in human teratocarcinoma cells *in vitro*. Anticancer Res *23*: 1313-1316, 2003.
10 Thompson S, Stren PL, Webb M, Walsh FS, Engström W, Evans EP, Shi WK, Hopkins B and Graham CF: Cloned human teratoma cells differentiate into neuron like cells and other cell types in retinoic acid. J Cell Sci *72*: 37-65, 1984.
11 Biddle C, Li CH, Schofield PN, tate VE, Hopkins B, Engström W, Huskisson NS and Graham CF: Insulin like growth factors and the multiplication of Tera 2 – a human teratoma derived cell line. J Cell Sci *90*: 475-485, 1988.
12 Chamberlain CG and McAvoy JW: Induction of lens fibre differentiation by acidic and basic fibroblast growth factor. Growth Factors *1*: 125-134, 1989.
13 McAvoy JW and Chamberlain CG: Basic effects on chick lens epithelial cells differ at different concentrations. Development *107*: 221-228, 1989.
14 McAvoy JW and Chamberlain CG: Growth factors in the eye. Prog Growth Fact Res *2*: 29-43, 1990.
15 Granerus M and Engstrröm W: Growth Factors in apoptosis. Cell Proliferation *29*: 309-314, 1996.
16 Harmer NJ, Pelelgrini L, Chirmagze D, Fernandez-Recio J and Blundell TL: The crystal structure of fibroblast growth factor (FGF) 19 reveals novel features of the FGF family and offers a structural basis for its unusual receptor affinity. Biochemistry *43*: 629-640, 2004.
17 Nakamura T, Mochizuki Y, Kanetake H and Kendsa S: Signals *via* FGF-receptor 2 regulate migration of endothelial cells. Biochem Biophys Res Comm *289*: 801-806, 2001.
18 Johnston CL, Cox HC, Gomm JJ and Coombes RC: bFGF and aFGF induce membrane ruffling in breast cancer cells but not in normal breast epithelial cells; FGF-R4 involvement. Biochem J *306*: 609-616, 1995.

Received April 7, 2006
Accepted June 15, 2006