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Differentiation associated modulation of K-FGF expression in a human teratocarcinoma cell line and in primary germ cell tumours

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The human teratocarcinoma cell line Tera 2 can be induced to differentiate in vitro after exposure to retinoic acid. We show in this paper that whereas the K-FGF oncogene is expressed in undifferentiated cells, addition of retinoic acid rapidly (< 60 min) downregulates the expression of this gene. However, when cells are cultured in RA for an extended period of time (> 15 days) K-FGF transcripts reappear. We also report that K-FGF is expressed in approximately one-third of primary human germ cell tumours but not in the corresponding normal testicular tissue.

K-FGF; Teratocarcinoma

1. INTRODUCTION

The heparin binding growth factor family now comprises a number of structurally related polypeptides which influence many aspects of cellular physiology, differentiation and proliferation. The acidic and basic fibroblast growth factors, originally isolated from pituitary and central nervous system respectively, are potent mitogens, both in vitro and in vivo [1] and since their discovery have been shown to stimulate proliferation of a wide variety of cells in tissue and organ culture [2,3]. The FGFs are also interesting in the light of their diverse developmental effects. Synthesis of heparin binding growth factors occurs both in perimplantation stages of mammalian embryogenesis and later during organogenesis. In both these situations there is circumstantial evidence for a pleiotropic response of target cells to the factor: different concentrations of the factor leading to proliferation, cell migration and differentiation [4,5]. Not only are these processes important in morphogenesis but also in the metastasis and angiogenic properties of tumours, and there is strong evidence that FGF or related molecules are potent stimulators of the capillary endothelial cell response to tumours [3].

Within recent years the FGF family has expanded to include proteins encoded by at least 7 different genes. In addition, genes encoding FGF-receptors have been recently characterized. Genes encoding the FGF homologues K-FGF/hst, int-2 and FGF-5 were originally isolated as oncogenes by DNA transfection assay, and sequence analysis has revealed the homology of the predicted proteins with prototype FGFs [6–11]. The expression of int-2 and K-FGF during embryogenesis is confined to distinct process related time windows [12,13] suggesting a potential regulatory role in development. Maintenance of the balance between differentiation and proliferation is essential for the progressive growth of stem cell driven differentiating systems such as the embryo or indeed a tumour. We have attempted to assess the contribution of auto or paracrine K-FGF action in a model system derived from a human developmental tumour.

The teratocarcinoma cell line Tera 2 cl-13 [14] was clonally derived from Tera 2 [15] which was itself established from a pulmonary metastasis of a testicular teratocarcinoma. In response to retinoic acid cells in monolayer culture terminally differentiate to form a mixture of cell types expressing characteristic markers. Among these cells are neurone-like cells that express tetanus toxin receptors and neurofilaments [14]. When the undifferentiated cells are injected into nude mice, connective tissue, epithelia and neurones can be detected in the tumours that develop. This and other evidence [16] suggests that Tera 2 can express the critical properties which might be expected of a cell line reflecting an early human embryonic stem cell.

We have established that Tera-2 expresses mRNA for K-FGF and have examined the expression of the K-FGF gene in differentiating Tera-2 cells. We report here that treatment with retinoic acid rapidly downregulates the gene but that transcripts reappear after 15 days.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Cells were maintained in alpha modified minimum essential

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medium lacking nucleosides and deoxynucleosides (Gibco, UK) and containing 10% heat-inactivated fetal calf serum. They were cultured as described by Thompson et al. [14]. Undifferentiated cells were routinely plated at 1×10^5 cells per 90 mm dish for growth assays. Induction of differentiation was achieved by plating cells in the presence of 2×10^{-5} *all trans* retinoic acid (Eastman Kodak) diluted from a 0.1 M stock in dimethylsulphoxide.

2.2. *Immunofluorescence*

Cells were harvested by rinsing with Ca^{2+}/Mg^{2+}-free phosphate buffered saline and incubation in a solution of 0.125% (w/v) trypsin in PBS containing 0.5 mM EDTA. The cells were spun in Eppendorf tubes to give approximately 1 × 106 cells per tube. The cells were then incubated in 20 μl of monoclonal antibody W6/32 culture supernatant [14] that had been diluted previously to 1/10 of its original volume with Ca^{2+}/Mg^{2+}-free PBS containing sodium azide and 2% (v/v) fetal calf serum. After incubation for 45 min at 4°C, 0.5 ml of the above azide/serum/PBS solution was added and the samples spun. The cell pellet was resuspended in 20 μl of a 1/30 dilution of rabbit anti mouse immunoglobulin G-fluorescein isothiocyanate for 45 min and viewed under a fluorescence microscope. At least 200 cells were counted for each sample.

The human EC-cell specific antibody GCTM-2 [17] was used as described at a final dilution of 1:50 on cells grown in parallel to experimental cultures on coverslips gelatinised by overnight treatment with 0.5% w/v gelatin (Sigma).

2.3. *Isolation of mRNA*

Cells were washed in situ with 5 ml of pre-warmed PBS and lysed in 2 ml per dish of 4 M guanidinium thiocyanate (Fluka) 25 mM trisodiumcitrate (pH 7.0), 0.1 M β-mercaptoethanol, 0.5% Sarkosyl (BDH) (GuSCN-buffer). Tissue samples from testicular tumours were homogenized in GusSCN-buffer with a high speed Ultra Turrax homogenizer. The lysates were layered onto a 2.2 ml cushion of 5.7 ml CsCl, 0.1 M EDTA (pH 8.0) in a Beckman SW40 tube and centrifuged at 33 000 rpm in a SW40Ti rotor for 20 h at 18°C. The resulting pellet was washed, taken up in 100 μl of filtered diethylpyrocarbonate-treated water and precipitated with 0.3 M sodium acetate and 20 μl of ethanol. Polyadenylated RNA was subsequently extracted by the use of oligo (dT) cellulose as described in detail in [18].

2.4. *Northern blotting*

5 μg of polyadenylated RNA from each sample was analysed by Northern blotting as previously described [19].

2.5. *DNA-probes*

The K-FGF/hst probe was 285 bp *Sac*I/*Hin*dIII fragment from pORF1 [20] generously provided by Professor Takashi Sugimura. The glyceraldehyde 3-phosphate dehydrogenase probe (GAP) was a murine cDNA and was the kind gift of Dr Peter Curtis, Wistar Institute.

3. RESULTS AND DISCUSSION

Undifferentiated Tera 2 cells in monolayer culture multiply exponentially with an approximate population doubling time of 24 h [20]. In contrast, if cells are plated in the presence of 2×10^{-5} M retinoic acid, overall cell numbers cease to increase by 12 to 18 days and the majority of cells become arrested in G1 as estimated by cytophotometry [20]. In the experiments described here less than 6% of the undifferentiated cells expressed W6/32 reactivity ($n > 200$) and the general lack of expression of HLA-A,B,C common determinants is consistent with their undifferentiated

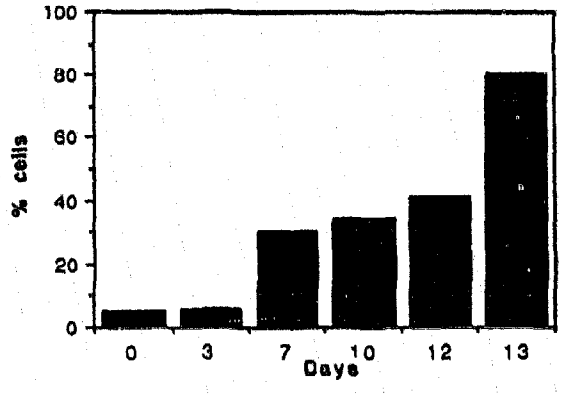

Fig. 1. The kinetics of appearance of the differentiation marker W6/32 reactivity after exposure of Tera 2 cells to 2×10^{-5} M retinoic acid.

phenotype. After addition of retinoic acid, the cell cycle rapidly lengthens. Fig. 1 shows an estimate of the rate of acquisition of HLA-A,B,C common determinants during a 14 day exposure to retinoic acid. By 12 days approximately 40% of the cells expressed antigens that reacted with the W6/32 antibody and by 13 days 80% of the cells reacted. These figures were confirmed by staining populations of undifferentiated and differentiated (15 d) cells using the antibody GCTM-2 which reacts only with a heparan sulphate proteoglycan on the surface of teratocarcinoma stem cells. The undifferentiated component of the 15 d RA-treated population was confined to less than 10% of the cells examined (>200).

Poly A^+ RNA was prepared from cultures exposed to retinoic acid for 60 min, 5, 12 and 15 days together with samples of undifferentiated cells. Fig. 2 shows that the K-FGF transcript has almost disappeared after 60 min exposure to retinoic acid, indicating a very rapid downregulation of this gene. This finding confirms a previous preliminary report [21] and is also consistent with a recent report that another human embryonal carcinoma cell line NT2/D1 downregulates K-FGF-expression after 5 days exposure to retinoic acid [22]. However, if the cells were exposed to retinoic acid for an extended period of time (i.e. 15 days) the 3 kb K-FGF transcript reappears. This finding has not been previously reported and suggests that K-FGF gene expression may represent a multifaceted function in the control of growth and differentiation. By using two separate biochemical criteria for differentiation it is clear that the reappearance of transcripts after 15 days of differentiation is not the result of a small RA unresponsive undifferentiated population taking over the cultures. Addition of retinoic acid in different patterns, either once at the initiation of differentiation or on alternate days through the experiment did not affect the results (data not shown). It has indeed been shown that K-FGF expression in the embryo is stage-specific and our observations on an embryonal carcinoma cell

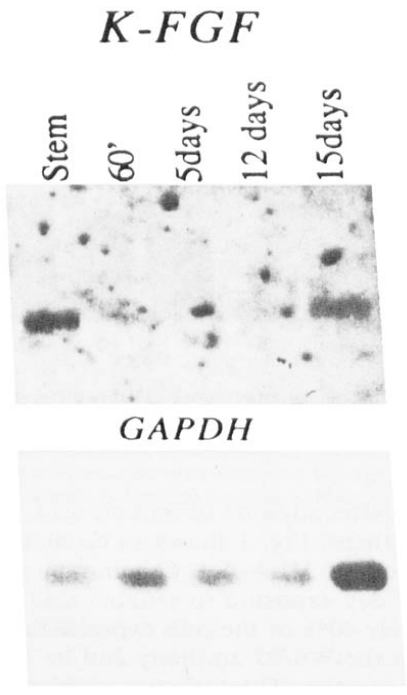

Fig. 2. Expression of K-FGF in cells exposed to retinoic acid for different periods of time as examined by Northern blotting. After probing with K-FGF cDNA, the filter was stripped of bound probe and rehybridised with a cDNA-probe for glyceraldehyde 3-phosphate dehydrogenase (GAP).

line in vitro may well indirectly reveal crucial embryonic developmental pathways.

To compare the expression of K-FGF in Tera 2 cells with the phenotype of primary germ cell tumours we purified polyA$^+$RNA from 10 surgical specimens of human testicular tumours (9 seminomas and 1 embryonal carcinoma) and from normal testis. Fig. 3

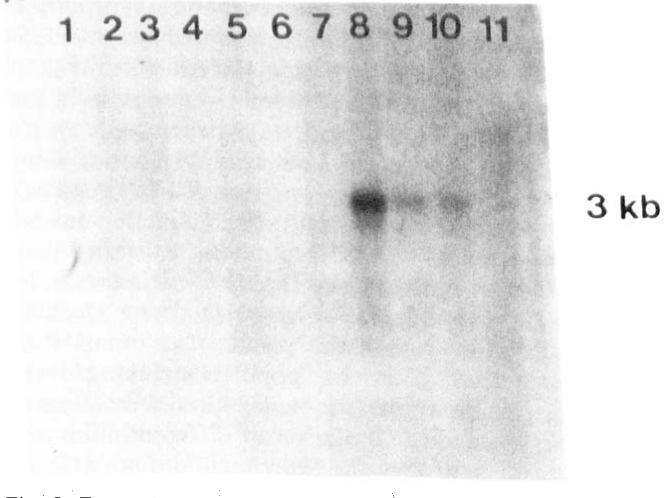

Fig. 3. Expression of K-FGF in a panel of surgical specimens from human germ cell tumours; 1–7, and 9–10 seminomas, 8, embryonal carcinoma.

shows that the expected 3 kb transcript could be observed in two seminomas and one embryonal carcinoma. No K-FGF transcripts could be detected in 7 seminomas or in normal testicular tissue. These data confirm a preliminary report by Yoshida et al. [23] who failed to detect transcripts from this gene in any other tissue but in 5 human germ cell tumours. Taken together these data suggest that K-FGF plays a pivotal role in embryogenesis as well as in the development of germ cell tumours.

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