Differential effects of fibroblast growth factor (FGF) 9 and FGF2 on proliferation, differentiation and terminal differentiation of chondrocytic cells *in vitro*

Nicole B. WEKSLER*, Gregory P. LUNSTRUM*, Eric S. REID* and William A. HORTON*†¹

*Research Department, Portland Shriners Hospital, Portland, OR 97201, U.S.A., and †Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland OR 97201, U.S.A.

Fibroblast growth factor (FGF) 9 was compared with FGF2 in its ability to influence proliferation, differentiation, terminal differentiation and apoptosis in a rat calvaria-derived cell line (RCJ 3.1C5.18) that spontaneously undergoes chondrocyte differentiation *in vitro*. Like FGF2, FGF9 promoted proliferation, but to a lesser extent. In contrast to FGF2, which blocked chondrocytic differentiation, FGF9 had no effect on differentiation but inhibited terminal differentiation. FGF9 also

stimulated expression of the mitotic inhibitor p21 to a greater extent than FGF2. Neither ligand influenced apoptosis. The results indicate that FGF9 could account for many of the physiological responses attributed to FGF-receptor activation in the growth plate.

Key words: apoptosis, chondrogenesis, receptors, signal transduction.

INTRODUCTION

The growth plate is a structure in which cartilage cells synthesize matrix that serves as template for endochondral bone growth. The key events in this process are chondrocyte proliferation, which determines the number of cells that participate in the process, and terminal differentiation, which creates space through cell enlargement and alters the cartilage template so that it can be replaced by bone. Terminal differentiation refers to the final stage of the chondrocytic developmental pathway in which chondrocytes considered fully differentiated enlarge (hypertrophy), alter their pattern of gene expression and ultimately die [1].

Several growth factors are known to regulate these events locally through specific receptors on chondrocyte cell surfaces. These include parathyroid-hormone-related hormone and Indian hedgehog, which constitute an inhibitory pathway that regulates terminal differentiation. Another involves fibroblast growth factor (FGF) ligands and receptors (FGFRs). Although three of the four known FGFRs (FGFR1, FGFR2 and FGFR3) are expressed in the growth plate [2,3], most attention has been focused on FGFR3 because the most common forms of dwarfism in humans are due to mutations of this receptor [4–6]. Indeed, mutations predicted to activate FGFR3 are found in achondroplasia, hypochondroplasia and thanatophoric dysplasia. This, coupled with observations that gene-targeted mice null for FGFR3 have abnormally long bones [7,8], indicates that FGFR3 signals also inhibit events in the growth plate.

The mechanism by which FGFR3 inhibits bone growth is not well defined. It is expressed in the growth-plate region where chondrocytes stop proliferating and begin to terminally differentiate as hypertrophic chondrocytes [3,8]. This has led to the view that FGFR3 signals block proliferation and promote terminal differentiation. The consequence would be fewer hypertrophic chondrocytes available to participate in endochondral ossification and presumably reduced bone growth [9]. Consistent with this idea are observations in the thanatophoric dysplasia growth plate that the number of hypertrophic chondrocytes is much reduced and levels of the mitotic inhibitor, p21, are

increased [10]. Moreover, p21 expression is induced in 293 cells transfected with a constitutively active FGFR3 mutant [10]. The reduction in hypertrophic chondrocyte number has also been attributed to FGFR3-induced apoptosis [11].

The physiological FGF ligand(s) for FGFR3 in the growth plate is (are) not known. Most attention to date has focused on FGF2 (basic FGF). It promotes outgrowth of embryonic limbs [12], it has been immunolocalized to the growth plate with a broader distribution than FGFR3 [13,14], it binds to the relevant (IIIc) isoform of FGFR3 with high affinity [15] and it is active in a wide range of assays *in vitro* [16–21]. FGF2 promotes cell proliferation and inhibits chondrocytic differentiation, especially terminal differentiation, under most cell-culture conditions and also *in vivo* [22–24].

FGF9 has been proposed recently as another possible physiological ligand for FGFR3 [25]. It binds with high affinity to the IIIc isoform of FGFR3, and its transcripts localize to the growth-plate regions of developing mouse cranial bones [26]. FGF9 has also been shown to activate mitogen-activated protein kinase pathways in human chondrocytes in culture in a fashion similar to that observed in cells harbouring constitutively active FGFR3 [11].

This investigation was carried out to compare FGF9 with FGF2, as well as with FGF1, as potential regulators of key growth-plate events. We used a culture scheme described previously in which rat calvaria-derived RCJ 3.1C5.18 cells (C5.18 cells) progress through the differentiation pathway exhibited by growth-plate chondrocytes, including the proliferation and terminal-differentiation steps [27–29]. Our results demonstrate substantial differences in how these FGF ligands influence chondrocyte differentiation.

EXPERIMENTAL

Antibodies and ligands

Recombinant human FGF1 and FGF2 were purchased from R&D Systems (Minneapolis, MN, U.S.A.), and recombinant

Abbreviations used: FGF, fibroblast growth factor; FGFR, FGF receptor; C5.18 cells, rat calvaria-derived RCJ 3.1C5.18 cells.

¹ To whom correspondence should be addressed (e-mail wah@shcc.org).

murine FGF9 was from Peprotech (Rocky Hill, NJ, U.S.A.). Polyclonal antibodies to FGFRs 1, 2 and 3 and p21 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal antibody against phosphotyrosine was provided by Dr. Brian Druker (Oregon Health Sciences University, Portland, OR, U.S.A.). Immunofluorescent staining of cell cultures with FGFR antibodies followed procedures supplied by the manufacturer.

Cell culture and stimulation

The rat clonal chondrogenic cell line C5.18 was obtained from Dr. Jane Aubin at the University of Toronto, Toronto, Canada. C5.18 cells were maintained in alpha minimal essential medium supplemented with 15 % heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM sodium pyruvate (Gibco BRL) and 10^{-7} M dexamethasone. After reaching confluence (3 days), cells began to differentiate spontaneously and cultures began to form nodules. At this point, fresh growth medium supplemented with both 50 µg/ml ascorbic acid phosphate (Wako Biochemicals, Richmond, VA, U.S.A.) and 10 mM β -glycerophosphate (Sigma) was added to support chondrogenesis. This supplemented medium was added again at 7 and 10 days. FGF1, FGF2 or FGF9 was also added with the fresh growth medium to give a final concentration of 10 ng/ml in the presence of heparin (2 µg/ml). Heparin was added to promote high-affinity receptor binding. Cultures were assayed as described below at 3, 7, 10 and 14 days.

Preparation of cell lysate

At each time point, cells were washed with cold PBS and solubilized in lysis buffer [1 % Nonidet P40, 150 mM NaCl, 20 mM Tris/HCl (pH 8.0), 1 mM EDTA and 10 % glycerol]. Protease inhibitors (10 µg/ml aprotinin and 1 mM PMSF) and a phosphatase inhibitor (1 mM sodium orthovanadate) were added immediately prior to use. After shaking for 30 min at 4 °C, cell lysates were cleared by centrifugation at 10000 *g* for 20 min. Protein concentrations were determined by the DC (detergent-component) protein assay (Bio-Rad), and aliquots were diluted in SDS sample buffer and heated.

Immunoblot analysis

Samples containing 10 µg of protein were loaded on to SDS/7.5 % polyacrylamide gels. After electrophoresis, proteins were transferred on to PVDF membranes (Millipore, Bedford, MA, U.S.A.). After incubation for 1 h at room temperature in blocking buffer (1 × Tris-buffered saline with 0.05 % Tween-20/4 % BSA), membranes were incubated overnight at 4 °C in primary antibody diluted in blocking buffer. Membranes were washed three times in blocking buffer and then incubated in secondary antibody (anti-rabbit IgG linked to horseradish peroxidase; Amersham) diluted in blocking buffer without BSA for 1 h at room temperature. After three more washes, membranes were developed for chemiluminescence using SuperSignal (Pierce).

Analysis of receptor activation

Cells were grown for 10 days in 60-mm tissue-culture plates in supplemented media as described above. At this time, cells were stimulated for 10 min with 10–20 ng/ml FGF1, FGF2 or FGF9 in the presence of heparin (2 µg/ml). Cell lysates were then prepared in 1 ml of lysis buffer and subjected to immunoprecipitation. Each 1 ml of lysate was precleared by adding

0.5 µg of normal rabbit IgG together with 10 µl of Protein A-agarose (Santa Cruz Biotechnology) and incubated on ice for 1 h. After centrifugation at 4 °C and 500 *g* for 5 min, supernatants were split into three 300-µl aliquots. Each aliquot was incubated with 1 µg of FGFR1, FGFR2 or FGFR3 antibody for 1 h on ice. Protein A-Agarose (10 µl) was then added and samples were rotated gently overnight at 4 °C. Immunoprecipitates were collected by centrifugation, washed three times with lysis buffer, and resuspended in 50 µl of SDS sample buffer. Immunoblot analysis was carried out as described above.

DNA quantification

At the desired time point, cells were washed with PBS and solubilized in cell-lysis solution (Puregene, Gentra Systems, Minneapolis, MN, U.S.A.). Aliquots of triplicate samples were diluted into TNE buffer [10 mM Tris/HCl, 0.2 M NaCl, 1 mM EDTA (pH 7.4)] containing 1 µg/ml Hoechst dye. Fluorescence was measured with a DyNAQuant 200 (Hoffman Pharmacia Biotech, San Francisco, CA, U.S.A.). The readings obtained were compared with a standard curve generated to determine DNA concentrations.

Alcian Blue staining

At each time point, cells were washed with PBS. After incubation in Alcian Blue stain (1 % Alcian Blue in 3 % acetic acid) for 20 min, cells were destained with three rinses in 3 % acetic acid and then washed once in water. To quantify stain incorporated, cells were solubilized in 1 % SDS by shaking for 30 min at room temperature and then heating to 90 °C for 1 h. Absorbances were taken at 605 nm for triplicate samples.

Alkaline phosphatase activity

After a wash with PBS, cells were incubated for 30 min at 4 °C in alkaline phosphatase extraction buffer [0.1 M Tris (pH 9.0), 200 mM NaCl, 0.2 % Nonidet P40, 0.2 % Triton X-100, 1 mM MgSO₄] to which protease inhibitors (10 µg/ml aprotinin and 1 mM PMSF) had been added immediately before use. Lysates were then cleared by centrifugation at 10000 *g* for 20 min. Aliquots of each lysate were added to Sigma-104 phosphatase substrate (Sigma) made up to 1 mg/ml in diethanolamine. Absorbances of triplicate samples were read at 405 nm after 30 min at room temperature.

RESULTS

The C5.18 cells were plated at densities that give confluent monolayers after 3 days of culture. At this point a second population of cells arises from and overgrows the monolayer, forming cartilaginous nodules that increase in size during the second week of culture [29]. Under these conditions, all three receptors were usually detected in the nodulated cultures beginning on day 7, although low levels of FGFR1 and FGFR2 were detected occasionally in 3-day cultures (Figure 1A). Figure 2 shows that FGFR3 localizes to cells within nodules and not to monolayer cells in a 14-day-old culture.

Treatment with FGF ligands, beginning on day 3 of culture, influenced the expression and activation of FGF receptors and the acquisition of differentiation and proliferation markers. Figure 1(B) shows expression of the receptors at days 7, 10 and 14 following ligand treatment. Treatment with FGF1 had little effect on receptor expression. FGF2 treatment suppressed expression of all three receptors below baseline levels. Receptor

Figure 1 FGFRs expressed in differentiating C5.18 cell cultures under baseline conditions (A) and after treatment with FGFs (B)

Cell extracts were prepared at the indicated times, fractionated on 7.5% SDS gels, and Western blotted with antibodies to FGFR1 (R1), FGFR2 (R2) and FGFR3 (R3). (A) Results from untreated cultures; (B) results following treatment with FGF1, FGF2 or FGF9.

Figure 2 Immunofluorescent staining of cartilaginous nodules in C5.18 cultures with FGFR antibodies

(A) Alcian Blue staining identifies a cartilaginous nodule amidst monolayer cells in a 10-day culture. (B) Anti-FGFR3 staining of cells within a nodule of a 10-day culture. (C) FGFR3 staining of nodules shown at higher magnification.

Figure 3 Effects of various FGFs on FGFR activation in C5.18 cell cultures

Cells were grown for 10 days without FGF treatment as described in the Experimental section. They were then treated for 10 min with FGF1 (F1), FGF2 (F2) or FGF9 (F9) and FGFRs were precipitated with antibodies specific for each receptor. Immunoprecipitates for FGFR1 (R1), FGFR2 (R2) and FGFR3 (R3) were fractionated on 7.5% gels and probed with antibody to phosphotyrosine. C, control.

levels of cultures treated with FGF9 on days 7 and 14 differed little from untreated cultures; however, a transient increase of FGFR2 was detected on day 10 of the treated cultures.

Western blots of cell extracts for FGFR1, FGFR2 and FGFR3 from 10-day-old untreated cultures showed similar

Figure 4 Quantification of total DNA in C5.18 cultures treated with various FGFs

Cell cultures were treated as described in Figure 3 and the amount of DNA in solubilized cell layers was quantified as described in the Experimental section. C, control.

Figure 5 Quantification of Alcian Blue staining in C5.18 cultures treated with various FGFs

Cell cultures were treated as described in Figure 3. At the indicated times cell layers were stained with Alcian Blue and, following solubilization, dye uptake was quantified at 605 nm.

levels of the respective receptors (Figure 1A). Probing receptor immunoprecipitates from FGF1-, FGF2- or FGF9-treated cultures with antibodies against phosphotyrosine showed activation of all three receptors by FGF2 and FGF9 treatment, but only minimal activation with FGF1 treatment (Figure 3). FGFR1 and FGFR2 were activated consistently by FGF2 or FGF9 at 10 ng/ml, whereas a 20 ng/ml concentration of either ligand was necessary to consistently demonstrate activation of FGFR3. FGF9 gave stronger activation of FGFR2 than FGF2.

Control as well as FGF-treated cells exhibited a several-fold increase in DNA over the first 3 days of culture (Figure 4). Afterwards, control cells showed a slight increase, which was also observed for cells treated with FGF1. Cells treated with FGF2 and FGF9 showed a substantial increase in DNA from day 3 to 7. The FGF9-treated cells peaked at day 7 and dropped off to control levels by day 14. FGF2-treated cells continued the

Figure 6 Quantification of alkaline phosphatase in C5.18 cultures treated with various FGFs

Cell cultures were treated as described in Figure 3 and alkaline phosphatase activity in solubilized cell layers was quantified as described in the Experimental section. C, control.

sharp increase in DNA to day 10 and then dropped off, but not to the extent seen for FGF9-treated cells.

There was a steady increase in staining for Alcian Blue in the absence of ligands (Figure 5). Both FGF1- and FGF9-treated cells exhibited a similar increase, although cells treated with FGF1 displayed a slightly greater increase than controls and the FGF9-treated cells showed reduced staining at 10 days even though they were comparable with controls at 14 days. In contrast, FGF2-treated cells showed essentially no increase after day 7. Together, these data suggest that about 50% of cells in control, FGF1-treated and FGF9-treated cultures express the chondrocyte phenotype, whereas essentially none of the FGF2-treated cells assumed this phenotype.

As shown in Figure 6, alkaline phosphatase activity increased slowly in control cultures over the first 10 days and then rose sharply from day 10 to 14. Cells treated with FGF1 exhibited a similar pattern. FGF9-treated cell cultures showed the slow increase to day 10, but activity decreased slightly from day 10 to 14. Essentially no increase in alkaline phosphatase was observed for FGF2-treated cultures.

To determine if cells treated with FGF2 were prevented permanently from differentiating as chondrocytes or blocked temporarily by the treatment, cultures were given FGF2 for up to 7 days and monitored by Alcian Blue staining for up to 14 more days. The cultures stained progressively, beginning about 7 days after FGF2 treatment was stopped (results not shown).

p21 was not expressed during the first week of culture, except in FGF9-treated cultures, in which it was detected on day 7 (Figure 7). p21 was found in all cultures on day 10. Whereas the levels observed for FGF1- and FGF2-treated cultures were comparable with controls, the level was higher for FGF9-treated cultures. At day 14, substantially more p21 was detected in FGF9-treated cultures compared with cultures treated with FGF2 or FGF1.

Poly(ADP-ribose) polymerase assays were done to determine if the drop in DNA concentration during the second week of treatment with FGF2 and FGF9 was associated with apoptosis. An increase in the ratio of 84- to 116-kDa poly(ADP-ribose) polymerase fragments, indicative of apoptosis, was observed in

Figure 7 Analysis of p21 expression in C5.18 cultures treated with various FGFs

Cell cultures were treated as described in Figure 3. Cell lysates prepared in SDS-gel sample buffer were fractionated on 10% gels and Western blotted with antibody against p21.

all cultures from day 10 to 14, but FGF-ligand-treated cells did not differ from untreated cells (results not shown).

DISCUSSION

Our results indicate that post-confluent C5.18 cells progress through the differentiation pathway in a way typical of growth-plate chondrocytes and that FGF9 affects this progression differently from other FGF ligands that may be physiologically relevant to bone growth. In the absence of exogenous FGF ligands, the cells form nodules within which they assume the appearance of chondrocytes. These cells express FGFR1, FGFR2 and FGFR3 coincidentally with the sequential expression of markers of chondrocyte differentiation and terminal-chondrocyte differentiation. Addition of FGF1 had little effect on this behaviour, and FGF2 promoted proliferation and limited chondrocyte differentiation as described previously [16–21]. FGF9 also stimulated limited proliferation. However, in contrast to FGF2, FGF9 treatment was conducive to chondrocytic differentiation even though it blocked terminal differentiation. FGF9 treatment also stimulated expression of p21, which was only minimal for FGF2. None of the ligands appeared to influence apoptosis.

The proliferative response to FGF2 involved non-chondrocytic monolayer cells, since there was virtually no chondrocytic differentiation judging from the absence of nodules and Alcian Blue staining in the FGF2-treated cultures. The FGF9-treated cultures contained two populations of cells: non-chondrocytic monolayer cells and chondrocytic cells that occupied the nodules. We attributed most of the proliferative response in these cultures to the non-chondrocytic cells, although we could not be certain of this because we did not specifically assess the chondrocytic cells for proliferation. If this is true, then the proliferative attributes of FGF9 are probably comparable with those of FGF2.

Mancilla et al. recently clarified the nature of the proliferative response of the growth plate to FGF2 [24]. They demonstrated that proliferation in rat tarsal organ cultures treated with FGF2 was restricted primarily to perichondrial cells and did not involve growth-plate chondrocytes. They suggested that FGF2 promotes appositional growth of cartilage by stimulating the formation of new cells at the perichondrial-epiphyseal cartilage boundary. FGF9 may thus have the same capability; however, this remains to be tested.

In our culture system, FGF9 inhibited only terminal-chondrocyte differentiation, in contrast to FGF2, which exerted its

inhibitory effect at the earlier stage of chondrocyte differentiation, i.e. at the entrance into the pathway. Although others have reported that FGF2 directly blocks terminal-chondrocytic differentiation [19], we cannot confirm this finding since there were no differentiated cells available to terminally differentiate in the FGF2-treated cultures. This distinction is important with regard to FGFR3 activation since defective terminal differentiation of growth-plate chondrocytes occurs in disorders resulting from constitutive activation of FGFR3.

Thus our results suggest that FGF9 could have two roles in the growth plate. First, it may promote appositional growth of epiphyseal cartilage. Second, it may slow linear growth by inhibiting terminal-chondrocyte differentiation. The latter is consistent with observations of short limb bones and a decrease in the number of hypertrophic chondrocytes in the growth plate of transgenic mice in which FGF9 was over-expressed in cartilage (S. Garofalo and W. A. Horton, unpublished work).

Expression of p21 is often associated with exit from the cell cycle and terminal differentiation [30,31]. In this case, however, the two are dissociated. High levels of p21 were detected in FGF9-treated cultures at 10 days, after which proliferation decreased, judging from DNA measurements. However, cells in these cultures did not terminally differentiate even though they continued to express p21. This result is similar to that reported recently by Di Cunto et al., in which forced expression of p21 inhibited terminal differentiation of keratinocytes [32].

FGF ligands appear to influence expression of the receptors that transduce their signals. For example, FGF2, which suppressed chondrocyte differentiation, reduced expression of all three receptors studied, implying that receptor expression is associated with chondrocyte differentiation. FGF9 treatment had relatively little effect on FGFR1 and FGFR3 expression, but influenced FGFR2 differentially during the culture period. There was a marked increase on day 10 relative to days 7 and 14, which was the opposite of the pattern for untreated cultures. The meaning of this observation is unclear, although the timing coincides with the block of terminal differentiation and the high levels of p21 expression associated with FGF9.

All three receptors were activated by FGF2 and FGF9, although activation of FGFR3 was less marked than for FGFR1 and FGFR2 and higher doses of ligands were needed to demonstrate activation. It is not clear if this difference is an artifact of our culture system, i.e. diffusion of ligands to cells is better for monolayer cells than for cells within nodules, or if it has biological significance. Further studies are under way to address this issue.

Since all three receptors were activated, we could not determine which receptor(s) mediated the observed responses. FGFR1 may be the best candidate for transducing the mitotic signals since it has been assigned this function in many cell types [33]. Similarly, FGFR3 may be the best candidate for transmitting signals that inhibit terminal differentiation since constitutive activation of FGFR3 is associated with markedly reduced terminal differentiation in the human dwarfing condition, thanatophoric dysplasia [9,34,35]. We have also observed a decrease in the number of hypertrophic chondrocytes in the growth plates of transgenic mice in which FGF9 was over-expressed in cartilage (S. Garofalo and W. A. Horton, unpublished work). It is possible that one or more of the observed responses were transduced by heterodimers of different receptor molecules.

Apoptosis has been proposed as a mechanism to explain how constitutive activation of FGFR3 reduces the number of terminally differentiated chondrocytes in the growth plate and thereby disrupts bone growth in patients with thanatophoric dysplasia [11]. We searched for evidence of enhanced apoptosis

in the FGF2- and FGF9-treated cultures, especially after detecting the substantial drop in DNA concentration during the latter stages of culture. Poly(ADP-ribose) polymerase assays suggested a modest increase in apoptosis in all cultures over the course of the experiment, but FGF-ligand treatment had little additional effect.

Many studies have used much higher ligand concentrations than we used to study chondrocytic responses to FGFs. We employed the lowest concentrations that produced consistent responses, which were 10–20 ng/ml for all ligands; others have used concentrations as high as 1000 ng/ml. One could argue that we would have obtained different results if we had used higher concentrations. However, one could counter that higher concentrations foster non-physiological ligand–receptor interactions. Since FGF-ligand–receptor interactions are influenced by many factors other than ligand concentration, such as heparin and heparin sulphate moieties of matrix and membrane-bound proteoglycans and other cell-surface proteoglycans, i.e. glypican and CD44 [12,36–38], it is impossible to determine what ligand concentrations *in vitro* will mimic physiological conditions. Exogenous heparin was added to our experiments to ensure high-affinity ligand–receptor binding, as is commonly done in such experiments. We cannot exclude that this over-rode natural responses of the cells, but the differential responses observed argue this was not a major factor.

Our results suggest that FGF9 could function as a physiological ligand for FGFR3 in the growth plate. Against this possibility is the recent observation that mice null for FGF9 exhibit normal bone growth (D. M. Ornitz, personal communication). Redundancy involving other FGF ligands, such as FGF2, could compensate for FGF9 under these conditions. An interesting variation of this explanation is that FGFR3 is activated by heterodimers, of which one monomer is FGF9. The recent finding that heparin sulphate can facilitate formation and presentation of hetero- as well as homo- FGF dimers to FGFRs supports this possibility [39]. Further speculation is not warranted.

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