Fibroblast growth factor (FGF) and its receptor (FGFR) are thought to be negative regulators of chondrocytic growth, as exemplified by achondroplasia and related chondrodysplasias, which are caused by constitutively active mutations in FGFR3. To understand the growth-inhibitory mechanisms of FGF, we examined the effects of FGF2 on cell cycle-regulating molecules in chondrocytes. FGF2 dramatically inhibited proliferation of rat chondrosarcoma (RCS) cells and arrested their cell cycle at the G1 phase. FGF2 increased p21 expression in RCS cells, which assembled with the cyclin E-Cdk2 complexes, although the expression of neither cyclin E nor Cdk2 increased. In addition, the kinase activity of immunoprecipitated cyclin E or Cdk2, assessed with retinoblastoma protein (pRb) as substrate, was dramatically reduced by FGF-2. Moreover, FGF2 shifted pRb to its underphosphorylated, active form in RCS cells. FGF2 not only induced p21 protein expression in proliferating chondrocytes in mouse fetal limbs cultured in vitro but also decreased their proliferation as assessed by the expression of histone H4 mRNA, a marker for cells in S phase. Furthermore, inhibitory effects of FGF2 on chondrocytic proliferation were partially reduced in p21-null limbs, compared with those in wild-type limbs in vitro. Taken together, FGF's growth inhibitory effects of chondrocytes appear to be mediated at least partially through p21 induction and the subsequent inactivation of cyclin E-Cdk2 and activation of pRb.

FGFs are a large family of at least 23 related polypeptides that bind to and activate a family of four tyrosine kinase receptors, FGFRs. They play important roles in regulating proliferation and differentiation of various types of cells, including those involved in limb development and long bone formation (1, 2). Long bones form by endochondral ossification, which is characterized by mesenchymal condensation, chondrogenic differentiation, chondrocytic proliferation, synthesis of cartilage matrix, hypertrophic differentiation, and replacement by bone. These sequential growth and differentiation processes are regulated by numerous growth factors and their receptors, such as parathyroid hormone-related protein, Indian hedgehog, and insulin-like growth factor-I (3, 4). Recently, achondroplasia, thanatophoric dysplasia, and hypochondroplasia have been shown to be caused by constitutively active mutations in the FGFR3 gene (5). Also, FGFR3-deficient mice displayed overgrowth of long bones (6, 7), and mice carrying dominant active FGFR3 genes exhibited dwarfism similar to that in patients with achondroplasia and thanatophoric dysplasia (8–13). Moreover, targeted overexpression of FGF9 in cartilage results in dwarfism in mice similar to the dwarfism in achondroplasia (14). Although these findings support the hypothesis that FGF and its receptors are negative regulators of endochondral bone development, little is known about the mechanisms by which FGF inhibits chondrocytic growth.

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ment of p21 in FGF’s growth inhibition by comparing the effects of FGF2 on cartilaginous growth in vitro in limbs isolated from p21-deficient and wild-type mice.

EXPERIMENTAL PROCEDURES

Cell Culture—RCS cells were grown in high glucose Dulbecco’s modiﬁed Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 μg/ml streptomycin (23). Cells were passaged using trypsin-EDTA (Life Technologies) and were split at a 1:5 ratio on a 100-mm dish (Falcon; Becton and Dickinson).

Cell Proliferation Assay—Cell proliferation was assessed by [%H]thymidine incorporation assay as well as by measuring DNA contents. RCS cells were plated at a density of 3 × 10^5 cells/24-well plate (Falcon), and growing cells (70% confluent) were treated with vehicle or 0.5 mM NaCl, 0.1% bovine serum albumin/phosphate-buffered saline) or the indicated dose of FGF2 (10−E7 systems) for 24 h. Cells were labeled with 0.5 μCi/ml [%H]thymidine for the last 2 h of culture and dissolved with 0.1 N NaOH, 0.01% bovine serum albumin, 1% Triton X-100 (Sigma). Samples were mixed with the same volume of ice-cold 10% trichloroacetic acid and centrifuged at 14,000 rpm at 4 °C for 10 min. After washing with 5% trichloroacetic acid, pellets were dissolved in 0.1 N NaOH, 1% Triton X-100, and their radioactivity was measured by a scintillation counter (Beckman).

DNA was measured by a fluorometric method. Briefly, RCS cells were plated at a density of 1 × 10^6 cells/24-well plate and treated with vehicle or indicated doses of FGF2. Cells were digested with 20 μg/ml of papain (Sigma) in 0.1 N sodium acetate, 50 mM EDTA, 10 mM cysteine-HCl (pH 5.53) at 55 °C for 16 h. The DNA content in digested lysates was measured using PicoGreen DNA quantitation kit (Molecular Probes, Inc., Eugene, OR).

Flow Cytometry and Western Blot Analysis—RCS cells were plated at a density of 8 × 10^4 cells/60-mm culture dishes, and subconﬂuent cells were treated with 3 nM FGF2 for 6, 12, and 24 h. Cells were then trypsinized, washed twice with phosphate-buffered saline, ﬁxed in 70% ethanol, and stained with propidium iodide (50 μg/ml; Sigma), RNase A (200 μg/ml; Sigma), and 132 μg/ml aprotinin (Sigma). Protein concentration of the lysates was determined using protein assay kit (Bio-Rad). Samples were then diluted by adding the same volume of 2× dye containing 20% glycerol, 4% 2-mercaptoethanol (Sigma), and equal amounts of protein were resolved on 10% SDS gel for pRb or 12.5% gel for all other proteins. Proteins were then transferred onto Immobilon-P membranes (Millipore Corp.). After being washed twice with 0.1% Tween 20, 10 mM Tris-HCl, 150 mM NaCl (pH 8.0) (T-TBS), blots were incubated with 5% nonfat dry milk in T-TBS (1 h) at room temperature and then incubated with primary antibodies diluted in blocking buffer (1 h) at room temperature or overnight at 4 °C. Antibodies used for Western blotting were diluted as follows: anti-p21 (F-5, 1:750), anti-p27 (F-8, 1:200), anti-cyclin D2 (M-20, 1:2000), anti-pRb (clone G3–245, 1:750, Pharmingen). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Santa Cruz Biotechnology) diluted in blocking buffer at 1:10,000. Western blots were developed with chemiluminescence reagent (PerkinElmer Life Sciences). In some experiments, blots were incubated with 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM 2-mercaptoethanol at 50 °C for 30 min and reprobed with other primary antibodies.

Immunoprecipitation and Cdk Kinase Assay—RCS cells growing on 100-mm dishes were treated with vehicle or 3 μM FGF2 for 12 h. Cells were then lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100 containing 10 μM β-glycerophosphate, phosphate buffer inhibitor mixtures I and II (Sigma), 2 mM phenylmethylsulfonyl ﬂuoride (Sigma), and 132 μg/ml aprotinin (Sigma). Protein concentration of the lysates was determined using protein assay kit (Bio-Rad). Samples were then diluted by adding the same volume of 2× dye containing 20% glycerol, 4% 2-mercaptoethanol (Sigma), and equal amounts of protein were resolved on 10% SDS gel for pRb or 12.5% gel for all other proteins. Proteins were then transferred onto Immobilon-P membranes (Millipore Corp.). After being washed twice with 0.1% Tween 20, 10 mM Tris-HCl, 150 mM NaCl (pH 8.0) (T-TBS), blots were incubated with 5% nonfat dry milk in T-TBS (1 h) at room temperature and then incubated with primary antibodies diluted in blocking buffer (1 h) at room temperature or overnight at 4 °C. Antibodies used for Western blotting were diluted as follows: anti-p21 (F-5, 1:750), anti-p27 (F-8, 1:200), anti-cyclin D2 (M-20, 1:2000), anti-pRb (clone G3–245, 1:750, Pharmingen). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Santa Cruz Biotechnology) diluted in blocking buffer at 1:10,000. Western blots were developed with chemiluminescence reagent (PerkinElmer Life Sciences). In some experiments, blots were incubated with 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM 2-mercaptoethanol at 50 °C for 30 min and reprobed with other primary antibodies.

RESULTS

Effects of FGF2 on Chondrocyte Proliferation—RCS cells display numerous chondrocytic characteristics, including expression of type II and type IX collagens and production of cartilaginous matrix (23). In preliminary studies, we conﬁrmed that RCS cells expressed FGFR2 and R3, which were phosphorylated at tyrosine residues upon treatment with FGF2 (data not shown). First, we assessed the effects of FGF2 on cellular proliferation by measuring [%H]thymidine incorporation as well as DNA content. As shown in Fig. 1A, FGF22 suppressed thymidine incorporation in a dose-dependent manner from 10 to 1000 pM. Growth inhibition by FGF22 was conﬁrmed by dose-dependent decreases of DNA content in RCS cells continuously treated with 3–100 μM FGF22 (Fig. 1B).

Then we analyzed the effects of FGF2 on cell cycle distribution of RCS cells. As shown in Fig. 2, vehicle-treated cells showed an almost identical distribution of cells across the cycle throughout the time course studied (G1, 58.9–63.4%; S, 16.3–17.8%; G2/M, 16.9–19.7%) except for a slight increase in the G1 population at 24 h. FGF22-treated cells ﬁrst accumulated in G1 phase at 12 and 24 h (73.90 and 77.94%, respectively), which was associated with a further reduction of cells in S phase (1.17% at 24 h). The G2/M population of FGF22-treated cells decreased dramatically between 6 and 12 h and remained at this level at 24 h. The distribution pattern of FGF22-treated cells at 24 h remained unchanged at least up to 36 h (data not shown).
FGF Induces p21 in Chondrocytes

Effects of FGF2 on Expression of G_{1}/S Cell Cycle Proteins—To understand the mechanisms of FGF-induced G_{1} arrest of RCS cells, we examined the effects of FGF2 on expression of G_{1}/S cell cycle proteins. As shown in Fig. 3, FGF2 induced p21 protein expression by 6 h, and its expression peaked at 12 h. In contrast, p57 was undetectable either in the presence or absence of FGF2 (data not shown). Also, whereas p27 expression was detectable at 12 and 24 h, FGF2 did not affect the level of its expression. We then examined the phosphorylation state of pRb by Western blotting, using an antibody to pRb that recognizes both hyperphosphorylated (ppRb) and underphosphorylated pRb in Fig. 3). FGF2 shifted pRb from hyperphosphorylated, inactive forms to underphosphorylated, active forms by 6 h. By 12 h and thereafter, the majority of pRb was in the underphosphorylated, active form in FGF2-treated cells. This paralleled the increased p21 protein expression as well as the increase of cells in G_{1}. A partial shift of pRb to underphosphorylated forms was observed in vehicle-treated cells also at 12 and 24 h, which might be related to the slight increase in G_{1} cells in vehicle-treated RCS at 24 h. Parallel to the shift of pRb to its active form, expression of cyclin A, whose expression is regulated by E2F, was dramatically reduced at 12 and 24 h. In contrast, whereas cyclin D2 expression was dramatically increased by FGF2, cyclin E expression was unaffected during the time studied. Furthermore, FGF2 did not change the expression levels of Cdk2 or Cdk4.

Effects of FGF2 on Expression of G_{1/S} Cell Cycle Proteins—To understand the mechanisms of FGF-induced G_{1} arrest of RCS cells, we examined the effects of FGF2 on G_{1/S} cell cycle proteins. We then examined the effects of FGF2 on expression of G_{1/S} cell cycle proteins. As shown in Fig. 3, FGF2 induced p21 protein expression by 6 h, and its expression peaked at 12 h. In contrast, p57 was undetectable either in the presence or absence of FGF2 (data not shown). Also, whereas p27 expression was detectable at 12 and 24 h, FGF2 did not affect the level of its expression. We then examined the phosphorylation state of pRb by Western blotting, using an antibody to pRb that recognizes both hyperphosphorylated (ppRb) and underphosphorylated pRb in Fig. 3). FGF2 shifted pRb from hyperphosphorylated, inactive forms to underphosphorylated, active forms by 6 h. By 12 h and thereafter, the majority of pRb was in the underphosphorylated, active form in FGF2-treated cells. This paralleled the increased p21 protein expression as well as the increase of cells in G_{1}. A partial shift of pRb to underphosphorylated forms was observed in vehicle-treated cells also at 12 and 24 h, which might be related to the slight increase in G_{1} cells in vehicle-treated RCS at 24 h.
substrate, as described under “Experimental Procedures.” Protein complexes were assayed for kinase activities using GST-Rb as a substrate, as described under “Experimental Procedures.”

Effects of FGF on Cip/Kip Expression in Limb Explants—We then examined whether FGF2 induces p21 in chondrocytes in the growth plate in limbs isolated from mouse fetuses. As shown in Fig. 5, p21 protein expression was limited to hypertrophic chondrocytes and some proliferating chondrocytes residing in the peripheral region of the lower half of the proliferating zone in vehicle-treated limbs (Fig. 5a). In contrast, after 8-h treatment with FGF2, p21 immunoreactivity was seen in almost all of the chondrocytes (Fig. 5, b and c). This widespread pattern of p21 immunoreactivity in growth plate chondrocytes was observed also at 16 and 24 h after FGF2 treatment (data not shown).

Effects of FGF on Chondrocytic Proliferation in p21−/− Limb Explants—Next, we examined whether inhibitory effects of FGF2 on chondrocytic proliferation differ in the growth plate of p21−/− mice, compared with WT mice, by comparing mRNA expression for H4 histone (H4), a marker for cells in S phase (27). To avoid the interference caused by developmental variability among littersmates, we treated one hind limb with vehicle alone and the contralateral hind limb with the indicated amounts of FGF2. As shown in Fig. 6, H4 expression was detected in cells in the proliferating zone but not in the hypertrophic zone in vehicle-treated tibia from WT and p21−/− mice. In both WT and p21−/− tibias, FGF2 dose-dependently decreased the number of H4-positive cells in the proliferating zone. However, whereas 1.5 nM FGF2 dramatically decreased the number of H4-positive chondrocytes in the proliferating zone in WT limbs, the number of H4-positive chondrocytes in the proliferating zone in p21−/− limbs was unaffected. Moreover, whereas 6 and 24 nM FGF2 completely suppressed the chondrocytic proliferation in WT tibia, as evidenced by the absence of H4 mRNA expression, chondrocytic proliferative response was only partially inhibited in p21−/− tibias at these doses of FGF2.

As shown in Fig. 7, when tibias were treated with 1.5 nM FGF2 or vehicle alone for 2 days, FGF2 dramatically reduced both the overall size of the growth plate and the thickness of the hypertrophic zone in WT tibia, compared with the vehicle-treated control. In contrast, neither the growth plate size nor the thickness of the hypertrophic zone in p21−/− tibia was affected as dramatically as WT tibia. 2 days of 24 nM FGF2 treatment decreased both of these in p21−/− tibias as it did in WT (data not shown), consistent with the effects on H4 expression.

**DISCUSSION**

Although FGF and its receptor are known to negatively regulate cartilage proliferation, little is known about the mechanisms involved. In this study, we examined the effects of FGF2 on the cell cycle inhibitor, p21, in both cell and fetal limb cultures to understand the growth-inhibitory pathway of FGF signaling in chondrocytes.

Unlike the effects of FGF2 on primary chondrocytes and ATDC5 cells, FGF2 inhibits growth of RCS cells, thus mimicking its effects *in vivo*. In this study, we took advantage of the antiproliferative response of RCS cells to analyze the growth-inhibitory effects of FGF2.
inhibitory mechanisms of FGF in chondrocytes. FGF2 arrests the cell cycle of RCS cells mainly at G1, a stage known to be controlled by pRb activity (28). pRb is underphosphorylated and exerts its antiproliferative function in G1, and hyperphosphorylation of pRb by cyclin D-Cdk4 and cyclin E-Cdk2 complexes inactivates it at the G1/S transition, allowing the cell to proceed into S phase (29–32). Several lines of evidence indicate that the cell cycle arrest at G1 is linked to the decreased kinase activity of cyclin D-Cdk4 or cyclin E-Cdk2 complexes as well as to the underphosphorylation of pRb (32–36). Consistent with these, the time course of G1 accumulation and exclusion from S phase in RCS cells after FGF2 treatment was synchronized to the underphosphorylation of pRb and decrease in cyclin A, whose expression is regulated by E2F family members through inactivation (hyperphosphorylation) of pRb (16). Moreover, all of these events (i.e. G1 accumulation, underphosphorylation of pRb, and decrease in cyclin A) were associated with p21 expression but not with p27, cyclin D2, cyclin E, Cdk2, and Cdk4 (Figs. 2 and 3).

On the other hand, a slight increase in G1 population and partial underphosphorylation of pRb also was observed in vehicle-treated RCS cells. These events were associated with the expression of p21 and p27 at 12 and 24 h (Fig. 3). Since RCS cells grow very rapidly, with the doubling time shorter than 16 h, and their growth is contact-inhibited, and because we started the experiment when cells were 70% confluent, it is highly likely that the apparent G1 arrest and the associated increase in p21 and p27 expression at later time points were caused by contact inhibition.

The involvement of p21 in G1 cell cycle arrest of RCS cells was further examined by coimmunoprecipitation and cyclin-dependent kinase assay. We found that FGF2 induced the assembly of cyclin E-Cdk2-p21, cyclin D2-Cdk4-p21, and cyclin D2-Cdk2-p21 complexes. Also, we demonstrated that FGF2 dramatically reduced the activity of Cdk2 and cyclin E-dependent kinases. Although FGF2 increased the expression of cyclin D2 in RCS cells, cyclin D2-dependent kinase activity was not affected by FGF2.
Our findings with limb explants show that the increase in p21 expression induced by FGF2 in vitro also is evident in whole growth plates. Moreover, higher doses of FGF2 are required to inhibit cartilaginous growth in limbs from p21−/− mice compared with those from WT mice. This further highlights the importance of p21 in the mechanisms by which FGF2 mediates inhibition of cartilage growth.

In contrast to our data, Li et al. (9) have reported that Ink4 family proteins but not p21 were activated in postnatal growth plates of mice carrying the mutant FGFR3 gene (K644E). We did not examine Ink4 protein expression in this study, because FGF2 did not change the activity of Cdk4 or cyclin D2-dependent kinase in RCS cells. The apparent discrepancy between our in vitro data and their in vivo data may be explained by a possible qualitative difference in signaling pathways in ligand-induced activation of WT FGFR and ligand-independent activation of mutant FGFR3 (K644E), or FGF's cell cycle regulation may involve different cell cycle inhibitors in fetal and postnatal growth plates.

In summary, our data indicate that FGF's inhibitory effects on chondrocytic proliferation appear to be mediated at least partially through p21 induction and the subsequent inactivation of cyclin E-Cdk2 and activation of pRb.

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