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ARTICLES

Fibroblast Growth Factor-2 (FGF-2) Increases N-Cadherin Expression Through Protein Kinase C and Src-Kinase Pathways in Human Calvaria Osteoblasts

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Abstract Fibroblast growth factors (FGFs) are important factors regulating osteogenesis. However, the early mechanisms and signaling pathways involved in FGF actions in osteoblasts are unknown. We investigated the effects of FGF-2 on cell–cell adhesion and cadherin expression and the underlying signaling pathways in immortalized human neonatal calvaria (IHNC) cells. These cells express E- and N-cadherins, as shown by immunocytochemical and Western blot analyses. rhFGF-2 increased cell–cell adhesion at 24–72 h, as measured in a cell aggregation assay, and this effect was blocked by specific neutralizing anti-N-cadherin, but not anti-E-cadherin antibodies. Accordingly, ELISA and Western blot analyses showed that rhFGF-2 (10–100 ng/ml) dose dependently increased N-cadherin but not E-cadherin protein levels. RT-PCR analysis showed that rhFGF-2 transiently increased N-cadherin mRNA levels in IHNC cells. The RNA polymerase II inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole prevented the rhFGF-2-induced up-regulation of N-cadherin mRNA, suggesting that transcription is necessary for this effect. Analysis of signaling molecules showed evidence that PLCγ-PKC, Src, Erk 1/2 and p38 MAPK pathways are activated by rhFGF-2 in IHNC cells. The selective PKC inhibitors calphostin C, Ro-31-8220, Gö6976 and Gö6983 abrogated the stimulatory effect of rhFGF-2 on N-cadherin mRNA levels. The src-family tyrosine kinase inhibitor PP1 also blocked rhFGF-2-promoted N-cadherin expression. In contrast, the p38 MAP kinase inhibitor SB 203580 or the MEK inhibitor PD98059 had no effect on rhFGF-2-induced N-cadherin mRNA levels. Our data indicate that FGF-2 increases N-cadherin expression and function in human calvaria osteoblasts via activation of PKC and src-kinase pathways. This study identifies N-cadherin as a previously unrecognized target gene for FGF-2 signaling pathway that regulates cell–cell adhesion in human osteoblasts. J. Cell. Biochem. 81:68–81, 2001. © 2001 Wiley-Liss, Inc.

Key words: N-cadherin; osteoblasts; FGF-2; PKC; src

Fibroblast growth factors (FGFs) are a family of polypeptides that are important factors controlling cell proliferation, differentiation and survival in a variety of tissues [Baird and Klagsbrun, 1991]. The skeleton is an important target tissue for FGFs as these factors are involved in bone development, growth, remodeling and repair [Rodan et al., 1989; Hurley and Florkiewitz, 1996; Marie et al., 2000]. FGF-2, a prototype of the FGF family, stimulates proliferation of osteoblastic cells derived from calvaria [Canalis et al., 1988; Globus et al., 1988; McCarthy et al., 1989; Debiais et al., 1998] or bone marrow stroma [Noff et al., 1989; Pitaru et al., 1993; Scutt and Bertram, 1999]. FGF-2 also regulates osteoblast differentiation genes [Hurley et al., 1993; Schedlich et al., 1994; Boudreaux and Towler, 1996; Debiais et al., 1998; D'Souza et al., 1999] although the effects are dependent on the stage of osteoblast maturation [Debiais et al., 1998]. The cellular actions of FGFs are known to be mediated by interactions with fibroblast growth factor receptors (FGFRs), a family of tyrosine kinase receptors [Jaye et al., 1992; Johnson and Williams, 1993; Wang et al., 1994]. In several cell types, FGF binding to FGFR induces receptor dimerization, intrinsic tyrosine phosphorylation and

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activation of multiple signal transduction pathways including Raf, mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinases (Erks), src and p38 MAP kinases, phospholipase Cγ (PLCγ), and protein kinase C (PKC) [Burgess et al., 1990; Zhan et al., 1994; Kuo et al., 1997; Maher, 1999]. Several PKC isoforms have been described according to their ability to be activated by diaglycerol (DAG), phosphatidylserine or calcium [Nishizuka, 1986]. They have been classified into calcium- and DAG-dependent isoforms (α, βI, βII and γ isoforms), calcium-independent and DAG-dependent isoforms (θ, η, δ and ε isoforms) and calcium- and DAG-independent isoforms (ζ, λ and μ isoforms) [Newton, 1995]. In osteoblasts, α, βI, ε, ζ, and λ PKC isoenzymes have been identified [Sanders and Stern, 1996]. Although FGF-2 was shown to activate Erk 1,2 and p38 MAP kinases in osteoblasts [Chaudhary and Avioli, 1997; Kozawa et al., 1999; Suzuki et al., 2000], the signaling pathways that mediate the biological actions of FGF-2 in osteoblasts remain largely unknown.

Cadherins are single chain transmembrane glycoproteins that mediate homophilic, calcium-dependent cell–cell adhesion [Takeichi, 1990]. Cadherins are crucial for cell–cell interactions and their spatiotemporal expression is essential in controlling early stages of morphogenesis during embryogenesis and differentiation [Takeichi, 1991; Grunwald, 1993]. A variety of cadherins are expressed in osteoblastic cells, albeit this varies with the cell type. In vivo, chicken and human calvaria osteoblasts express the classical members of the cadherin family, N-cadherin, E-cadherin, and neural cell adhesion molecule (N-CAM) [Lee and Chuong, 1992, Lemonnier et al., 1998]. In vitro, human osteoblasts were found to express N-CAM, cadherin-4 and cadherin-11 [Cheng et al., 1998; Lemonnier et al., 1998]. N-cadherin and E-cadherin are also expressed in rat, mouse or human osteoblasts in culture [Babich and Foti, 1994; Lemonnier et al., 1998; Tsutsumimoto et al., 1999; Haÿ et al., 2000], whereas OB N-cadherin has been described in murine and human osteoblastic cells [Okazaki et al., 1994; Cheng et al., 1998]. Interestingly, some cadherins were recently found to be regulated by local factors in osteoblastic cells [Cheng et al., 1998; Tsutsumimoto et al., 1999; Haÿ et al., 2000]. Moreover, recent data point to a role of cadherins in osteoblast differentiation [Cheng et al., 1998; Lemonnier et al., 1998; Ferrari et al., 2000; Haÿ et al., 2000]. We previously reported that activating FGFR-2 mutations in Apert craniosynostosis [Lomri et al., 1998] induce over-expression of E- and N-cadherins in human calvaria cells [Lemonnier et al., 1998], suggesting that FGFR activation may control E- and N-cadherin expression in human osteoblasts. This prompted us to determine the effect of FGF-2 on cell–cell adhesion and E- and N-cadherin expression and to analyze the signaling pathways involved in the effects of FGF-2 in human calvaria osteoblastic cells. In this paper, we provide the first evidence that FGF-2 increases N-cadherin expression and function in human calvaria osteoblasts. We also show that this effect involves PKC and src signaling pathways, which identifies N-cadherin as a previously unrecognized target gene downstream of the FGF-2 signaling pathway in human osteoblasts.

MATERIALS AND METHODS

Cell Cultures and Treatments

Normal coronal sutures were obtained from 1-day neonate undergoing local surgery, according to the French ethical recommendations. Calvaria cells were obtained by collagenase digestion from the coronal sample as described previously [De Pollak et al., 1997]. The cells were immortalized by transfection with the SV-40 oncogene as described [Lomri et al., 1999] and called immortalized human neonatal calvaria (IHNC) cells. These cells express the large T-antigen and are stable at least until the last passage tested (13th). Cells at the 6–9th passage were used for the experiments described below. Previous studies showed that these cells express alkaline phosphatase, type 1 collagen, the specific osteoblast transcription factor CBFA1 and osteocalcin at the mRNA level [Haÿ et al., 2000]. Part of the cell population (50%) showed alkaline phosphatase activity whereas all cells (100%) expressed immunoreactive type 1 collagen and osteopontin in basal conditions [Haÿ et al., 2000]. These osteoblast phenotypic characteristics are similar to those of non-immortalized neonatal human calvaria cells of the same age [Haÿ et al., 1999].

The cells were cultured in DMEM supplemented with glutamin (292 mg/L), 10% heat inactivated FCS, and antibiotics (100 IU/ml

penicillin and 100 μg/ml streptomycin). In preliminary experiments, the response of IHNC cells to recombinant human (rh) FGF-2 (Sigma) was tested by treating pre-confluent cells cultured in DMEM with 1% FCS with grading concentrations of rhFGF-2 (1–100 ng/ml) and measuring cell proliferation (determined by cell number and DNA synthesis), changes in alkaline phosphatase (ALP) activity and type 1 collagen. The maximal stimulatory effect for cell proliferation and inhibitory effect for ALP and type 1 collagen activity was obtained at 50–100 ng/ml (data not shown) and subsequent studies were performed at the optimal dose of 50 ng/ml.

Immunocytochemical Analysis

For immunocytochemical analysis of cadherins, preconfluent cells were cultured in serum-free medium for 24 h, then fixed in acetone/methanol (1:1), washed in PBS/0.1% Triton X-100, incubated 1 h at 37°C with 3% BSA/goat serum to block unspecific binding, then exposed 2 h at 37°C to mouse monoclonal antibodies against N-cadherin (1/100; Sigma), E-cadherin (1/200; Zymed) or N-CAM (1/100; Sigma) in PBS/1% BSA. These antibodies do not show cross-reactivity with other cadherins [Volk and Geiger, 1984]. Control sections were incubated with PBS/1% BSA. After 2 h exposure at 37°C, the cells were washed three times in PBS and exposed 1 h at room temperature to goat anti-mouse IgG (1:50) linked to colloidal gold particles (IntenSETMM, Amersham, Arlington Heights, IL). After washing, the gold particle staining was enhanced by precipitation of metallic silver (ImmunoGold Silver Staining). Microphotographs of representative cells were performed in dark field using a BH2 Olympus microscope.

Cell Aggregation Assay

For cell aggregation assays, confluent IHNC cells were treated with rhFGF-2 (50 ng/ml) or the vehicle for 24–72 h, then the cells were cultured in aggregates by pooling 10^6 cells/well on bacteriological grade culture dishes (Falcon, Becton-Dickenson), and cell–cell adhesion was assayed as described [Hamaguchy et al., 1993] with minor modifications [Haÿ et al., 2000]. Briefly, IHNC cells were allowed to aggregate for 60 min at 37°C on a gyratory shaker, and cell aggregation was evaluated by the ratio of the total cell number plated to the number of total particles (i.e., single cells and cell clusters) per well after incubation.

To determine the role of N- and E-cadherins in the effect of rhFGF-2 on cell aggregation mediated by these cell–cell adhesion molecules, IHNC cells treated with rhFGF-2 (50 ng/ml, 48 h) or the vehicle were allowed to aggregate as described above, in the presence of neutralizing anti-N-cadherin or anti-E-cadherin antibodies (10 μg/ml) or a non-specific IgG (Dako, Denmark), and cell aggregation was determined as described above.

Elisa and Western Blot Analyses

To determine the effects of rhFGF-2 on N-cadherin, E-cadherin and N-CAM synthesis, the levels of these proteins were determined by ELISA. IHNC cells plated in 96-well plates were cultured in 1% FCS with 0.1% BSA for 24 h and then treated with rhFGF-2 (50 ng/ml) or the vehicle. After 24–48 h, the cells were fixed in acetone/methanol for 20 min at −20°C, washed in PBS, then incubated in PBS and 3% BSA to saturate non-specific binding sites. After rinsing in PBS, the cells were exposed for 1 h to anti-N-cadherin, anti-E-cadherin or anti-N-CAM antibodies diluted 1/150 in PBS/1% BSA. Control cells were incubated in PBS/1% BSA. After rinsing, appropriate anti-murine second antibody linked to peroxydase and diluted 1/500 in PBS/1% BSA was added for 1 h. The cells were washed, and specific immunoreactivity for N-cadherin, E-cadherin and N-CAM was revealed using *o*-phenylenediamine dihydrochloride/Na citrate (pH 5) and H_2O_2 and measured optically at 492 nm.

To further document the selective effect of FGF-2 on N-cadherin synthesis, IHNC cells were treated with rhFGF-2 (50 ng/ml), and N-cadherin and E-cadherin protein levels were determined by Western blot analysis. A dose–response analysis was performed by treating IHNC cells with rhFGF-2 (10–100 ng/ml) or the vehicle for 24 or 36 h, and N-cadherin protein levels were determined. Cell extracts were prepared by adding 1 ml of lysis buffer (10 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na_3VO_4, 10% glycerol, 0.5% Triton X-100 and protease inhibitors). Lysates were clarified by centrifugation at 12,000*g* for 10 min at 4°C and the protein content of supernatants was determined using the DC Protein Assay (Bio-

Rad Laboratories, CA). Proteins were subjected to electrophoresis on SDS-polyacrylamide gel and actin was used as internal control for protein loading. Proteins were transferred onto PVDF membranes (Hybond-P, Amersham) in buffer containing 20% methanol. The membranes were incubated overnight with 1% blocking buffer (Boehringer) in TBS (50 mM Tris–HCl, 150 mM NaCl) containing 0.1% Tween 20, and then for 1 h at room temperature with N-cadherin antibody, E-cadherin antibody or a polyclonal antibody against β-actin (Sigma, St Louis, MI) in 0.5% blocking buffer, washed three times for 10 min with TBS/0.1% Tween 20, then once in 0.5% blocking buffer, and incubated for 1 h with horseradish peroxydase (HRP)-conjugated secondary antibody (Amersham). After four washes of the membranes in TBS/0.1% Tween 20, the signal was visualized with BM chemiluminescence blotting substrate (Boehringer Mannheim, Germany).

RT-PCR and Southern Blot Analyses

To determine the early effects of FGF-2 on N-cadherin mRNA levels, IHNC cells were treated with rhFGF-2 (50 ng/ml) or the vehicle for 4–48 h. At different time points, the cells were washed with PBS and lysed with Extract-All (Eurobio) reagent according to manufacturer's instructions. The levels of N-cadherin mRNA were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Optimization of RT-PCR results was carried out by generating saturations curves of RT-PCR products against cycle number (0–30 cycles) in order to allow semi-quantitative variation of product levels [Haÿ et al., 2000]. The same cycle number (20 cycles) was used for N-cadherin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for which the amplification was linear. Three micrograms of total cellular RNA from each sample were reverse transcribed and the cDNA samples were divided and amplified using specific primers (20 pmol/tube) for N-cadherin, E-cadherin or GAPDH [Haÿ et al., 2000]. In semi-quantitative RT-PCR analysis, the amount of primers for GAPDH was reduced to 1 pmol/tube in order to avoid saturation. The primer sequences used for N-cadherin are sense: 5′-CAAGTGCCATTAGCCAAGG-3′, antisense: 5′-TTAAGCCGAGTGATGGTCC-3′, internal: 5′-GTTGTCAACAT GGTACCGGC-3′. Southern blots were performed by running aliquots of amplified cDNA on 1.3% agarose gel followed by transfer onto nylon membrane according to the manufacturer's protocol. Hybridization of blots was carried out overnight at 55°C with (γ^{32}P)ATP-labeled internal primers. Membranes were washed twice in $2 \times$ SSC/0.1% SDS at room temperature for 15 min, once in $0.1 \times$ SSC/0.1% SDS at 55°C for 5 min, then the filters were exposed to X-ray films. Autoradiographic signals were quantified using a scanner densitometer (Transydine General Corporation, Ann Arbor, MI). The signal for each gene was corrected for GAPDH.

To determine whether transcription was required for the up-regulation of N-cadherin gene expression by FGF-2, IHNC cells were pre-treated with 25 µg/ml of 5,6-dichloro-1β D-ribofuranosyl benzimidazole (DRB, Sigma), a selective inhibitor of RNA polymerase-II elongation [Chodosh et al., 1989]. Cells were exposed to DRB for 15 min before and throughout a 16 h treatment with rhFGF-2 or the vehicle, and semi-quantitative RT-PCR analysis of N-cadherin and GAPDH was performed as described above.

Signal Transduction Analyses

To determine the signal transduction pathways that are activated by FGF-2 in cells, IHNC cells were cultured in DMEM in 1% FCS/0.1% BSA in the presence of rhFGF-2 (50 ng/ml) or the vehicle for the indicated time. Cells were then frozen in liquid nitrogen and proteins were extracted in lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP 40, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 nM calyculin A, 50 nM microcystin LR, 2 mM Na_3VO_4). Western blot analysis was performed as described previously [Suzuki et al., 2000]. Essentially, equal amounts of lysate proteins were incubated with agarose-conjugated anti-phosphotyrosine antibody (4G10; Upstate Biotechnology Inc., Lake Placid, NY) for 18 h at 4°C. Immunoprecipitates were washed three times in lysis buffer and proteins were solubilized in reducing sample buffer containing 125 mM Tris, pH 6.8, 100 mM dithiothreitol, 10% glycerol, 0.025% bromophenol blue and 2% SDS. Proteins were then fractionated by reducing SDS/PAGE acrylamide gradient gels and transferred to Immobilon P membranes according to the manufacturer's instructions (Millipore Corporation, Bedford, MA). Western blot analysis was performed using recombinant

anti-phosphotyrosine antibody RC20 linked to horseradish peroxidase (Transduction Laboratories, Lexingthon, KY), anti-PLCγ, anti-Src kinases (SRC 2), anti-phospho-Erk1,2 and anti-phospho-p38 (Santa Cruz, CA).

For direct analysis of PKC activation, confluent IHNC cells were cultured in DMEM with 1% FCS/0.1% BSA for 24 h, then treated with rhFGF-2 (50 ng/ml) or the vehicle for 10–60 min. The cells were lysed in lysis buffer (25 mM Tris–HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 mM PMSF). PKC activity was determined by measuring the transfer of ^{32}P-labeled phosphate to a biotinylated peptide substrate (AAKIQAS*FRGHMARKK) that is specific for PKC activity [Chen et al., 1993] using the Signa TECT PKC Assay System (Promega).

Selective Inhibition of Signaling Pathways

To determine the signal transduction pathways involved in the effect of FGF-2 on N-cadherin expression, we used selective inhibitors of the signaling pathways that we found to be activated by FGF-2 in IHNC cells. We used calphostin C (Biomol Research Laboratories, Plymouth, PA; 0.1–2 μM), a potent and selective inhibitor of PKC [Tamaoki and Nakano, 1990], Ro-31-8220 (10–100 nM; Calbiochem, San Diego, CA) a selective inhibitor of membrane-bound and cytosolic PKC, Gö6976 (1–1,000 nM; Calbiochem) a PKC inhibitor with high selectivity for the PKCα isoform [Martiny-Baron et al., 1993; Shih et al., 1999] and Gö6983 (1–1000 nM; Calbiochem), a selective inhibitor of PKCα, β and γ isotypes [Wang et al., 1998]. For other signaling pathways, we used 2′-amino-3-methoxyflavone (PD-98059; Biomol), a specific inhibitor of MEK activation [Alessi et al., 1995], 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazol (SB 203580; Calbiochem), a highly specific inhibitor of p38 MAP kinase [Kramer et al., 1996] and PP1 (Calbiochem), a selective inhibitor of src kinases. IHNC cells were pretreated for 2 h with the indicated signaling inhibitor or the vehicle, then treated with rhFGF-2 (50 ng/ml) or the vehicle in the presence of the inhibitor or the vehicle for 16 h. N-cadherin mRNA levels were determined by RT-PCR analysis using GAPDH as control as described above.

Data Analysis

All experiments were repeated 2–4 times and the results presented are representative of all experiments. The data are expressed as the mean ± SEM and were analyzed using the statistical package super-ANOVA (Macintosh, Abacus concepts, Inc., Berkeley, CA). Differences between the mean values were evaluated with a minimal significance of $P < 0.05$.

RESULTS

Human Neonatal Calvaria Cells Express N- and E-Cadherins

Our previous studies showed that N-cadherin, E-cadherin and N-CAM are expressed in human preosteoblasts and osteoblasts in the normal human calvaria in vivo [Lemonnier et al., 1998]. We examined whether these cadherins are also expressed in human calvaria cells in vitro. In basal conditions, most IHNC cells cultured in monolayer expressed E-cadherin (Fig. 1B) and N-cadherin immunoreactivity (Fig. 1C). Control cells were negative (Fig. 1A). IHNC cells also expressed N-CAM, albeit to a lower extent (not shown). E- and N-cadherin immunoreactivity was localized at the membrane level and was higher in cells that are densely connected with other cells, consistent with the role of these cell–cell adhesion molecules (Fig. 1B,C).

FGF-2 Increases Cell–Cell Adhesion

We then determined whether FGF-2 affected cell–cell adhesion in IHNC cells and whether this was dependent on N- or E-cadherins. Figure 2A shows that rhFGF-2 (50 ng/ml) significantly increased cell–cell adhesion by about 130% in IHNC cells compared to control cells, as measured in a cell aggregation assay. This FGF-2-induced promotion of cell–cell adhesion was found at 24, 48 and 72 h in several repeated experiments. To determine the respective role of N- and E-cadherins in this effect, we tested the effects of neutralizing anti-N-cadherin and E-cadherin antibodies. In basal conditions, N-cadherin and E-cadherin antibodies did not significantly decrease cell–cell adhesion (Fig. 2B). In cells treated with rhFGF-2 (50 ng/ml; 48 h), the anti-E-antibody induced a weak inhibitory effect on cell–cell adhesion, which did not differ from the effect of the non-specific IgG (Fig. 2B). In contrast, the anti-N-cadherin antibody decreased cell–cell aggregation com-

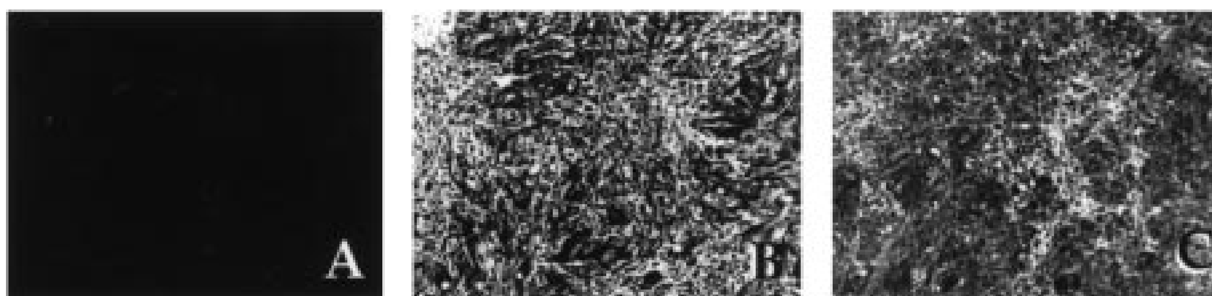


Fig. 1. IHNC cells express E-cadherin and N-cadherin. Cells were serum starved for 24 h, fixed and exposed to mouse monoclonal antibodies against E-cadherin (**B**), N-cadherin (**C**), or PBS/1% BSA (Control, **A**), exposed to goat antimouse IgG linked to colloidal gold particles, and gold particle staining was enhanced by precipitation of metallic silver and visualized in black field. Membrane-bound immunorective E- and N-cadherins are apparent on most cells. Original magnification × 125.

pared to the non-specific IgG, and abolished the stimulatory effect of rhFGF-2 on cell–cell adhesion (Fig. 2B). Similar data were consistently observed in repeated experiments. These results show that FGF-2 promotes cell–cell adhesion in IHNC cells and that N-cadherin is involved in the effect of rhFGF-2 on IHNC cell–cell adhesion.

FGF-2 Increases N-Cadherin Synthesis in Human Calvaria Cells

We then determined by ELISA whether FGF-2 affected N-cadherin, E-cadherin or N-CAM synthesis in IHNC cells. As shown in Figure 3A, rhFGF-2 (48 h) increased N-cadherin levels by about 130%. In contrast, rhFGF-2 had no significant effect on E-cadherin or N-CAM protein levels (Fig. 3A). To confirm this finding, a Western blot analysis of N- and E-cadherins was performed in IHNC cells after rhFGF-2 stimulation. As shown in Figure 3B, rhFGF-2 (50 ng/ml, 48 h) increased N-cadherin but not E-cadherin protein levels in IHNC cells. A dose–response study was performed to further determine the early effect of rhFGF-2 on N-cadherin protein levels analysed by Western blot. As shown in Figure 4, rhFGF-2 induced a dose-dependent effect on N-cadherin protein levels. N-cadherin levels rose by 125% at 24 h and increased by 2.2–2.4-fold at 36 h exposure by rhFGF-2 (50–100 ng/ml) (Fig. 4B). These results show that FGF-2 dose-dependently increases N-cadherin protein levels in human calvaria cells.

FGF-2 Increases N-Cadherin mRNA in IHNC Cells

We then determined the effect of FGF-2 on the expression of N-cadherin mRNA levels. The

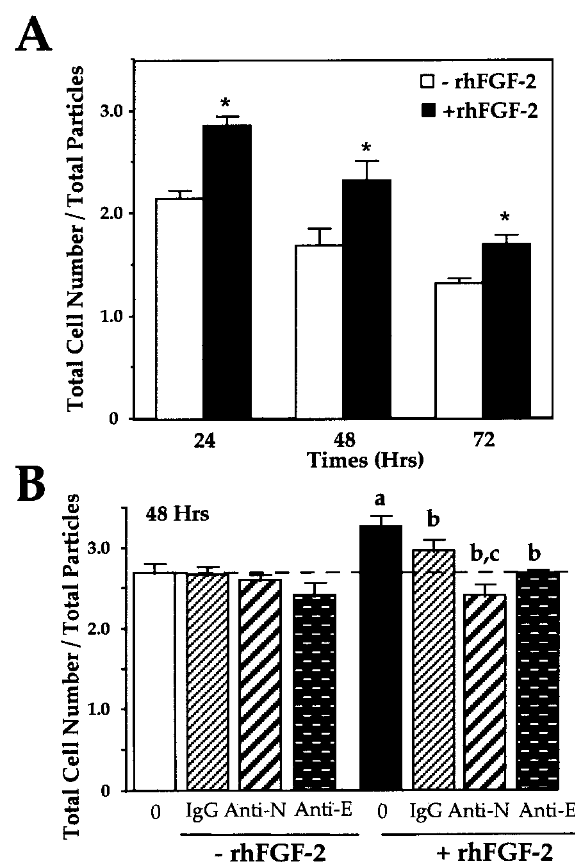


Fig. 2. rhFGF-2-increases cell–cell adhesion mediated by N-cadherin in IHNC cells. **A**: Cells treated with rhFGF-2 (50 ng/ml, 24–72 h) or the vehicle were allowed to aggregate and cell aggregation was determined as described in Section 2. The data are the mean ± SEM of five values; $^{*}P < 0.001$–0.05 vs. control cells. **B**: Cells treated with rhFGF-2 (50 ng/ml, 48 h) or the vehicle (0) were allowed to aggregate in the presence of anti-N-cadherin or anti-E-cadherin neutralizing antibodies or a non-specific IgG, and cell aggregation was determined. The data are the mean ± SEM of four values (a: $P < 0.01$ vs. control cells; b: $P < 0.01$ vs. cells treated with rhFGF-2 alone; c: $P < 0.01$ vs. cells treated with non-specific IgG).

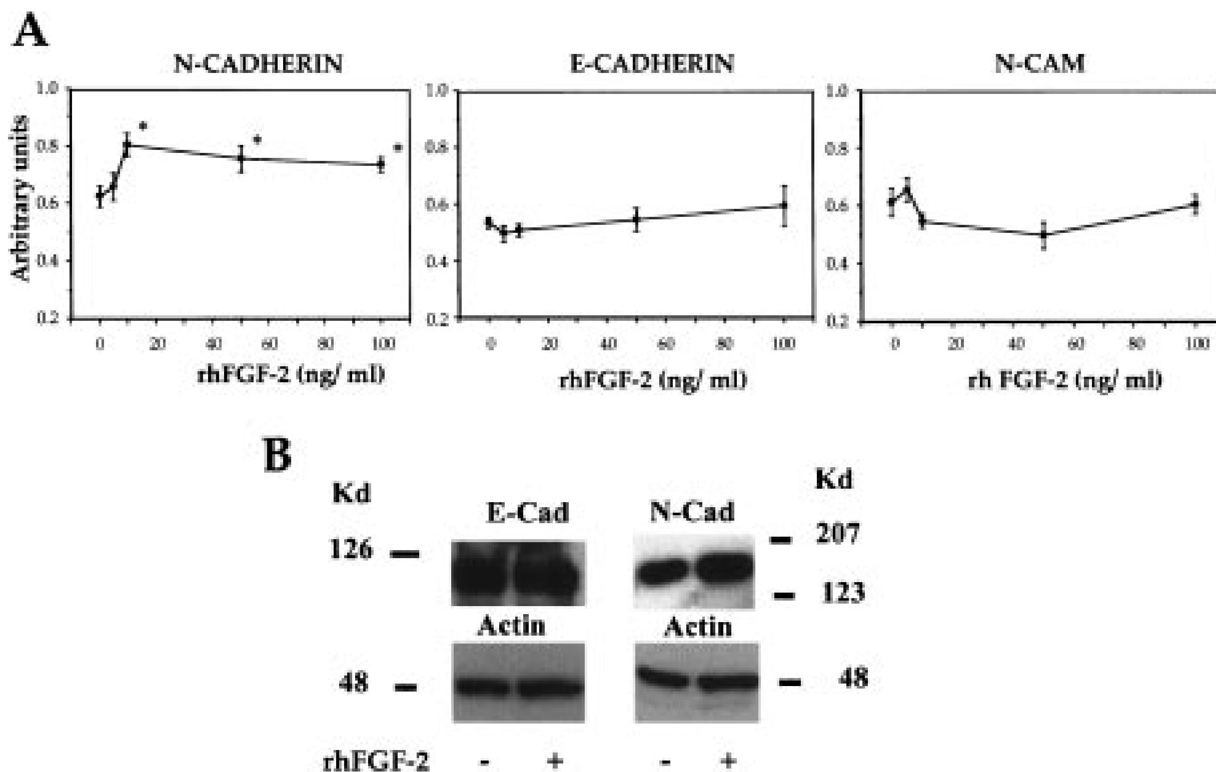


Fig. 3. rhFGF-2 increases N-cadherin protein levels in IHNC cells. The cells were treated with rhFGF-2 (50 ng/ml) or the vehicle (0) for 48 h and N-cadherin, E-cadherin and N-CAM protein levels were determined by ELISA (**A**) or Western blot analysis (**B**) Using β-actin as control of protein loading. The data are the mean ± SEM of four values (*$P < 0.01$ vs. vehicle-treated cells).

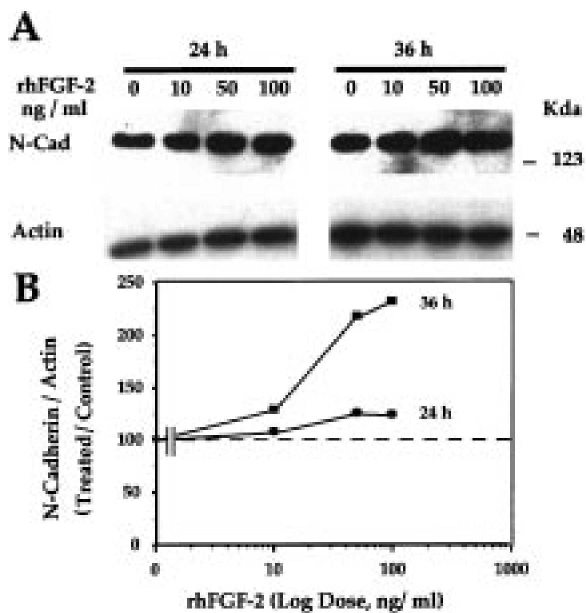


Fig. 4. Dose–response effect of rhFGF-2 on N-cadherin protein levels. IHNC cells were treated with rhFGF-2 or the vehicle (0) at the indicated dose, and N-cadherin levels were determined by Western blot analysis (**A**). Densitometric analysis was performed and the levels were corrected for protein loading using β-actin (**B**).

RT-PCR analysis showed that rhFGF-2 increased mRNA levels for N-cadherin. The time-course study showed that mRNA abundance increased at 4 h, peaked at 16 h and returned to control levels at 48 h of treatment (Fig. 5). In contrast, E-cadherin mRNA levels were not changed by rhFGF-2 (data not shown). Similar results were found in repeated experiments. To determine whether the stimulatory effect of FGF-2 on N-cadherin mRNA levels may involve transcriptional mechanisms, IHNC cells were pre-treated with DRB, a RNA polymerase II inhibitor [Chodosh et al., 1989], and N-cadherin mRNA levels were determined by semi-quantitative RT-PCR analysis in which cDNAs were amplified in the same aliquot [Haÿ et al., 2000]. As expected, rhFGF-2 (50 ng/ml, 16 h) increased N-cadherin mRNA levels by about 150% (Fig. 5A), confirming our previous results. In the presence of DRB, mRNA for N-cadherin decreased in control cells, and the stimulatory effect of rhFGF-2 was almost completely suppressed (Fig. 5B), suggesting that transcription is necessary for the observed increase in N-cadherin mRNA levels induced by rhFGF-2.

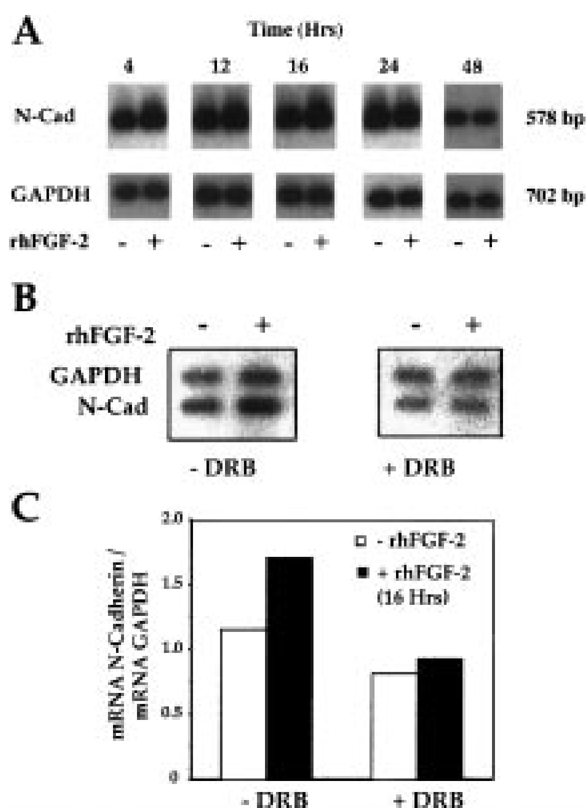


Fig. 5. The stimulatory effect of rhFGF-2 on N-cadherin mRNA levels is abrogated by the RNA polymerase DRB. **A**: IHNC cells were treated with rhFGF-2 (50 ng/ml) or the vehicle at the indicated time and N-cadherin mRNA levels were determined by RT-PCR analysis using GAPDH as internal control. **B**: Cells were exposed to DRB (25 μg/ml) for 15 min before and throughout a 16 h treatment with rhFGF-2 (50 ng/ml) or the vehicle, semi-quantitative RT-PCR analysis was performed as described in Section 2, and densitometric analysis of N-cadherin mRNA levels was performed using GAPDH used as internal control (**C**).

FGF Activates PLCγ, PKC and Src, Erk and p38 Kinases in IHNC Cells

To investigate which signaling pathways are involved in the stimulatory effect of FGF-2 on N-cadherin in IHNC cells, we analyzed alteration in signaling molecules known to be activated by FGF-2 in osteoblast-like cells using immunoprecipitation and Western blotting analyses [Suzuki et al., 2000]. Figure 6A shows that rhFGF-2 (50 ng/ml, 5–60 min) increased the tyrosine phosphorylation and/or the association with tyrosine phosphorylated proteins of multiple signaling molecules in IHNC cells including PLCγ and src kinases. Also shown in Figure 6A is the increased amount of phosphorylated Erk 1,2 and p38 MAP kinases, indicating their stimulation by rhFGF-2. In relation with evidences for activation of PLCγ by rhFGF-2 (Fig. 6A), changes in PKC activity was determined in IHNC cells treated with 50 ng/ml rhFGF-2 for 10–60 min. Figure 6B shows that rhFGF-2 increased PKC activity with a slow kinetic (+53% at 40 min; +98% at 60 min of treatment), which corresponds to the time-course of PLCγ stimulation induced by rhFGF -2 (Fig. 6A), suggesting the implication of a PLCγ-PKC pathway in rhFGF-2 signaling in IHNC cells.

PKC and Src are Involved in the Effect of FGF-2 on N-Cadherin in IHNC Cells

To further identify the role of signaling molecules in the stimulatory effect of FGF-2 on N-cadherin expression, IHNC cells were treated with specific inhibitors of FGF signaling pathways and the effect of rhFGF-2 (50 ng/ml, 16 h) on N-cadherin mRNA levels was determined by RT-PCR analysis. As shown in Figure 7A, rhFGF-2 increased N-cadherin mRNA levels (+131%) and calphostin C, a potent and selective inhibitor of PKC, abolished the stimulatory effect of rhFGF-2 on N-cadherin transcripts. In contrast, the p38 MAP kinase inhibitor SB 203580 (25 μM) or the MEK inhibitor PD98059 (1 μM) did not inhibit the stimulatory effect of rhFGF-2 on N-cadherin mRNA levels which were still 124 and 129% higher than in FGF-2 control cells, respectively (Fig. 7A, B). Similar effects were found at different doses (1–25 μM) of the inhibitors (not shown). Moreover, we found that the src-family tyrosine kinase inhibitor PP1 blocked the effect of rhFGF-2 on N-cadherin expression (Fig. 7A, B). As shown in Figure 8A and B, the inhibitory effect of PP1 on FGF-2-promoted N-cadherin expression was dose-dependent with a maximal inhibitory effect at 1 μM.

Since we found evidences for a PLCγ-PKC pathway in rhFGF-2 signaling in IHNC cells (Fig. 6), we tested the effect of various specific PKC inhibitors on the response induced by rhFGF-2. All specific PKC inhibitors significantly reduced rhFGF2-induced N-cadherin expression (Fig. 8). Calphostin C dose-dependently decreased the effect of rhFGF-2 on N-cadherin mRNA (Fig. 8A, B). Ro-31-8220, a selective inhibitor of membrane-bound and cytosolic PKC also inhibited the effect of rhFGF-2 on N-cadherin mRNA (Fig. 8A, B). Moreover, Gö6976, a potent PKCα inhibitor, abrogated

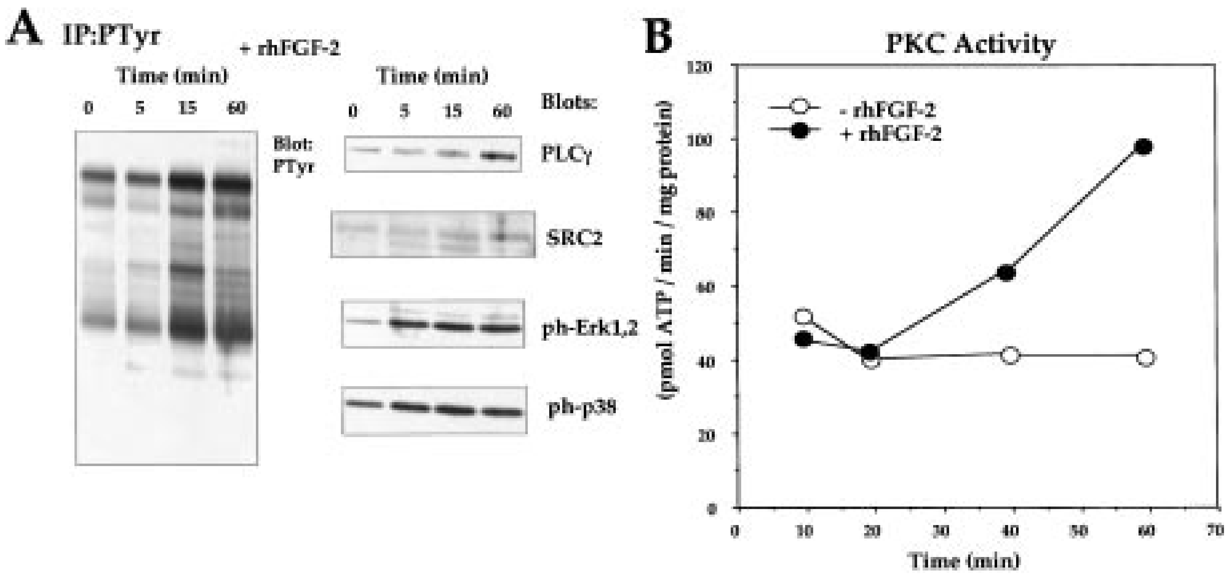
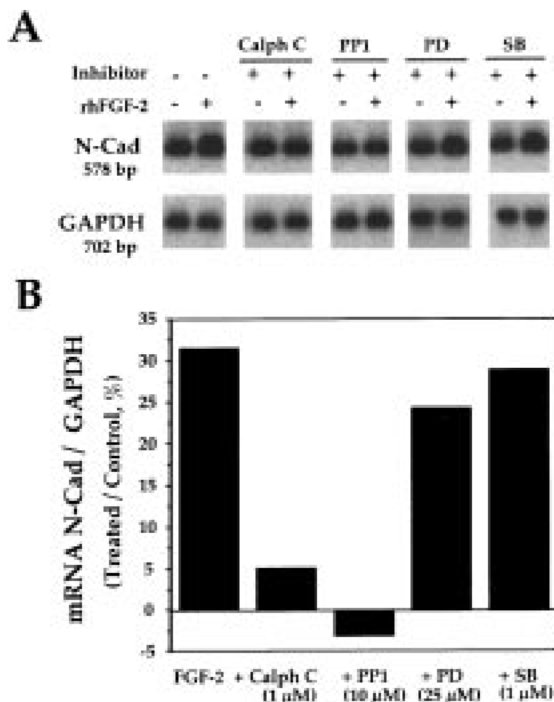


Fig. 6. FGF-2 activates multiple signaling pathways. **A**: IHNC cells were exposed to FGF-2 (50 ng/ml) or the vehicle for the indicated time points and proteins were extracted in lysis buffer. Changes in protein tyrosine phosphorylation induced by rhFGF-2 was analyzed by immunoprecipitation and Western blotting techniques using a specific P-Tyr antibody. Changes in the amounts of PLCγ_and Sr-like kinase in P-Tyr-immunoprecipitates was obtained using specific antibodies whereas activation of Erk and p38 MAP kinases was determined using specific antibodies against phosphorylated (ph) proteins, as described in Section 2. **B**: Changes in PKC activity induced by rhFGF-2 (50 ng/ml) or the vehicle was determined by the transfer of ^{32}P-labeled phosphate to a biotinylated peptide substrate that is specific for PKC activity.

the effect of rhFGF-2 on N-cadherin transcripts. Gö6983, a less sensitive PKCα inhibitor, also reduced the effect of rhFGF-2 on N-cadherin transcripts albeit to a lesser extent (Fig. 8C, D), suggesting that PKCα is involved in the promoting effect of rhFGF-2 on N-cadherin expression. Altogether, these data indicate that FGF-2 enhances N-cadherin mRNA and protein expression and N-cadherin function likely through activation of PKC and src kinases in IHNC cells.



DISCUSSION

In vitro and in vivo studies implicate FGFs as regulatory factors of osteoblast growth and differentiation. However, the early mechanisms of action of FGF-2 in osteoblasts are poorly known. In this study, we show that FGF-2 regulates N-cadherin and function in human calvaria osteoblastic cells. We also show that promotion of N-cadherin expression by FGF-2 requires activation of PKC and src kinase signaling pathways.

Fig. 7. The stimulatory effect of rhFGF-2 on N-cadherin is inhibited selectively by PKC and src kinase inhibitors. **A**: IHNC cells were pretreated for 2 h with the PKC inhibitor calphostin C, the src kinase inhibitor PP1, the MEK inhibitor PD-98059 or the p38 inhibitor SB 203580, then treated with rhFGF-2 (50 ng/ml) or the vehicle for 16 h in the presence of the inhibitors, and mRNA levels for N-cadherin were determined by semi-quantitative RT-PCR analysis. **B**: Densitometric analysis of mRNA levels was performed using GAPDH as control.

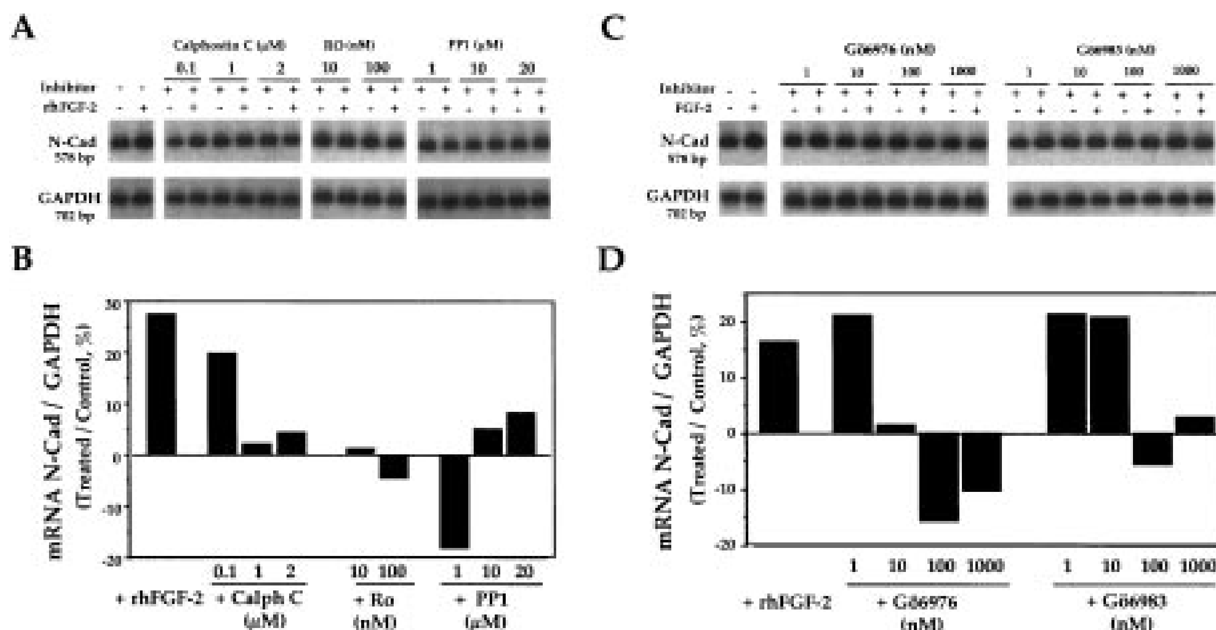


Fig. 8. Dose-dependent effects of selective PKC and src inhibitors on the rhFGF-2-promoted N-cadherin mRNA levels in IHNC cells. The cells were pretreated for 2 h with the PKC inhibitor calphostin C, the selective inhibitor of membrane-bound and cytosolic PKC Ro-31-8220, or the src kinase inhibitor PP1 (**A**), or in the presence of Gö6976, a PKC inhibitor with high selectivity for PKCα, or Gö6983, a selective inhibitor of PKCα, β and γ isotypes (**C**). Cells were then treated with rhFGF-2 (50 ng/ml) or the vehicle for 16 h in the presence of the inhibitors. N-cadherin mRNA levels were determined by RT-PCR analysis and densitometric analysis was performed using GAPDH as control (**B**, **D**).

Although several cadherins were reported to be expressed by osteoblasts, the expression of these molecules varies with the cell type or the cell origin [Babich and Foti, 1994; Okazaki et al., 1994; Cheng et al., 1998; Lemonnier et al., 1998; Tsutsumimoto et al., 1999; Ferrari et al., 2000; Haÿ et al., 2000]. We found that the human calvaria-derived IHNC osteoblastic cells express N-cadherin and E-cadherin under basal conditions. The expression of N- and E-cadherins was localized in cell membranes, consistent with a role of these membrane molecules in cell–cell adhesion, as found in non-osteogenic cell types [Takeichi, 1991; Grunwald, 1993]. The in vitro expression of N-cadherin and E-cadherin in human calvaria cells is of particular interest since these molecules are expressed in vivo in preosteoblasts and osteoblasts in the normal human calvaria [Lemonnier et al., 1998]. We then tested our hypothesis that cadherin expression and function may be modulated by FGF, an important regulatory factor of calvaria cells. Our data indicate that N-cadherin is a target gene for FGF-2 in human calvaria osteoblasts. Indeed, FGF-2 increased N-cadherin, but not E-cadherin mRNA levels in IHNC cells. Moreover, we found that the stimulatory effect of FGF-2 on N-cadherin mRNA levels was rapid and transient and was inhibited by DRB, indicating that transcription is required for this effect. Based on the lack of complete inhibition of N-cadherin mRNA levels by DRB in control cells, it appears that endogenous N-cadherin transcripts are rather stable in osteoblasts. Consistent with the increased N-cadherin mRNA levels, we found that FGF-2 increased N-cadherin protein levels. Since N-cadherin protein levels were sustained for up to 36–48 h after rhFGF-2 exposure, it can be postulated that FGF-2 may also act on post-transcriptional mechanisms to maintain sustained N-cadherin protein levels long enough to increase cell–cell aggregation in human osteoblasts. In addition, N-cadherin function might be modulated by post-translational ways. N-cadherin phosphorylation appears unlikely since the major targets of tyrosine kinases within the cadherin complex are p120, β-catenin and plakoglobulin [Daniel and Reynolds, 1997]. The possible effects of FGFs on other post-translational regulatory pathways remains to be determined.

The stimulatory effect of FGF-2 on N-cadherin expression presents some similarities with those of BMP-2 that we recently reported in human osteoblastic cells [Haÿ et al., 2000]. Both factors transiently induced N-cadherin mRNA and protein levels in IHNC cells. This common effect on the modulation of N-cadherin had similar immediate functional consequences on osteoblastic cell–cell adhesion. However, in contrast to FGF-2 [Debiais et al., 1998], BMP-2 increased both N- and E-cadherin expression and promoted osteoblast differentiation in human calvaria cells [Haÿ et al., 2000]. These different effects may perhaps be related to the fact that BMP-2 induced a greater and longer increase in cadherin expression than FGF-2 in IHNC cells, or that only N-cadherin, but not E-cadherin expression was increased in response to FGF-2. Although we cannot rule out the possibility that other cell–cell adhesion molecules might be regulated by FGF-2, our data show that N-cadherin is a previously unrecognized gene that is regulated by FGF-2 in human osteoblasts.

We then started to identify the signaling molecules that are involved in the FGF-2-promoted N-cadherin expression in human osteoblasts. It is well documented that FGF binding to its receptor leads to activation of several signaling molecules including PLCγ, PKC, src, Erk 1,2 and other kinases [Burgess et al., 1990; Wang et al., 1994; Zhan et al., 1994; Kuo et al., 1997; Maher, 1999]. Few data, however, have been reported on signaling molecules involved in the effects of FGF-2 in osteoblasts. MAP kinases were shown to be activated by FGF-2 in osteoblasts [Chaudhary and Avioli, 1997]. FGF-2 was also shown to activate p38 MAP kinase in murine osteoblasts [Kozawa et al., 1999; Suzuki et al., 2000]. In this study, we provide evidence for activation of several signaling pathways by FGF-2 in IHNC cells, including Erk 1,2 and p38 MAP kinases, PLCγ-PKC and src kinases. Data obtained with selective inhibitors of these signaling pathways indicate that only some of these pathways are involved in the effect of FGF-2-induced N-cadherin expression. Although Erk 1,2 and p38 are activated by FGF-2 in IHNC cells, the p38 MAP kinase inhibitor SB 203580 or the MEK inhibitor PD98059 did not inhibit the FGF-2-stimulated N-cadherin mRNA levels, indicating that the effect of FGF-2 on N-cadherin does not involve these MAP kinases. In contrast, we showed that several selective PKC inhibitors inhibited the induction of N-cadherin mRNA expression by FGF-2, showing that activation of PLCγ-PKC pathway is likely to be involved in this effect. We presently do not know which PKC isoforms are expressed in IHNC cells and which one is activated by FGF-2. However, based on the inhibition of FGF-2-induced promotion of N-cadherin expression by Gö6976 and Gö6983, which are preferential inhibitors of PKCα, it is tempting to speculate that this PKC isoform plays an important role in mediating this effect of FGF-2. Interestingly, our data also indicate that PP1, an inhibitor of the src-family tyrosine kinase, significantly reduced the stimulatory effect of FGF-2 on N-cadherin mRNA expression. This indicates that PKC and src signaling pathways are required for the stimulation of N-cadherin expression in IHNC cells.

The stimulatory effect of FGF-2 on N-cadherin protein levels had functional consequences, because we found that FGF-2 promoted cell–cell adhesion for up to 72 h in IHNC cells. Specific anti-N-cadherin inhibited the cell aggregation induced by FGF-2, showing that N-cadherin is involved in the promoting effect of FGF-2 on cell–cell adhesion. These experiments support a functional role for N-cadherin in IHNC cell aggregation induced by FGF-2. Such a role for N-cadherin in osteoblasts is consistent with studies showing that N-cadherin is expressed in mesenchymal cells that are undergoing active cellular condensation [Hatta et al., 1987], and with data showing that N-cadherin mediates the cell–cell interactions that are important for chondrogenesis [Oberlender and Tuan, 1994]. In support of a role of FGF/FGFR interaction in cell–cell adhesion induced by N-cadherin is our recent finding that constitutive activation of FGFR-2 by the Ser252Trp FGFR-2 mutation increases expression of N-cadherin as well as cell–cell adhesion in human calvaria mutant osteoblasts [Lemonnier et al., 1998]. We can then postulate that one of the role of FGF-2 may be to increase N-cadherin expression and thereby cell–cell adhesion in the normal human calvaria during the early steps of mesenchyme condensation. One possible biological consequence of the FGF-2-induced increase in N-cadherin and cell–cell adhesion may be the induction of cell regulatory signals via the catenins that are linked to the cadherins [Barth et al., 1997; Aplin et al., 1998]. Such N-cadherin-mediated cell–cell con-

tacts may subsequently induce immediate and early intracellular signaling events leading to gene expression [Knudsen et al., 1998]. Further studies will help to determine the nature of the genes that may be induced following the increased N-cadherin expression and cell–cell adhesion induced by FGF-2 in human osteoblasts.

In conclusion, our study shows that FGF-2 enhances N-cadherin expression and that this effect has functional implications on cell–cell adhesion in human calvaria osteoblasts. Furthermore, our study indicates that N-cadherin is a target gene for PKC and src signaling pathways resulting from FGF/FGFR activation. This study identifies N-cadherin as a previously unrecognized target gene for FGF-2 signaling pathway in osteoblasts.

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