Mechanisms of GDF-5 action during skeletal development

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This paper is dedicated in memory of Peter Thorogood, a friend and colleague

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SUMMARY

Mutations in GDF-5, a member of the TGF-β superfamily, result in the autosomal recessive syndromes brachypod (bp) in mice and Hunter-Thompson and Grebe-type chondrodysplasias in humans. These syndromes are all characterised by the shortening of the appendicular skeleton and loss or abnormal development of some joints. To investigate how GDF-5 controls skeletogenesis, we overexpressed GDF-5 during chick limb development using the retrovirus, RCASBP. This resulted in up to a 37.5% increase in length of the skeletal elements, which was predominantly due to an increase in the number of chondrocytes. By injecting virus at different stages of development, we show that GDF-5 can increase both the size of the early cartilage condensation and the later developing skeletal element. Using in vitro micromass cultures as a model system to study the early steps of chondrogenesis, we show that GDF-5 increases chondrogenesis in a dose-dependent manner. We did not detect changes in proliferation. However, cell suspension cultures showed that GDF-5 might act at these stages by increasing cell adhesion, a critical determinant of early chondrogenesis. In contrast, pulse labelling experiments of GDF-5-infected limbs showed that at later stages of skeletal development GDF-5 can increase proliferation of chondrocytes. Thus, here we show two mechanisms of how GDF-5 may control different stages of skeletogenesis. Finally, our data show that levels of GDF-5 expression/activity are important in controlling the size of skeletal elements and provides a possible explanation for the variation in the severity of skeletal defects resulting from mutations in GDF-5.

Key words: GDF-5, joint, chondrogenesis, proliferation, cell adhesion

INTRODUCTION

The vertebrate appendicular skeleton consists of a series of elements that are separated by joints. Initially, the elements arise in the limb bud from the condensation of prechondrogenic mesenchyme, which subsequently differentiates to form cartilaginous templates of the respective elements, separated by joints (see Hinchliffe and Johnson, 1980 for review). In prospective long-bone elements, the chondrocytes are arranged in three zones: a central diaphyseal hypertrophic zone, flanked by two outer metaphyseal flattened cell zones and two proliferative epiphyseal rounded cell zones (Fell, 1925). The elements are surrounded by a fibrous perichondrium, which also possesses chondrogenic potential.

Members of the TGF-β superfamily such as the Bone Morphogenetic Proteins (BMPs) and the closely related Growth and Differentiation Factors (GDFs) are fundamental players controlling skeletal development. This notion is based on three main lines of evidence. First, loss-of-function or gain-of-function studies of either the proteins themselves or their antagonists in the mouse and chick result in changes in skeletal development that appear to be a primary consequence of defective cartilage development rather than changes in embryonic patterning (Kingsley et al., 1992; Storm et al., 1994; Duprez et al., 1996; Storm and Kingsley, 1996; Zou et al., 1997; Brunet et al., 1998; Capdevila and Johnson, 1998; reviewed by Kingsley, 1994). Second, the ability of BMPs to promote endochondral bone formation, when implanted subcutaneously into adult rats, shows that some members of the family are able to initiate a cascade of skeletogenesis (reviewed by Carrington and Reddi, 1991; Rosen and Thies, 1992; Reddi, 1992). Finally, in vitro studies have shown that these factors can modulate chondrogenesis (for examples in the developing chick limb see Roark and Greer, 1994; Chen et al., 1991).

GDF-5 (also known as CDMP-1) is one member of the family that has received particular attention as there are naturally occurring mutations in both humans and mice (Grüneberg and Lee, 1973; Storm et al., 1994; Storm and Kingsley, 1996; Thomas et al., 1996, 1997; Polinkovsky et al., 1997). These result in defects in the development of the
appendicular skeleton. The autosomal recessive syndromes, brachypod (bp) in mice, chondrodysplasias Grebe type (CGT) and Hunter-Thompson (CHTT) in humans are all characterised by pronounced shortening of the skeletal elements, with more severe effects distally and the loss of one or more joints. CHTT and brachypodism are due to missense mutations in GDF-5 resulting in total loss of GDF-5 function (Storm et al., 1994; Thomas et al., 1996). In contrast, CGT is due to mutation CDMPC400Y in the conserved cysteine in the functional domain of the protein (Thomas et al., 1997). CGT is more severe than CHTT and this is thought to be due to loss-of-function of other members of the TGF-β family with which GDF-5 heterodimerises (Thomas et al., 1997). In addition, there are syndromes due to haploinsufficiency of GDF-5 such as Brachydactyly type C, which is characterised by the shortening, and occasionally, the loss, of some phalanges (Polinkovsky et al., 1997). Brachydactyly has also been noted in humans that are heterozygous for the mutation CDMPC400Y (Thomas et al., 1997). This has led to the proposal that skeletal elements vary in their sensitivity to the loss of GDF-5 function (Polinkovsky et al., 1997). In mice, the defects are first apparent at day 12 of embryonic development prior to overt chondrogenesis and, by day 14, chondrogenesis of the autopod is clearly delayed (Grüneberg and Lee, 1973). Thus, GDF-5 has a major role in the development of the appendicular skeleton, particularly at the early stages of chondrogenesis. Here, we use gain-of-function approaches to examine the mechanisms by which GDF-5 controls the development and growth of skeletal elements.

MATERIALS AND METHODS

Cloning of chick Gdf-5

Total RNA from day 17 chick sternal chondrocytes was extracted using a modified acid guanidine-phenol-chloroform method (Trizol, Life Technologies). Poly(A)+ RNA was isolated using magnetic beads using a modified acid guanidine-phenol-chloroform method (Trizol, Life Technologies). Poly(A)+ RNA was isolated using magnetic beads (PolyA Tract, Promega, Madison, WI) and first-strand cDNA synthesis was performed using random hexanucleotide primers. Two degenerate oligonucleotide primers corresponding to highly conserved motifs in the C-terminal region of the BMPs were used: S1, 5’-GGI-TGG-(C/A)AI-GA-(C/T)-TGG-AT/(A/C/T) -TA/(A/G)TGC-(A/C/G/T)-CC3’, corresponding to amino acids [GW(Q/N)DWI(I/V)AP], and AS1, 5’-(A/G)-GT/-C/(T)/TG-(A/C/G/T)AC-(A/G)AT-(A/G)- GC-(A/G)TG-(A/G)TT-3’, corresponding to amino-acids [NHAIVQTL]. Nucleotides in parenthesis denote degeneracy and I denotes inosine. The PCR amplifications with the degenerate primers were performed using conditions described previously (Chang et al., 1994). The reaction products were electrophoresed on 1.2% agarose gels, and DNA fragments of the appropriate size were excised and purified using the Magic PCR prep DNA purification system (Promega, WI). Reamplification was performed with the same primers and each PCR product was subcloned into the PCRII vector using the TA cloning system (InVitrogen Corporation, San Diego, CA). A 120 bp PCR fragment encoding part of the C-terminal domain was used to screen approximately 1x10^6 plaques of a 10 day chicken embryo λgt11 cDNA library (Clonetech, Palo Alto, CA) by standard procedures. Hybridisations were performed for 20 hours at 42°C in 6× SSC, 1× Denhardt’s solution, 0.01% tRNA, 0.05% sodium pyrophosphate and the membranes (Dupont 137 mm nylon membranes, New England Nuclear, MA) were washed to a final stringency of 6× SSC, 0.1% SDS at 55°C for 20 minutes.

All sequencing was done by dideoxy DNA sequencing of both strands using Sequenase Version 2.0 DNA polymerase according to the manufacturer’s instructions (USB, Cleveland, OH). Confirmatory data in ambiguous regions were obtained by automated thermal cycle sequencing with an Applied Biosystems Model 370A sequencer and by using 7-deaza-GTP (USB, Cleveland, OH). The sequencing data were obtained from restriction fragments subcloned into pBluescript using either M13 forward and reverse primers or synthetic oligonucleotide primers.

Gdf-5 in situ probes

The full-length cDNA encoding chick Gdf-5 was subcloned into pBluescript SK+. For in situ analysis, antisense riboprobes to 700 bp of the 3’ UTR were synthesized with T7 RNA polymerase using Psfl-digested DNA as a template whilst 890 bp sense control riboprobes to the 5’ UTR and part of the GDF-5 coding sequence were synthesized with T3 RNA polymerase, using HindIII-digested DNA as a template.

Chick embryos

Fertilised wild-type chicken eggs (White Leghorn; Poyndon Farm, Waltham Cross, Hertfordshire, UK) were incubated at 38(±1)°C and staged according to Hamburger and Hamilton (1951).

In situ hybridisation to whole mounts and tissue sections

Embryos were fixed in 4% (w/v) paraformaldehyde, processed to methanol for whole-mount in situ hybridisation or embedded in wax for in situ hybridisation of tissue sections as previously described (Francis-West et al., 1995).

Construction of the retroviruses

The mouse GDF-5 coding region was inserted into the retrovector, RCASBP(A), via a cloning step in the adaptor plasmid, Cla12 Nco (Hughes et al., 1987). The inserted fragment was generated from a mouse Gdf-5 cDNA clone (Storm et al., 1994) by PCR, using oligonucleotide primers with the sequences, 5’GAC CAT GGG ACT CCC CAA ACT CCT CCT C3’ and 5’CCG GAT CCT ACC TGC ACG CAC AAG ATT CCC3’ to the 5’ and 3’ end of the GDF-5 coding region respectively. The sequences underlined in the 5’ and 3’ oligos encode a NcoI and BamHI site, respectively, and were introduced to facilitate cloning and expression. To introduce a NcoI site into the first ATG (methionine), the mouse Gdf-5 cDNA was mutated at the nucleotide shown in bold in the 5’ oligo from an A to a G, changing the second amino-acid in GDF-5 from an arginine to a glycine. To produce the functional GDF-5 protein, the N-terminal region is cleaved in vivo; therefore, the mutation that is in the signal sequence of the protein should not affect the functional activity of the final protein. As a negative control, we used a virus that encodes mGdf-5 inserted in the antisense orientation or alkaline phosphatase (kind gift of Professor Cliff Tabin, Department of Genetics, Harvard Medical School).

Culture and grafting of the retrovirus

Viral-infected cells for grafting and virus concentrates (titre between 3-7x10^5 pfu/ml) for injections were obtained and introduced into the developing embryo as described in Logan and Francis-West (1999). Following manipulation, embryos were fixed at various time points, for skeletal analysis by Alcian green staining or to determine viral spread by in situ hybridisation using a mouse GDF-5 probe or by detection of ectopic alkaline phosphatase activity as described by Morgan and Fekete (1995). The lengths of the humeri were measured in the treated and contralateral control limbs and the differences in the lengths were analysed using a paired Student’s t-test. For histological examination, the whole mounts were embedded in wax, sectioned at 8 μm and stained with Mallory’s triple stain.

Autoradiography of S-phase chondrocytes

Proliferative chondrocytes were localised as described by Goff and Tabin (1997). Cell counts were made from representative sections of 2 pairs of control and manipulated humeri. Differences in cell proliferation were analysed using a paired Student’s t-test.

Quantitation of DNA and sulphated glycosaminoglycans

Humeri were solubilised in a solution containing 1 ml papain.
Micromass culture

The micromass cultures were carried out as described by Cottrill et al. (1987) using stage 23 forelimb and hindlimb buds. Recombinant human GDF-5 protein was supplied by the Genetics Institute and was included in the micromass cultures at various concentrations (10, 50 and 100 ng/ml). Media and GDF-5 were changed daily. Micromasses were cultured in the presence of 2% fetal calf serum and were fixed after 1 or 3 days for analysis of cartilage production. Under these conditions, there is little muscle survival/development and we are directly testing the action of GDF-5 on a relatively undifferentiated population of mesenchymal cells (Archer et al., 1990). Total DNA was analysed in 3 day micromasses by papain digests as described above. Because of the sensitivity limitations of the DMBB assay, production of GAGs was determined by modification of an Alcian blue binding assay. Briefly, cultures were fixed in 10% formal saline, washed and stained with Alcian blue (pH 1) overnight. The cultures were then washed in running tap water and unbound Alcian blue was extracted with 1% acetic acid in 70% ethanol. The cultures were washed exhaustively and the bound Alcian blue was extracted with 1 ml 4M guanidine hydrochloride in 33% isopropanol overnight (Masuda et al., 1994). The extracted supernatant was then read on a spectrophotometer at A630 nm. Results were converted to a percentage difference compared to the control values.

To determine the effects of GDF-5 on cell proliferation, micromass cultures were grown in the presence or absence of GDF-5 (100 ng/ml) for 8 or 50 hours and were then pulsed with [\(^{3}H\)]thymidine (10 \(\mu\)Ci/ml) for 3 and 2 hours, respectively. To determine [\(^{3}H\)]thymidine incorporation in the 8 hour micromasses, the cultures were extracted in papain as described above and the DNA was precipitated with trichloroacetic acid (5% final volume) for 20 minutes at 4°C. The precipitated DNA was collected by centrifugation, washed twice in 95% ethanol and bound isotope was solubilised for 30 minutes at 37°C in 0.1 M NaOH, 2% Na2CO3, 1% SDS and counted in a scintillation counter. Data were expressed as incorporated counts/minute per culture. The 50 hour cultures were fixed and processed through to wax and 7 μm sections were cut through the depth of the culture. Sections were dewaxed and dipped in LM-1 autoradiographic emulsion (Amersham, UK) and exposed for 9 days at 4°C. The slides were developed in D19 (Kodak) for 5 minutes, fixed and counterstained with half-strength Mayer’s haematoxylin and eosin. Sections were examined microscopically and the fraction of labelled cells counted. Results were analysed for their significance using the Student’s t-test.

Cell suspension cultures

Stage 23 forelimbs and hindlimbs were placed in 0.25% trypsin for 15 minutes, dispersed using a rotary mixer and filtered twice through Nitex (40 mesh) to obtain a single cell suspension. Cells (125,000) were placed in 2 ml of medium as described above but containing 1% suspension (0.56 units/ml; type III. Sigma, Poole, UK) in 1 ml PBS supplemented with 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetra-acetic acid (EDTA) at pH 5.7 overnight at 60°C. Glycosaminoglycan (GAG) concentration in the papain digest was measured by a modification of the 1, 9-dimethylmethylen blue (DMBB) dye binding assay of Farndale et al. (1982). An aliquot of the papain digest was diluted ten-fold and duplicate 40 μl samples were then pipetted into the well of a 96-well plate. To this, 200 μl of DMBB was added and the mixture was read immediately at an absorbance of 525 nm. The samples were measured against known standards of shark chondroitin-4-sulphate (5, 10, 15, 20 and 30 mg/ml). DNA concentration was calculated by fluorescent dye-binding to Hoechst 33258 and read in a fluorimeter (Hoefer Dynaquant 200). Triplicate 20 μl samples of the papain digest were diluted into 2 ml of low range assay solution (0.1 μg/ml Hoechst 33258; 0.2 M sodium chloride, 10 mM Tris; 1 mM EDTA pH 7.4). Calf thymus DNA was used to construct a standard curve. Differences in DNA and GAG levels were analysed using a paired Student’s t-test.
Hepes buffer in a 5 ml bijou bottle. They were then placed on a roller mix (30 revs/minute) for 18 hours in the presence or absence of GDF-5 (10 ng/ml). The cultures were collected by centrifugation (18,000 g) for 4 minutes, the medium was aspirated off and the cells were gently resuspended and fixed in 10% formalin containing 5 ng/ml of propidium iodide for 30 minutes to label the nuclear DNA. The cells were recentrifuged, resuspended in 100 µl of 70% ethanol and transferred to well slides with the aid of a plastic pipette that had the end cut off to allow passage of larger cell aggregates. Cultures were examined under fluorescence microscopy and the number and size of the aggregates were recorded. These experiments were carried out in triplicate.

RESULTS

Cloning of the chick homologue of GDF-5

Following RT-PCR with poly(A)+ RNA from day 17 chick sternal chondrocytes as a template, amplification products of 120 bp were obtained (data not shown). Subcloning and sequencing showed that these products encoded CDMP-1/GDF-5 and OP-1 (BMP-7), and several other BMP-like sequences. Cloned inserts with CDMP-1/GDF-5 homologous sequences were used to screen a cDNA library. Several clones were isolated and the size of their inserts (2.3 kb) and their restriction maps were found to be identical. One of the clones was sequenced and shown to encode the chick homologue of CDMP-1/GDF-5. The open reading frame of chick GDF-5 contains 500 amino-acids consisting of a pro-region of 376 amino-acids, a typical cleavage site (RXXR/A), and a C-terminal domain of 120 amino-acids containing the seven highly conserved cysteines characteristic of the TGF-β
superfamily. Alignment of the human, mouse, chick and zebrafish GDF-5 proteins is shown in Fig. 1.

Expression of Gdf-5 in developing limb buds
The expression of Gdf-5 during chick limb bud development was determined by in situ hybridisation to whole embryos or tissue sections (Fig. 2). The pattern of expression is the same in the developing wings and legs. Initial studies by whole-mount in situ hybridisation showed that Gdf-5 is expressed in early developing joints and later is detectable at the lateral edge of the joints (Fig. 2A,B; see also Storm et al., 1994; Storm and Kingsley, 1996; Wolfman et al., 1997; Brunet et al., 1998). However, more detailed analysis by radioactive in situ hybridisation revealed that Gdf-5 is also expressed in the condensing mesenchyme of the cartilage elements, prior to joint formation (Fig. 2C,D; see also Chang et al., 1994). Later during development, Gdf-5 is expressed in the early perichondrial layer around the cartilage elements in addition to the joint interzone (Fig. 2A,E,F; see also Storm et al., 1994; Brunet et al., 1998). Finally, expression becomes localised to the joint interzone and is downregulated as the joint cavitates (Fig. 2G,H and data not shown). The apparent discrepancy between the whole-mount and radioactive in situ hybridisation studies presumably reflects the greater sensitivity of the latter technique and may also be due to the inability of the probe to penetrate cartilage matrix in the whole-mount studies. In situ hybridisation using sense Gdf-5 probes showed background levels of hybridisation (data not shown).

Overexpression of GDF-5 increases the length of skeletal elements
Mutation studies in mice have shown that GDF-5 controls both the growth of developing skeletal elements and the formation of joints. To further examine the roles and mechanisms of GDF-5 action, we constructed a replication competent retrovirus, RCASBP, encoding mouse GDF-5 which shares 98.3% amino-acid identity with chick GDF-5. A pellet of cells infected with the retrovirus was grafted into the early developing chick limb bud and the embryos were allowed to develop until 10 days when viral spread and/or skeletal phenotype were determined. Alternatively, the developing limb bud was injected with concentrated virus particles. Analysis of viral spread showed that the virus had spread through the developing limb bud by 5 days of development (Fig. 3A; for more detailed analysis of viral spread see Duprez et al., 1996).

Examination of 10 day skeletal elements from infected limbs by Alcian green staining showed that overexpression of GDF-5 increased the length and the width of one or more of the developing skeletal elements and affected humeri were up to 37.5% longer (Fig. 3C,D; n=33; average, 16.1%; range 2.5-37.5%; P<0.001). The joints were usually fused or partially fused between affected elements (Fig. 3C,D). In contrast, in limbs that had been infected with the control virus, there was no significant difference between the length of the humeri in the treated and contralateral control limbs (n=13; data not shown).

GDF-5 can increase the size of skeletal elements at two stages of development
Gdf-5 is expressed in the early developing cartilage condensations and later in the developing joint interzone. This suggests that GDF-5 may act at two distinct stages of development, first to control the development of the early cartilage condensation and second in the joint interzone where GDF-5 may signal from the joints to the epiphyses of adjacent skeletal elements. To investigate these possibilities, limbs were infected at stage 17-20 with concentrated virus particles. The embryos were allowed to develop until stage 26/27, which is 24 hours after the initial cartilage condensation has been laid down but just prior to the consolidation of the diaphyseal perichondrium. Staining with Alcian green revealed that there was a detectable increase in the size of the cartilage condensation in GDF-5-infected limbs (Fig. 3E,F; n=4). Histological analysis of transversely sectioned limbs showed that there was an average 28% increase in the number of cells across the width of the developing humeri (n=2; P<0.001). In limbs that had been infected with the control virus, there was no detectable change in the size of the developing humeri at stage 26/27 (n=4; data not shown) or the number of cells across the width of the cartilage condensation (n=2; +0.3% increase).

To determine if GDF-5 could increase the length of skeletal elements and the formation of joints, we constructed a replication competent retrovirus, RCASBP, encoding mouse GDF-5. A pellet of cells infected with the retrovirus was grafted into the early developing chick limb bud and the embryos were allowed to develop until stage 26/27, which is 24 hours after the initial cartilage condensation has been laid down but just prior to the consolidation of the diaphyseal perichondrium. Staining with Alcian green revealed that there was a detectable increase in the size of the cartilage condensation in GDF-5-infected limbs (Fig. 3E,F; n=4). Histological analysis of transversely sectioned limbs showed that there was an average 28% increase in the number of cells across the width of the developing humeri (n=2; P<0.001). In limbs that had been infected with the control virus, there was no detectable change in the size of the developing humeri at stage 26/27 (n=4; data not shown) or the number of cells across the width of the cartilage condensation (n=2; +0.3% increase).
elements during later development, concentrated virus stocks were injected into the developing elbow region at stage 26-27. At this stage of development, the elbow joint is evident and expresses Gdf-5 but the joint has yet to cavitate. Following injection, the embryos were allowed to develop until 10 days. Using this protocol, the virus typically spread around the adjacent developing cartilage elements (Fig. 3B). Analysis of the humeri by Alcian green staining showed that overexpression of GDF-5 increased the width at the epiphyses and overall length of the humerus at these later stages (Fig. 3G,H; \( n = 9 \), average 12% increase in length; range 2.4-32.7%). The localised effect on the epiphyses is probably related to the inability of virus to penetrate cartilage matrix, particularly in regions of the more differentiated perichondrium around the diaphysis (Fig. 3B). In limbs that were injected with the control virus, there was no change in the length of the developing humerus (\( n = 9 \); data not shown).

**Mechanism of increase in size**

The increase in size of GDF-5-infected elements may be due to an increase in the number of chondrocytes, an increase in the quantity of matrix or, at later stages of development, due to an increase in the number of hypertrophic chondrocytes. To first investigate these possibilities, manipulated elements and their contralateral controls were sectioned longitudinally and examined histologically. This showed that the chondrocytes were arranged as normal, with no major changes in the levels of matrix (Fig. 4). Therefore, morphologically, cell differentiation appeared normal. To confirm this, infected limbs were probed for the expression of type X collagen, a marker of terminal differentiation and \( \beta \)-III procollagen, a marker of the flattened, prehypertrophic zone. This confirmed that there was no change in the pattern or state of differentiation of the chondrocytes in GDF-5-infected limbs (data not shown).

To quantitatively assess changes in matrix levels and chondrocyte number, humeri from 10 day infected and contralateral control limbs were dissected out, care being taken to remove all adjacent soft tissue, and digested with papain. The total number of cells was determined by measuring total DNA levels by fluorescence spectroscopy and the levels of matrix were analysed by DMMB assay to determine the amount of sulphated glycosaminoglycans (GAGs), which are the principal matrix component of the skeletal elements. In GDF-5 manipulated limbs, there was an average 64.5% increase in the DNA content of the cartilage elements (\( n = 8 \), \( P = 0.005 \)) and an average 81.1% increase in the amount of sulphated GAGs (\( n = 8 \), \( P = 0.011 \)). In contrast, in humeri from limbs that had been infected with the control virus, there was no significant change in the quantity of DNA and sulphated GAGs per element (~3.5% in DNA content, \( P = 0.17 \); +1.9% in the levels of sulphated GAGs, \( P = 0.35 \)). There were no significant differences in the GAG/DNA ratio in either GDF-5 or control humeri (+11.8% in GDF-5-infected humeri (\( P = 0.63 \); +2.9% increase in the control infected humeri (\( P = 0.37 \))). Thus, the increase in size of GDF-5 affected skeletal elements appears to be predominantly due to an increase in the number of cells rather than due to major changes in matrix metabolism. This increase in the number of cells could be due to increased proliferation of chondrocytes or recruitment of cells from the surrounding mesenchyme into the condensation or later from the perichondral layer around the skeletal element (Ede and Agerbak, 1968; Holfreter, 1968; Ede and Flint, 1972; Thorogood and Hinchliffe, 1975).

**GDF-5 acts at the initial steps of chondrogenesis**

The overexpression studies showed that GDF-5 could increase the size of the prechondrogenic condensation. At these stages of development, the number of cells in the cartilage condensation increases either through cell proliferation and/or recruitment where cells from the surrounding mesenchyme, or later from the forming perichondrium, are recruited into the chondrogenic lineage. To determine how GDF-5 controls the initial stages of chondrogenesis, we used a micromass culture system. This is a standard in vitro model for chondrogenesis and has been used to determine mechanisms of action of TGF-\( \beta \)-1 and several members of the TGF-\( \beta \)-1 subfamily (for examples, see Kulyk et al., 1989; Leonard et al., 1991; Jiang et al., 1993; Roark and Greer, 1994).

Micromass cultures were made from stage 22-23 limb buds i.e just prior to the initiation of chondrogenesis. They were cultured in the presence of various concentrations of GDF-5 (0, 10, 50, 100 ng/ml) and were analysed for changes in chondrogenesis by Alcian blue staining. After 1 day, control cultures stained very lightly for Alcian blue within the developing cartilage nodules (Fig. 5). In contrast, cultures containing GDF-5 showed an enhancement in Alcian blue staining (Fig. 5). After 3 days, a dose-dependent increase in chondrogenesis was clearly evident (Fig. 5). This was confirmed by extracting the bound Alcian blue and assaying spectrophotometrically. At 10 ng/ml, there was an average increase in GAG content of 108% (\( n = 2 \); 92% and 124%) relative to the controls, at 50 ng/ml, there was an average 404% increase in GAG content in both control and GDF-5 treated cultures (Fig. 5). To determine if GDF-5 was increasing the condensation, we stained fixed cultures with Alcian blue to determine cartilage morphology. C marks the control cultures. Scale bar, 250 \( \mu \)m.
increase \((n=2; 328\% \text{ and } 481\%)\) whilst, at 100 ng/ml, there was an average 654\% increase \((n=2; 592\% \text{ and } 716\%)\). Analysis of total DNA in these cultures showed that there was no overall increase of DNA in response to GDF-5 at any concentration (controls, \(2.3\pm0.3\) mg, \(n=3\); 10 ng/ml, \(2\pm0.1\) mg, \(n=3\); 50 ng/ml, \(2\pm0.1\) mg, \(n=4\); 100 ng/ml, \(2.7\pm0.3\) mg, \(n=3\)).

To determine whether GDF-5 could induce the initial steps of chondrogenesis, the number of Alcian-blue-stained nodules was analysed at day 1. In micromasses cultured with GDF-5 (10 ng/ml), there was a 30\% increase in the number of nodules \((n=4; 370\pm24 \text{ control, } 481\pm25, \text{ GDF-5 (10 ng/ml); } P=0.01; \text{ Student’s } t\)-test). GDF-5 may increase the number and size of nodules in micromass culture by increasing cell proliferation and/or increasing cell adhesion (Abbot and Holtzer, 1966; reviewed by Thorogood, 1983). To investigate this, micromasses were cultured either alone or in the presence of GDF-5 and were pulsed with \[^{3}H\]thymidine after 8 or 50 hours i.e whilst the initial condensations are forming and later during cartilage formation. Cells in S-phase were determined by autoradiography or scintillation counting of the incorporated \[^{3}H\]thymidine. After 8 hours, GDF-5-treated micromasses incorporated an average of \(15,150\pm4,860 \text{ counts/minute/culture compared to the controls which had a mean value of } 13,960\pm3040 \text{ counts/minute/culture (} P>0.05\). After 50 hours, there was also comparable \[^{3}H\]thymidine incorporation as determined by the labelling indices of 0.12 and 0.11 (1,800 cells counted) for controls and GDF-5-treated micromasses, respectively. These studies showed that there is no significant change in proliferation and are consistent with the DNA analysis of the micromass cultures, which show that there is no increase in the DNA content of GDF-5-treated micromasses compared to controls.

To investigate if GDF-5 can increase cell adhesion, single cell suspensions of stage 23 limb buds were roller cultured in the presence or absence of GDF-5 (10 ng/ml). This technique has been used to determine cell surface changes following WNT misexpression in other cell systems (Bradley et al., 1993). After 18 hours, the number and size of the aggregates was determined. The aggregates were grouped into the two classes: small aggregates of 5-20 cells whilst large aggregates consisted of over 100 cells. In GDF-5-treated cultures, there was a 2.3-fold increase in the number of small aggregates relative to the control cultures and several large aggregates were observed which were very rarely observed in control cultures (Fig. 6).

**Table 1. GDF-5 increases proliferation of chondrocytes**

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<th>Total number of cells</th>
<th>Number of labelled cells</th>
<th>Percentage of labelled cells</th>
<th>Percentage change in labelled cells on the treated side</th>
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<td>Gdf-5-infected embryos</td>
<td>Control humeri</td>
<td>67,371</td>
<td>18,592</td>
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<td></td>
<td>Treated humeri</td>
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Total number of cells and the number of \[^{3}H\]thymidine-labelled cells in stage 27/28 humeri that had been infected with a retrovirus expressing either GDF-5 \((n=2)\) or alkaline phosphatase (control; \(n=2\)) and the equivalent numbers in their contralateral control humeri.

**GDF-5 can also increase proliferation of chondrocytes**

Injection of the GDF-5 virus at later stages of development, when recruitment of cells is limited to the epiphyses and perichondrium, also resulted in significant increase in the size of the cartilage elements. It is impossible to investigate recruitment at this stage of development, but Gdf-5 expression is negligible in the perichondrium at these stages suggesting that normally GDF-5 does not recruit perichondrial cells into the chondrogenic lineage. However, at this stage Gdf-5 is expressed in the developing joints and it is possible that GDF-5 signals to the adjacent skeletal elements, controlling proliferation of the chondrocytes at the epiphyses. To investigate this, developing limbs were infected with the GDF-5 retrovirus between stages 17 and 20 and were pulsed for 8 hours with \[^{3}H\]thymidine at stage 27/28 to label proliferating cells. The skeletal elements were sectioned and \[^{3}H\] incorporation was determined by autoradiography. The percentage of proliferating cells was determined and compared to that in the contralateral control element. In the GDF-5-infected humeri, there was an average increase of 53.6\% in S-
phase cells (Table 1; n=2). In contrast, limbs that had been infected with the control virus showed no significant change in the percentage of labelled cells (Table 1; n=2).

**DISCUSSION**

The development of the skeleton starts with an initial condensation of mesenchymal cells that is thought to be formed and grow as a result of increases in cell adhesion and/or proliferation. Subsequently, the condensations undergo differentiation and morphogenesis to form the chondrogenic skeletal elements, surrounded by a fibrous perichondrium, which are separated by joints. Growth at these latter stages is due to hypertrophy of the chondrocytes, which promotes growth predominantly along the long axis, matrix production and proliferation of chondrocytes at the epiphyses of the elements. In addition there is recruitment of cells from the perichondrium, which contributes to an increase in the width of the skeletal element. Here we show that GDF-5 can act at two stages of skeletal development by two distinct mechanisms. First, GDF-5 promotes the initial condensation step by increasing cell adhesion. Later, when its expression is restricted to the joints, it may signal to the epiphyses of the adjacent skeletal elements controlling chondrocyte proliferation.

**Role of GDF-5 during cartilage condensation**

The developing cartilage element starts as a condensation of mesenchymal cells. Comparison of cartilage condensations has shown that their initial size is one of the key factors determining the dimensions of the final skeletal element (Atchley and Hall, 1991). Here we show that overexpression of GDF-5, which is normally expressed in the prechondrogenic condensation, increases the size of the early skeletal element. This is the complementary phenotype to that seen in the *bp* mouse mutant where the early prechondrogenic condensations are thinner and malformed at embryonic day 12 (Grüneberg and Lee, 1973). The combined data suggest that the expression of GDF-5 is a significant physiological factor controlling early chondrogenic development and that regulating the expression, distribution or activity of GDF-5 may be simple mechanisms to change the size of appendicular skeletal elements.

**Role of GDF-5 in the developing joint**

Later, Gdf-5 expression becomes localised to the developing joint interzone. As older cartilage elements still respond to GDF-5 signalling this suggests that GDF-5 expression in the joints may control the growth of adjacent skeletal elements by increasing chondrocyte proliferation.

Not only may GDF-5 expression in the joint have an effect on neighbouring skeletal structures but analysis of the *bp* and double *bp/Bmp-5* mouse mutants, which lack some joints, has been taken as evidence that GDF-5 may specify these joints (Hinchliffe and Johnson, 1980; Storm and Kingsley, 1996). In our overexpression studies, there is no evidence of ectopic joint formation and, by 10 days of development, the joints were usually partially fused. This fusion of joints is possibly due to down-regulation of the synthesis of Hyaluronan (HA), an essential component of cavitating joints (reviewed by Pitsillides, 1999). This latter possibility is supported by the fact that application of recombinant GDF-5 to 17 day chick fibroarticular cells in vitro downregulates the activity of uridine diphosphoglucose dehydrogenase, an enzyme involved in HA synthesis (Pitsillides et al., 1995; G. Dowthwaite, A. A. Pitsillides and C. W. A., unpublished). Therefore, misexpression of GDF-5 in the cavitating joint may prevent cavitation, simply by downregulating HA synthesis.

Although our overexpression data alone cannot be taken as definite proof, we suggest that GDF-5 itself does not specify joints. This idea is also based on two other lines of evidence. First, our data have shown that Gdf-5 is expressed throughout the early cartilage condensation in both humans and chicks, not only in area of the future joint but also in regions of the future skeletal elements (see also Chang et al., 1994). Second, in *bp* mice there is evidence that the joint interzone starts to develop but the joint fails to cavitate (Grüneberg and Lee, 1973). The loss of joints in *bp* mice may be due to several factors. One model of joint development is that synthesis of matrix components by the adjacent skeletal elements, results in the flattening of the interzone cells, in a manner analogous to the formation of the perichondrium (Fell, 1925; Rooney et al., 1984). Thus the lower levels of matrix production in *bp* skeletal elements may indirectly prevent the formation of the joint interzone cells (Duke and Elmer, 1979; Kwagiroch et al., 1992). Alternatively, as movement is essential for joint cavitation but not joint interzone formation, the tendon and muscle abnormalities in *bp* mice may prevent complete joint development resulting in subsequent chondrogenesis across the joint (Grüneberg and Lee, 1973; reviewed by Pitsillides, 1998).

**Mechanism of action**

Our studies show that GDF-5 can modulate the initial stages of chondrogenesis, i.e. the mesenchymal condensations, by increasing cell adhesion, and later during skeletal development can increase chondrocyte proliferation. These mechanisms of action are consistent with the expression pattern of Gdf-5, the skeletal changes in the chick following overexpression of GDF-5 and the complementary phenotypes due to loss of GDF-5 function in *bp* mice and in the GDF5 human syndromes (Grüneberg and Lee, 1973; Storm et al., 1994; Thomas et al., 1996, 1997; Polinkovsky et al., 1997).

Micromass and cell suspension cultures were used to analyse the mechanism of GDF-5 action during the early steps of chondrogenesis. These showed that GDF-5 can both accelerate and induce chondrogenesis, acting on the undifferentiated mesenchyme to enhance the number and size of nodules. We did not detect a change in proliferation at any time point tested. However, the cell suspension cultures showed that GDF-5 directly modulates cell adhesion. This suggests that normally in vivo, GDF-5 increases chondrogenesis by recruiting cells into the chondrogenic lineage initially from the surrounding mesenchyme and later from the early perichondrium by increasing cell adhesion (Ede and Agerbak, 1968; Hofreiter, 1968; Ede and Flint, 1972; Thorogood and Hinchliffe, 1975). Cell surface differences have been observed in the *bp* mouse and, in future, it will be important to determine fully how GDF-5 modulates expression of cell surface molecules (Hewitt and Elmer, 1976; Duke and Elmer, 1977, 1978, 1979; Elmer and Wright, 1983; Elmer et al., 1988). In addition to altering cell adhesion, our other studies have shown that GDF-5 can downregulate HA synthesis and it is also possible that, in
micromass culture and in vivo, GDF-5 may downregulate HA synthesis promoting cell contact. Indeed, in hp mice there are increased levels of HA during early chondrogenesis which have been suggested to be, in part, responsible for the delay in appendicular skeletogenesis (Shambaugh and Elmer, 1980).

Despite the ability to induce the formation of extra nodules in vitro, ectopic chondrogenesis was rarely observed in vivo and if so, was found localised at the site of the injection. The inability of GDF-5 to induce chondrogenesis in all limb bud cells in vivo is most likely due to the inhibition of chondrogenesis by ectodermal signals and also may be attributable to the absence of the appropriate receptors on the cells (Zanetti and Solursh, 1986; Solursh and Reiter, 1988). This latter idea is reinforced by the inability of GDF-5 to initially induce chondrogenesis in all cells in micromass culture, in the absence of ectodermal signals and by the fact that in single cell suspension culture, GDF-5 enhanced aggregation in a subpopulation of cells. Finally, the in vitro studies showed that GDF-5, as with many factors, can act in a dose-dependent way. Therefore, it is possible that, in vivo, the virus does not express GDF-5 at effective concentrations to induce ectopic chondrogenesis.

Later during development, Gdf-5 expression is restricted to the joints and its main mechanism of action during growth of the skeletal elements is likely to be signalling to the epiphyses, controlling proliferation in the rounded zone. Consistent with this possibility, Alk6, the type I receptor for GDF-5, is expressed in the developing cartilage epiphyses and overexpression of constitutively activated Alk6 receptor can increase chondrocyte proliferation (Rosen et al., 1996; Nishitoh et al., 1996; Zou et al., 1997; Erlacher et al., 1998; Merino et al., 1998).

Comparison with other members of the TGF-β1 family

Equivalent studies have been done with a few other members of the TGF-β1 family. Tgf-β1, like Gdf-5, is expressed in the prechondrogenic mesenchyme and can induce/promote the formation of the initial condensations in micromass cultures (Heine et al., 1987; Leonard et al., 1991). In contrast, BMP-2, BMP-3 and activin a at the later steps of chondrogenesis (Carrington et al., 1991; Jiang et al., 1993; Roark and Greer, 1994). Although activin a appears to act at a different stage, it also promotes chondrogenesis by increasing cell adhesion and recruitment of mesenchymal cells into condensations (Jiang et al., 1993).

In vivo studies have shown that overexpression of BMP-4, the constitutively activated Alk6 or Alk3 receptors in the chick and knockout of noggin function in mice, all increase chondrogenesis. Overexpression of the constitutively activated Alk6 and Alk3 receptors, like GDF-5 but unlike BMP-4, also increase chondrocyte proliferation (Duprez et al., 1996; Zou et al., 1997). Also, similarly to GDF-5, overexpression of constitutively activated Alk6 accelerates the early steps of chondrogenesis and increases the number of nodules in micromass culture, again consistent with Alk6 being the type I receptor for GDF-5 (Nishitoh et al., 1996; Zou et al., 1997; Erlacher et al., 1998). However, in contrast to these previous studies, GDF-5 increased the length of the skeletal elements suggesting that we are observing some specific effects due to overexpression of GDF-5.

Timing

One possibility from our data is that GDF-5 acts by accelerating the initial rates of chondrogenesis. This would be consistent with the hp phenotype where chondrogenesis and subsequent differentiation are initially delayed but eventually almost all the bones of the appendicular skeleton ossify, catching up with their normal counterparts (Grüneberg and Lee, 1973; Hewitt and Elmer, 1976; Shambaugh and Elmer, 1980; Elmer et al., 1988). We propose that, in the absence of GDF-5, there is delay in the initiation/condensation of cells but once the skeletal elements are a sufficient size the normal programme of skeletogenesis can be implemented.

Dose dependence

The in vitro micromass studies demonstrated that GDF-5 could act in a dose-dependent manner, suggesting that levels of GDF-5 may be important in determining the size of skeletal elements. Hence, the reason that some skeletal elements are more susceptible to loss of GDF-5 function is that they may require higher levels of GDF-5 activity, or prolonged expression of GDF-5, during normal development. In addition, the ability to promote the formation of extra nodules in micromass culture suggests that, in the absence of GDF-5, some cells would normally fail to chondrify and that in vivo there may be variation in the ability of cells to differentiate in the absence of or in low levels of GDF-5. The differential sensitivity of cells to GDF-5 signalling may be supported by analysis of the human syndromes. Hunter-Thompson chondrodysplasia is characterised by the pronounced shortening of the appendicular skeleton, with more severe effects distally and is thought to be due to complete loss of GDF-5 function (Thomas et al., 1996). In contrast, haploinsufficiency of GDF-5 can result in Brachydactyly type C, characterised only by a reduction in the length, or the loss, of some phalanges with the other appendicular skeletal elements being relatively unaffected (Polinkovsky et al., 1997). However, another possibility for the differential effect of GDF5 mutations on skeletal development may be that, in some regions, there is compensation from other members of the BMP/GDF family or that the GDF-5 mutations have differential activity (for more discussion, see Storm and Kingsley, 1996). However, it is also noted that the dose-dependent effect of GDF-5 in vitro culture may be due to GDF-5 activating receptors normally bound by other members of the TGF-β1 family.

Conclusion

Our data show that GDF-5 controls the development of skeletal elements in part by accelerating the initial steps of chondrogenesis, via increases in cell adhesion, and later GDF-5 may control chondrocyte proliferation. The mechanisms, inferred from our overexpression studies, are consistent with the expression of Gdf-5 during normal development and the loss-of-function phenotypes and do not appear to be due to functional redundancy between GDF-5 and other BMPs. Furthermore, our data together with the complementary hp mouse mutant and human syndromes suggest modulation of GDF-5 activity is a critical physiological determinant of the size of developing skeletal elements. Finally, the data suggests that in the developing joints Gdf-5 signals to the adjacent
epiphyses, controlling cell proliferation. How factors expressed in the developing joints influence skeletal differentiation is an important question and it is possible that the joint is a ‘signalling centre’ that initially co-ordinates development of the adjacent skeletal structures. This idea is supported by the noggin knockout phenotypes whereby loss of joint results in changes in the differentiation pattern of the skeletal elements (Brunet et al., 1998).

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