Abnormal FGFR 3 expression in cartilage of thanatophoric dysplasia fetuses

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Thanatophoric dysplasia (TD), the commonest lethal skeletal dysplasia in humans, is accounted for by recurrent mutations in the fibroblast growth factor receptor 3 gene (FGFR 3), causing its constitutive activation in vitro. Taking advantage of medical abortion of 18 TD fetuses, cartilage sections were studied for FGFR 3 gene expression by in situ hybridization and immunohistochemistry. Specific antibodies revealed high amounts of FGFR 3 in cartilage of TD fetuses with no increased level of the corresponding mRNA. The specific signal was mainly detected in the nucleus of proliferative and hypertrophic chondrocytes. Based on this observation and the abnormal expression of collagen type X in hypertrophic TD chondrocytes, we suggest that constitutive activation of the receptor through formation of a stable dimer increases its stability and promotes its translocation into the nucleus, where it might interfere with terminal chondrocyte differentiation.

INTRODUCTION

Recurrent mutations of the fibroblast growth factor receptor 3 gene (FGFR 3) have been shown to account for achondroplasia (1,2), hypochondroplasia (3), two clinical forms of thanatophoric dysplasia (TD I and TD II; 4,5) and several craniosynostosis syndromes (6,7), suggesting that this gene plays a major role in endochondral and membranous ossification. Subsequently, transfection experiments provided convincing evidence of constitutive activation of the receptor in a ligand-independent manner in chondrodysplasias (8–10). Hitherto, however, the cellular consequences of abnormal gene expression in the growth plate of chondrodysplasia patients have remained poorly understood.

Taking advantage of a series of 18 affected fetuses, we have investigated the pattern of FGFR 3 gene expression in cartilage of TD I and age-matched control fetuses by immunohistochemical and in situ hybridization techniques. Here we report high amounts of FGFR 3 protein in hypertrophic chondrocytes of TD I fetuses, irrespective of the position of FGFR 3 mutations, with no alteration in the amount of FGFR 3 mRNA.

RESULTS

Expression of the FGFR 3 gene during early human development

In order to investigate the differential expression of FGFR 3 isoforms (IIIb and IIIc) during development, human probes specific for alternatively spliced exons 8 (IIIb) and 9 (IIIc) were used for in situ hybridization on normal human embryos. At the end of the sixth week of gestation FGFR 3 IIIc transcripts were first detected in the chondrifying mesenchyme (anlage) of the developing limbs, while FGFR 1 transcripts were mostly found in the condensed perichondral mesenchyme (Fig. 1). In contrast, no FGFR 3 labeling was observed in the precartilagenous sclerotomal mesenchyme at the early stages of embryogenesis (32–35 days; Fig. 2). Both FGFR 3 isoforms were strongly expressed in brain and spinal cord but the type IIIc-specific probe gave a stronger staining and was the only detectable isoform in the mesonephros.

At seven weeks of gestation, while hypertrophic chondrocytes of the growth plate are still lacking in long bones, both isoforms were expressed in the resting cartilage of ribs, vertebral bodies and long bones (particularly FGFR 3 IIIc). At later stages (from day 58–60), when hypertrophic chondrocytes are present (as attested by expression of the COL10A1 gene) and ossification centers become visible, FGFR 3 IIIc was detected mostly in the proliferative and hypertrophic cells of the growth plate, but at lower levels than FGFR 1, which in addition to perichondrium was also detected in hypertrophic and calcification zones (Fig. 3e). Analysis of mineralized fetal long bones (tibiae and femurs) at 15–35 weeks of gestation showed low levels of FGFR 3 IIIc isoforms in the resting zone, which stained less strongly than the proliferative and hypertrophic zones (Fig. 3h). Surprisingly, no staining was observed in the last array of terminally differentiating chondrocytes at the chondro-osseous junction, which nevertheless
gave a strong signal with a collagen type X riboprobe (Fig. 3g). The IIIb isoform was almost undetectable (not shown).

**Disorganization of the cartilage growth plate in TD fetuses**

Serial sections revealed a severe disorganization of the cartilage growth plate with a marked reduction of the hypertrophic zone in TD specimens. Since collagen type X expression is a marker of terminal differentiation in normal hypertrophic chondrocytes, we used a COL10A1 probe for studying chondrocyte differentiation in TD specimens. Distribution of COL10A1 transcripts was uneven, as some of the hypertrophic cells displayed no specific signal. Moreover, the proportion of collagen type X-expressing cells was markedly reduced as compared with normal growth plates (Fig. 4). In addition, COL10A1 was also expressed in the fibrous band specifically observed in TD patients and disrupting the growth plate. These results were regarded as evidence of markedly disturbed chondrocyte differentiation in TD.

**Abnormal expression of the FGFR 3 protein in the cartilage growth plate of TD fetuses**

Staining of serial cartilage sections with an anti-FGFR 3 antibody revealed a stronger signal in TD fetuses, as compared with age-matched controls, both in the hypertrophic and the proliferative zones of the growth plate (Fig. 5). Similarly, staining of the perichondrium and resting chondrocytes was more intense and homogeneous in TD fetuses than in age-matched controls. A large amount of immunoreactive material was observed both in the nucleus and cytoplasm of hypertrophic TD chondrocytes, irrespective of the position of FGFR 3 mutations and regardless of the technical procedure for cartilage sectioning (Fig. 5). Subsequent in situ hybridization using a FGFR 3 IIIc probe failed to detect significant differences between TD specimens and controls (Fig. 3h and j), suggesting that the FGFR 3 mutations did not alter the amount of specific mRNA but rather modified turnover of the protein.

**DISCUSSION**

In the developing mouse FGFR 3 gene expression is first detected in the cartilage rudiments at days 12.5–14.5 p.c. and then restricted to the resting zone during endochondral ossification at day 16 p.c. (11). At embryonic day 20 and in the newborn animal the specific transcripts are mainly expressed in the proliferative chondrocytes of the vertebrae (12,13). An alternative splicing of exons encoding the second half of the third immunoglobulin-like loop was shown to give rise to two isoforms, IIIb and IIIc, in both mouse and human (14–17). Hitherto, however, little was known
regarding the pattern of FGFR 3 gene expression during human embryonic development.

Here we report on absent FGFR 3 gene expression in primordial human sclerotomes and its early expression in human brain, spinal cord (5th week) and chondrification centers of developing limbs (6th–7th week of gestation). The present data on FGFR 3 gene expression in the human central nervous system are consistent with the pattern of Fgfr expression in mouse embryos (11,18,19). However, the absence of FGFR 3 expression in prevertebral sclerotomes is at variance with results of animal studies (13,20). Similarly, in contrast to mouse embryos and newborn mice, the level of gene expression in human ossifying long bones was higher in hypertrophic and proliferative chondrocytes than in the resting zone of the cartilage growth plate, albeit no FGFR 3 expression was observed in terminally differentiating chondrocytes of the chondro-osseous junction. Hence, FGFR 3 expression in hypertrophic human chondrocytes appears to be down-regulated only when resorption of cartilage occurs, i.e. in fully differentiated chondrocytes undergoing cell death. In keeping with this, loss of FGFR 1 expression was demonstrated to be required for terminal differentiation of myogenic cells in chicken limb (21). Discrepancies between human and mouse could possibly be accounted for by a higher level of FGFR 1 expression in the hypertrophic zone of mouse cartilage as compared with human and the reduction in FGFR 3 gene expression in mouse hypertrophic chondrocytes might occur earlier than in human. Whether FGFR 1, FGFs or other factors such as BMPs are involved in negative regulation of FGFR 3 expression during skeletal growth is still unknown.

Most interestingly, analysis of the cartilage growth plate using an anti-FGFR 3 antibody detected an intense staining in hypertrophic and proliferative chondrocytes of TD fetuses relative to age-matched controls (with a nuclear and/or perinuclear localization of the receptor). Although variations in the intensity of FGFR 3 staining were noted among TD fetuses, no significant correlation between the position of the mutations and immunohistochemical findings was found. Furthermore, no increased level of specific FGFR 3 mRNA was observed on cartilage sections. The significance of these observations is unclear, but might be related to ligand-independent constitutive dimerization of the receptor through disulfide bonding (9,10). Stabilization of the mutant receptor in a dimeric form could facilitate its translocation to the nucleus. In keeping with this, stimulation of NIH 3T3 cells by FGF 1 resulted in translocation of the FGFR 1 receptor to the nucleus (22) and incubating mouse fibroblasts with FGF 2 resulted in binding of the activated receptor to the nuclear matrix (23).

In normal cartilage terminal differentiation of hypertrophic chondrocytes is associated with collagen type X synthesis and programmed cell death. Our observation that only a fraction of hypertrophic chondrocytes expressed collagen type X in TD fetuses suggests that constitutive activation of the receptor interferes with terminal differentiation. This hypothesis is supported by the previous demonstration that application of FGF 2 to rabbit chondrocytes prevented terminal differentiation (24). Since achondroplasia and TD mutations consistently activate FGFR 3 by mimicking the presence of ligand, it is tempting to speculate that in TD patients chondrocytes undergo premature apoptosis prior to the hypertrophic stage, leading to reduced skeletal growth. The observation that targeted disruption of Bcl-2 (an anti-apoptotic factor that controls programmed cell death) led to accelerated maturation of chondrocytes with shortening of long bones (25) gives additional support to this model and suggests that FGFR 3 mutations alter chondrocyte differentiation rather than proliferation.
Table 1. FGFR 3 mutations in thanatophoric dysplasia (TD) fetuses and technical procedures used on cartilage samples

<table>
<thead>
<tr>
<th>Fetus no.</th>
<th>Gestational age (weeks)</th>
<th>FGFR 3 mutation</th>
<th>In situ hybridization</th>
<th>Immunohistochemistry</th>
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<td></td>
<td></td>
<td></td>
<td>demineralized</td>
<td>non demineralized</td>
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<tr>
<td>1</td>
<td>17</td>
<td>Y373C</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>J807G</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>R248C</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>S249C</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
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<td>21</td>
<td>J807C</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>Y373C</td>
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<tr>
<td>18</td>
<td>21</td>
<td>?</td>
<td>–</td>
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Control fetuses (7) were matched for gestational age in the range 13–35 weeks. No FGFR 3 mutation was found in one TD fetus (18) and no DNAs samples were available (n.a.) for fetuses 13–15.

In situ hybridization using sense and antisense FGFR 3 IIIc and COL10A1 riboprobes were performed in five TD fetuses (6–10).

For immunohistochemical staining, cartilage samples were demineralized or non-demineralized prior to fixation.

*MATERIALS AND METHODS*

**Cartilage samples**

Tibial and/or femoral cartilage fragments were obtained from 18 medically aborted TD fetuses following the informed consent of the parents. In all cases pregnancies were legally terminated after ultrasonographic and X-ray detection of severe dwarfism. Histological studies of cartilage sections and molecular detection of FGFR 3 mutations subsequently confirmed the diagnosis of TD I. The control group included spontaneously aborted fetuses showing no evidence of skeletal abnormalities. Embryos were obtained from legally terminated pregnancies according to the French ethical committee recommendations.

Detection of FGFR 3 mutations

Screening of FGFR 3 mutations on either white blood cell or cultured skin fibroblast DNA was performed by SSCP or restriction analyses of amplification products (26,27). Mutant genotypes included the R248C (5/18), S249C (2/18), Y373C (4/18) and stop codon mutations (3/18) (Table 1). DNA from 3/18 cases was not available and we failed to detect FGFR 3 mutations in 1/18 fetuses.

**Immunohistochemistry**

Bone fragments were demineralized by moderate nitric acid treatment for 48 h, then fixed and embedded in paraffin blocks. Other samples were directly fixed with 4% paraformaldehyde then embedded in paraffin. A polyclonal antibody raised against

Figure 3. FGFR 3 IIIc, FGFR 1 and COL10A1 gene expression during human endochondral bone formation (dark field photographs). (a–e) Serial sections of a 12-week-old human fetus developing hand. (a) Hematoxylin–eosin staining of the developing carpal bones and phalanges (P1–3). (b–e) Higher magnification of the inset in (a) showing COL10A1 (b), FGFR 3 IIIc (c) and FGFR 1 expression (e) in the hypertrophic chondrocytes of the P1 phalange growth plate (antisense probes). The FGFR 3-IIIc sense probe is shown in (d). (f–i) Serial sections of femoral cartilage growth plate of a 19-week-old control fetus. (f) Hematoxylin–eosin staining of proliferative (pr) and hypertrophic chondrocytes (hy). In situ hybridization with COL10A1 antisense probe (g), FGFR 3 IIIc antisense probe (h) and FGFR 3 IIIc sense probe (i). Note that hypertrophic chondrocytes located in the vicinity of the chondro-osseous junction (cj) do not express FGFR 3 IIIc transcripts. (j) In situ hybridization of FGFR 3 IIIc antisense probe to cartilage growth plate of a 19-week-old TD fetus (number 9, Table 1). hy, hypertrophic chondrocytes; cz, calcification zone; pr, proliferative chondrocytes; cj, chondro-osseous junction. Bars: (a) 500 µm; (b–j) 100 µm.
the C-terminal end of human FGFR 3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Its specificity for FGFR 3 has been previously documented (28). After a 1 h incubation of the serial sections with the specific antibody at room temperature (dilution 1:20) a streptavidin–biotin complex was added for signal amplification and specific detection was achieved by adding the chromogenic substrate dianinobenzidine. Serial sections were counterstained with methyl green and negative controls were obtained by omitting the specific antibody. A second anti-FGFR 3 antibody kindly provided by A. Yayon gave similar results. Higher antibody dilutions (1/50, 1/100 and 1/200) were tested on both control and TD cartilage sections. No signal was detected in control samples when dilution exceeded 1/50, while TD cells stained positively even at a 1/200 dilution.

**In situ hybridization conditions**

Total RNA from cultured human fetal chondrocytes was reverse transcribed using primers specific for the human FGFR 1, FGFR 3 and COL10A1 transcripts and the respective cDNAs were cloned into a pCR3 vector (Invitrogen). The FGFR 3 probe (438 bp) encompassed the extracellular (Ig III loop) and transmembrane domains of the cDNA (nt 860–1298) (29). Two additional probes specific for the FGFR 3 IIIB (151 bp) and IIIc alternative transcripts (132 bp) were obtained by PCR amplification of human genomic DNA using exonic primers (16). Forward primers: 5′-GATCATGTGAGAGTGTCGAGC-3′ (IIIB); 5′-CGCTAAACACCACCCAGAA-3′ (IIIc). Reverse primers: 5′-GAACGCTCAGCCAAAAGG-3′ (IIIB); 5′-AGCACCACCAGCCACGGCA-3′ (IIIc). The FGFR 1 probe (363 bp) encompassed part of the extracellular and transmembrane domains (nt 957–1320) (30). Finally, primers located in exon 3 of the COL10A1 gene (nt 277–633) (31) were used to amplify a 386 bp fragment encompassing part of the triple helical domain. Sequencing of cloned PCR products with the dye terminator sequencing kit (Perkin Elmer) confirmed the originally reported human sequences (29–31).

Sense and antisense riboprobes were generated using either T7 or SP6 RNA polymerases in the presence of [α-35S]UTP (1200 Ci/mmol; NEN). Labeled probes were purified on Sephadex G50. Hybridization and post-hybridization washes were carried out according to standard protocols. Slides were dehydrated, dipped in Kodak NTB 2 emulsion, exposed for 10–20 days, then
Figure 5. Detection of the FGFR 3 protein by immunohistochemical staining of demineralized and non-demineralized tibial cartilage from control and TD fetuses. (a–c) expression of the receptor in the proliferative and hypertrophic chondrocytes of the non-demineralized cartilage growth plate; (a) 24-week-old control fetus; (b and c) TD fetuses 11 and 8 (Table 1). (d and e) FGFR 3 expression in resting chondrocytes and perichondral cells of a 24-week-old control (d) and age-matched TD fetus (number 3, Table 1) demineralized cartilage (e). (f–h) FGFR 3 expression in the proliferative and hypertrophic chondrocytes of the growth plate in demineralized control cartilage (24-week-old fetus) (f) and in the demineralized cartilage of two TD fetuses (numbers 16 and 3, Table 1) (g and h). Note the nuclear and perinuclear staining of hypertrophic TD cells. pe, perichondrium; r, resting chondrocytes; p, proliferative chondrocytes; h, hypertrophic chondrocytes; cj, chondro-osseous junction; vc, vascular canal. Bars (a)–(h) 50 μm.
developed and counterstained with methyl green. The results were analyzed by dark and bright field microscopy.

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ABBREVIATIONS

TD, thanatophoric dysplasia; FGFR, fibroblast growth factor receptor; COL10A1, collagen type X gene; FGF-2, fibroblast growth factor-2, also called basic fibroblast growth factor.

REFERENCES