Can the growth factors PTHrP, Ihh and VEGF, together regulate the development of a long bone?

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Abstract

Endochondral ossification is the process of differentiation of cartilaginous into osseous tissue. Parathyroid hormone related protein (PTHrP), Indian hedgehog (Ihh) and vascular endothelial growth factor (VEGF), which are synthesized in different zones of the growth plate, were found to have crucial roles in regulating endochondral ossification. The aim of this study was to evaluate whether the three growth factors PTHrP, Ihh and VEGF, together, could regulate longitudinal growth in a normal human, fetal femur.

For this purpose, a one-dimensional finite element (FE) model, incorporating growth factor signaling, was developed of the human, distal, femoral growth plate. It included growth factor synthesis in the relevant zones, their transport and degradation and their effects. Simulations ran from initial hypertrophy in the center of the bone until secondary ossification starts at approximately 3.5 months postnatal. For clarity, we emphasize that no mechanical stresses were considered.

The FE model showed a stable growth plate in which the bone growth rate was constant and the number of cells per zone oscillated around an equilibrium. Simulations incorporating increased and decreased PTHrP and Ihh synthesis rates resulted, respectively, in more and less cells per zone and in increased and decreased bone growth rates.

The FE model correctly reflected the development of a growth plate and the rate of bone growth in the femur. Simulations incorporating increased and decreased PTHrP and Ihh synthesis rates reflected growth plate pathologies and growth plates in PTHrP-/- and Ihh-/- mice. The three growth factors, PTHrP, Ihh and VEGF, could potentially together regulate tissue differentiation.

Keywords: Growth plate; Endochondral ossification; Metabolic bone disease; Finite element model

1. Introduction

Many signaling molecules have been found to regulate endochondral ossification in growth plates (Kronenberg, 2003; Minina et al., 2001, 2002; Ornitz and Marie, 2002; Ortega et al., 2004; Vortkamp et al., 1996). Diminished or increased synthesis can lead to severe abnormalities (Ballock and O’Keefe, 2003; Schipani et al., 1999; Kirkpatrick et al., 2003; Wilkie, 2005). The effects of parathyroid hormone related protein (PTHrP), Indian hedgehog (Ihh) and vascular endothelial growth factor (VEGF) are considered critical for development and regulation of the growth plate. These are well documented in the literature and involve three important steps in growth plate morphogenesis: cell proliferation, cell differentiation and turnover of cartilage into bone (Kronenberg, 2003; Lanske et al., 1996; Long and Linsenmayer, 1998; Minina et al., 2001, 2002; Vortkamp et al., 1996). Ihh and PTHrP together control the stimuli for chondrocytes to leave the proliferative pool, through a feedback loop (Amizuka et al., 2004; Kronenberg, 2003; Vortkamp et al., 1996; Weiss et al., 2002). Ihh is synthesized by prehypertrophic chondrocytes and binds to its receptor Ptc-1 (Alvarez et al., 2002; Kronenberg, 2003; Medill et al., 2001; Volk and Leboy, 1999).
stimulates chondrocyte proliferation both directly and indirectly, by stimulating the synthesis of PTHrP (Amizuka et al., 2004; Karp et al., 2000; Kronenberg, 2003).

During fetal life, PTHrP is expressed in the periarticular perichondrium (Alvarez et al., 2002; Kronenberg, 2003; Long and Linsenmayer, 1998; Medill et al., 2001; Vortkamp et al., 1996). PTHrP presumably diffuses into the growth plate (Di Nino et al., 2001) and keeps chondrocytes proliferating. In addition, PTHrP has been shown to inhibit differentiation (Amizuka et al., 2004; Chung et al., 1998; Lee et al., 1996; Volk and Leboy, 1999). Ihh is expressed in the developing bone of the mouse as soon as chondrocytes become prehypertrophic and PTHrP is subsequently expressed in the perichondrium (Bitgood and McMahon, 1995).

VEGF, a potent angiogenic stimulator, is expressed by hypertrophic chondrocytes (Colnot et al., 2004; Ortega et al., 2004; Petersen et al., 2002). It can induce migration and differentiation of osteoblasts and osteoclasts (Maes et al., 2002; Ortega et al., 2004).

We hypothesized that the three growth factors PTHrP, Ihh and VEGF, together, could regulate the development of a fetal long bone. We therefore studied long-bone development, only controlled by these growth factors, using a quantitative numerical model. A mathematical, temporo-spatial model of bone fracture healing, which included signaling by generic growth factors, was developed earlier (Bailon-Plaza and van der Meulen, 2001) in addition to a finite element (FE) mechanoregulation model of endochondral ossification in a developing long bone (Stevens et al., 1999). However, since none of the currently available models study the interaction of growth factors, development of a model was part of the present study.

The mathematical formulas and their quantities were obtained from the literature, or, if necessary, estimated from literature data. For evaluation of growth factor-controlled fetal long-bone development in a temporal and spatial sense, these formulas were implemented in a one-dimensional FE model, representing the distal half of the human, fetal femur. The model was implemented to simulate normal fetal bone development. Subsequently, it was applied to pathological growth patterns caused by altered PTHrP or Ihh synthesis.

2. Methods

Synthesis, transport and degradation of growth factors were determined throughout the growth plate in the computational model. The concentrations of growth factors determined the bone development by affecting differentiation and proliferation rate. Once the fetal growth plate originated, the model represented the articular perichondrium, resting, proliferative, prehypertrophic, hypertrophic zones and bone (Fig. 1).

Individual cells were described in the proliferative, prehypertrophic and hypertrophic zones. The other zones were assumed to consist of homogeneous material. A cell and its surrounding extracellular matrix (ECM) were considered as a domain of elements, with homogeneous properties. These properties were growth factor synthesis rate, diffusion coefficient and half-life time, which depended on the type of cell the elements were representing. With increasing cell length, the number of elements describing the cell increased. PTHrP was assumed to be synthesized in the articular perichondrium, Ihh by prehypertrophic and VEGF by hypertrophic cells; their assumed effects are depicted in Fig. 2.

A cell and its ECM changed phenotype when its elements reached prescribed conditions, as discussed below.

The general differential equation for the concentrations of all growth factors was

$$\frac{\partial c_{gf}}{\partial t} = S_{gf} + T_{gf} + D_{gf},$$

(1)

![Fig. 1. 1-D mesh representing a femur after bone development has taken place. PC = perichondrium, RZ = resting zone, PZ = proliferative zone, pHZ = prehypertrophic zone, HZ = hypertrophic zone.](image)

![Fig. 2. Arrows between zones indicate cell differentiation. The position of the growth factor depicts its release site. A dotted arrow reflects an inhibitory effect. A solid arrow reflects an inducing effect.](image)
where synthesis is given by
\[
S_{gf} = \frac{a_{gf}}{\text{cell}},
\]
transport is given by
\[
T_{gf} = \nabla \cdot (d_{gf} \nabla c_{gf}),
\]
and degradation is given by
\[
D_{gf} = -\frac{\ln(2)}{\tau_{gf}} c_{gf},
\]
where \(c_{gf}\) is the concentration of a growth factor, in which subscript \(gf\) can be \(p, i\) and \(v\) for PTHrP, Ihh and VEGF, respectively, \(l_{\text{cell}}\) the length of one cell, \(a_{gf}\) the growth-factor synthesis rate, \(d_{gf}\) the diffusion coefficient and \(\tau_{gf}\) the half-life time of a growth factor.

Growth-factor synthesis rate per cell was assumed to be continuous and not to increase with hypertrophy, but constant (Farnum et al., 2002). For the synthesis of Ihh and VEGF, \(a_{gf}\) was a constant. The synthesis of PTHrP was dependent on the concentration of Ihh in the elements of the perichondrium according to the Hill equation
\[
a_p = b \frac{c_i^4}{c_i^4 + K_{mp}},
\]
where \(b\) is the maximal synthesis rate and \(K_m\) the concentration of Ihh at which half the maximal synthesis rate of PTHrP is reached. The conditions for a cell to change phenotype varied per zone. It was suggested that there is a threshold concentration below which PTHrP no longer prevents initiation of the hypertrophic program (St Jacques et al., 1999). Therefore, the elements of the cell most distal in the proliferative zone would become prehypertrophic when its PTHrP concentration, \(c_p\), was below a limit \(l_1\). Hence,
\[
c_p < l_1.
\]

It remains unclear what causes a cell to change from being prehypertrophic to hypertrophic. The most distal prehypertrophic cell became hypertrophic when it reached the length \(l_2\). Hence,
\[
l_{\text{cell}} < l_2.
\]

We assumed that when the concentration of VEGF, \(c_v\), in the bone reached a level \(l_3\), the ECM around the last cell in the hypertrophic zone was mineralized. Hence,
\[
c_v < l_3.
\]

Enlargement of prehypertrophic and hypertrophic cells depended on the local PTHrP concentration. It was assumed that when no PTHrP is present, hypertrophy takes place at a maximal rate \(f\). Since PTHrP inhibits the rate of differentiation, an adjusted Michaelis–Menten equation was chosen to describe the rate of hypertrophy as
\[
e = \frac{f \left(1 - \frac{c_p}{c_p + K_{mp}}\right)}{c_p + K_{mp}},
\]
where \(e\) is the hypertrophy rate and \(K_m\) the concentration of PTHrP at which half the maximal hypertrophy rate is reached.

2.1. Numerical implementation

The model was implemented in the SEPRAN FE-package (Ingenieursbureau SEPRA, Leidschendam, The Netherlands). A one-dimensional, 2-node linear line element for standard diffusion problems was used, yielding one unknown for concentration. Synthesis, transport and degradation of growth factors were taken into account using the accurate second-order Strang-operator splitting scheme (Sengers et al., 2004). The procedure consisted of three parts: For each time step, first the system of ordinary differential equations, based on synthesis and degradation of growth factors, was time-integrated. Then the decoupled linear-transport partial-differential equations were solved per growth factor separately. Finally, the first part was repeated again. Synthesis and degradation were solved by using an explicit Runge–Kutta time integration; diffusion was solved using Crank–Nicholson time integration.

The FE model contained 1215 elements, representing 3.375 mm (Fig. 1). The geometry of the mesh was fixed, but properties of the elements were allowed to change during the simulation. Each element in the perichondrium and the resting zone represented 15 \(\mu\)m. In this zone, the properties are constant. The properties of elements in the proliferative, prehypertrophic and hypertrophic zones changed during the simulation, depending on the zone they represented. To accurately describe these zones, elements represented only 1.3 \(\mu\)m. The number of elements that constituted one cell increased during hypertrophy. Upon proliferation, the proliferative zone increased with the number of elements representing one cell, and the prehypertrophic and hypertrophic zones shifted down over this distance. Hence, when cells hypertrophied or proliferated, the anlage and the growth plate increased in size and more elements of the mesh were included in the simulation. When a hypertrophic cell was turned into bone, bone growth was documented to monitor the actual bone length. The mineralized elements were then excluded from the computations to limit the number of elements in the simulations. Only elements within the current length of the anlage were taken into account; the unused part of the mesh acted as a buffer to accommodate for continuous change in the number of elements that represented the non-mineralized part of the anlage. At zero simulation time, the length of the anlage was 2.2 mm.
No mass could flow over the boundaries of the anlage; diffusion occurred only in the part of the mesh that made up the anlage. The time step was optimized to 100 s.

Every 12 time steps, cell enlargement was calculated for each cell. The number of elements per cell was expressed in continuous numbers, whereas the elements are discrete. Elements describing two cells were assigned average properties.

A base cell production rate of one per 10 h was assumed in the proliferative zone in the model, which was doubled in the presence of normal Ihh concentrations (Karp et al., 2000; Kember and Sissons, 1976; St Jacques et al., 1999). It has been found that proliferative cells positioned close to the prehypertrophic zone divide at a lower rate than those positioned proximally (Farnum and Wilsman, 1993). It was assumed that a new cell was always formed most proximally.

It was assumed that when a cell changes phenotype, this process takes 2 h. During this period, this cell would not synthesize growth factors. Between each zone, only two cells were allowed to transfer at the same time, resulting in a maximal in- and outflow of 18 cells/day.

2.2. Parameter values

We determined the apparent diffusion coefficient of dextran 10 and 40 kDa using FRAP (Sniekers and van Donkelaar, 2005) in the proliferative zone at values of 69 (+14%) and 57 (+14%) μm²/s, respectively. Based on molecular weights, PTHrP and Ihh were assigned a diffusion coefficient of 63 μm²/s and VEGF of 69 μm²/s in the proliferative zone. Assuming that the diffusion occurs in the ECM and not in the cells, the diffusion coefficient for all other zones was derived from the ECM/cell ratios (Fazzalari et al., 1997; Hunziker et al., 1987).

We assumed that the proliferative zone remained at 1.7 mm from the distal end of the bone (Gardner and Gray, 1970). The length of a proliferative cell and its ECM was constant at 10.5 μm. Cell enlargement started when it became prehypertrophic. The limit l₁ was set at 22.5 μm, as estimated from histology. Hypertrophic cells would then further enlarge until approximately 39 μm depending on cₑ (Fazzalari et al., 1997)(Hunziker et al., 1987).

The half-life times of all growth factors were assumed to be 10 min (Bailon-Plaza and van der Meulen, 2001). No degradation of PTHrP and Ihh during transport between perichondrium and proliferative zone was assumed.

Growth-factor synthesis rates are unknown. These were set by assuming that, in a fully developed growth plate, given the aforementioned parameters for transport and degradation, the concentration profiles were consistent with the concentration limits l₂ and l₃.

The simulation started with a cartilage anlage at 8 weeks of gestation, consisting of proliferative cells only. It was continued until secondary ossification starts, at approximately 3.5 months after birth. To show the applicability of the model, four additional simulations were run in which either PTHrP or Ihh synthesis was 50% increased or decreased. The results were compared qualitatively with literature data.

3. Results

The initial model of the human, distal, femoral growth plate contained only a proliferative zone. Hence, the Ihh concentration throughout the growth plate was zero and thus the PTHrP concentration also (Fig. 3A). Therefore, proliferative cells could become prehypertrophic. When these cells had reached the length l₂, they became hypertrophic, thus stopped synthesizing Ihh and started synthesizing VEGF. After approximately 8 days, a stable growth plate had evolved in which the length of all three zones oscillated around an equilibrium level (Figs. 3B and 4). The varying cell number coincided with oscillating growth factor concentrations (Fig. 5). The number of proliferative cells varied between 44 and 47, prehypertrophic cells between 17 and 21, and hypertrophic cells between 10 and 12.

The simulation ran for 400 cycles, corresponding with 48 weeks, ending at 3.5 months after birth, during which the growth plate remained stable. Hereafter, secondary ossification starts, which was not represented in the model. The cell division rate, after the growth plate was developed, was constant at a rate of 5.05 cells/day and the bone growth rate was 1.62 mm/day. Since it is known that the distal, human femoral growth plate accounts for 70% of the total femoral bone growth (Kember and Sissons, 1976), the total bone growth rate would be 2.31 mm/day. This corresponds well with experimental data (Fig. 6) (Chitty and Altman, 2002). The length of the total modeled femur at birth was 76 mm.

All four simulations incorporating alternative PTHrP or Ihh synthesis rates resulted in stable growth plates. Increased and decreased PTHrP synthesis rates resulted in, respectively, an increased and a decreased number of proliferative cells and bone growth rate (Table 1). The number of cells in the prehypertrophic and hypertrophic zones did not vary substantially between the normal, PTHrP increased and decreased situations. Increased and decreased Ihh synthesis rates, respectively, caused enlarged and shortened proliferative and prehypertrophic zones. It also resulted in, respectively, increased and decreased bone growth rates. The effects of changes in Ihh were more pronounced than those following PTHrP changes.
4. Discussion

A temporo-spatial computational model of the developing human distal femur was developed and implemented in a FE model. Growth factor signaling determined the proliferation and differentiation of cells, hereby determining which growth factor each cell produces. The model was able to simulate the initiation of the growth plate in an anlage. The growth plate remained stable, resulting in continuous, linear bone growth throughout the whole simulation period (48 weeks, 16 weeks after birth). The total bone growth rate as well as the number of cells predicted by the model corresponded to literature values (Chitty and Altman, 2002; Honarvar et al., 2001; Neufeld et al., 2004). Hence, we conclude that the growth factors PTHrP, Ihh and VEGF together could regulate linear growth in a long bone.

The synthesis rate per cell, $a_{gf}$, and the maximal hypertrophy rate, $f$, are unknown. Values for these parameters were derived from an already stable growth-plate model and subsequently applied to the anlage model. With these, the development of a bone from early anlage to fully developed growth plate could be described appropriately, despite the initial variations in number of cells (Fig. 4), implying that a regulatory system based on PTHrP, Ihh and VEGF is robust.

Transport, which is modeled by diffusion as a first approximate, is more complex. Growth-factor-to-receptor binding and the availability of receptors affects the effective transport of the growth factor and its active lifetime. Active transport of growth factors is also known to play a substantial role. Ihh has been found to have short- and long-range activities and heparan sulfate has been hypothesized to regulate long-distance transport of Ihh, between the prehypertrophic zone and the articular perichondrium (Gritli-Linde et al., 2001; Koziel et al., 2004). The strong growth factor gradient between the proliferative and resting zone predicted by the model would be challenging to test experimentally.

Simulations with increased or decreased PTHrP synthesis resulted in increased and decreased numbers of proliferative cells, respectively. When the PTHrP synthesis rate is increased, the PTHrP concentration in the growth plate subsequently increases. Therefore, the position where the PTHrP concentration crosses the level $l_1$, below which the most distal proliferative cell becomes prehypertrophic, moves farther away from the perichondrium, leading to an enlarged proliferative zone. This concurs with experiments on mice, in which overexpression of PTHrP is targeted (Weir et al., 1996) and with growth plates of patients suffering from
Jansen’s metaphyseal chondrodysplasia (Ballock and O’Keefe, 2003; Schipani et al., 1999).

When decreased PTHrP synthesis is incorporated, the opposite effect occurs, leading to a shortened proliferative zone. These results agree with data from the literature, showing that PTHrP-/- mice have a reduced domain of proliferating cells (Amizuka et al., 1994; Karaplis et al., 1994; Karp et al., 2000; Lanske et al., 1996; Lee et al., 1996), and with growth plates seen in Blomstrand chondrodysplasia (Jobert et al., 1998).

The number of cells in the prehypertrophic and hypertrophic zones did not vary substantially between the normal, PTHrP increased and decreased situations, which agrees with the literature (Lee et al., 1996). A new equilibrium is established at the point where the PTHrP concentration in the most distal proliferative cell crosses the level $l_i$. The PTHrP concentration profile after that point remains the same and therefore the degree of hypertrophy, controlled by PTHrP, is the same, leading

Fig. 5. (A) PTHrP concentration in the most distal proliferative cell is above the critical limit (horizontal dashed line). (B) Due to a prehypertrophic cell becoming hypertrophic, the Ihh concentration and thus the PTHrP concentration will decrease. The PTHrP concentration in the most distal proliferative cell is below the critical level and the cell will therefore become prehypertrophic. (C) Normal VEGF concentrations in the hypertrophic zone. (D) Due to a prehypertrophic cell becoming hypertrophic, the VEGF concentration in the hypertrophic zone and the bone increases above the critical level leading to a hypertrophic cell to become bone. Horizontal dashed lines indicate critical levels; vertical dashed lines indicate the position in bone where the VEGF concentration is compared with the critical level.

Fig. 6. Femur length from center to distal end and total femur length with gestation as computed with the model compared with experimental data from human femurs (Chitty and Altman, 2002).
to a similar number of cells in the prehypertrophic and hypertrophic zone.

The simulation with increased Ihh synthesis resulted in an increased number of both proliferative and prehypertrophic cells. The former was the result of the fact that an increased Ihh concentration leads to a higher PTHrP synthesis, which in turn leads to an enlarged proliferative zone. The latter was caused by increased cell-division rates in the proliferative zone, due to increased Ihh synthesis. Therefore, the overall cell in- and outflow for each zone, which is always equal in a stable growth plate, was increased compared to the normal situation. Because hypertrophy rate, based on the PTHrP concentration, was approximately the same in the prehypertrophic zone, the number of cells in the prehypertrophic zone increased. The bone growth rate also increased compared to the normal growth plate. The simulation with decreased Ihh synthesis rates showed the opposite effects, which agrees with data on Ihh-/- mice (St Jacques et al., 1999).

The effects of Ihh synthesis changes were larger than PTHrP changes, in terms of number of cells and proliferation rates. Karp et al. (2000) found that the proliferative zone and the proliferation rate in Ihh-/- mice is more reduced than in PTHrP-/- mice, which corresponds to our results. A simulation with no PTHrP or Ihh synthesis, however, resulted ultimately in disappearance of the growth plate (data not shown). This effect is not seen in transgenic mice, possibly due to other, non-modeled, growth factors which become more important in the absence of PTHrP or Ihh.

The number of cells in the model oscillates around an equilibrium (Fig 4). Although the assumptions made in the model affected the degree of oscillation, presumably, the number of cells in a column actually varies in time, as indicated by the variation in appearance of different columns in a growth plate. It would be challenging to quantify this oscillation per column and per zone experimentally.

In experiments using mice and rats, unfortunately, the distribution of growth factors and the internal structure of the growth plate cannot be monitored over time. Such dynamic analyses have now become possible with the finite element model presented.

In conclusion, it was found that the three growth factors PTHrP, Ihh and VEGF could potentially control developments in the growth plate, resulting in constant bone growth rates. The finite element model also showed that increased and decreased PTHrP and Ihh synthesis rates result in different numbers of cells and bone growth rates in stable growth plates. These numbers of cells and bone growth rates are in agreement with those seen in pathologies and transgenic mice.

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References


