Growth factor profile in calcified cartilage from the metaphysis of a calf costochondral junction, the site of initial bone formation

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Received November 3, 2020; Accepted March 11, 2021

DOI: 10.3892/br.2021.1430

Abstract. Endochondral bone formation is orchestrated by growth factors produced by chondrocytes and deposited in the cartilage matrix. Whilst some of these factors have been identified, the complete list and their relationship remains unknown. In the present study, the growth factors were isolated from non-calcified and calcified cartilage of costochondral junctions. Cartilage dissected from the ribs of 6-20-week-old calves was purchased from a local butcher within 24 h of the death of the animal. The isolation involved hyaluronidase digestion, guanidinium hydrochloride (GuHCl) extraction, HCl decalcification and GuHCl extraction of the decalcified matrix. Growth factors were purified by heparin chromatography and their quantities were estimated using ELISA. Decalcified cartilage was also used for protein sequence analysis (data are available via ProteomeXchange; ID, PXD021781). Bone morphogenetic protein-7 (BMP-7), growth/differentiation factor-5 (GDF-5) and NELL-like protein-1 (NELL-1), all known growth factors that stimulate bone formation, quantitatively accounted for the majority of the material obtained in all steps of isolation. Thus, cartilage serves as a store for growth factors. During initial bone formation septoclasts release osteoclastogenesis-stimulating factors deposited in non-calcified cartilage. Osteoclasts dissolve calcified cartilage and transport the released factors required for the stimulation of osteoprogenitor cells to deposit osteoid. High concentrations of BMP-7, GDF-5 and NELL-1 at the site of initial bone formation may suggest that their synergistic action favours osteogenesis.

Introduction

The isolation of growth factors stimulating the formation of bone from decalcified bone matrix has been shown in several studies, characterising their activity in intra- and extra-skeletal sites and their stimulatory/inhibitory effects on cell populations involved in bone formation (1). Growth factors are also essential for endochondral bone formation and they have been identified in various components of the epiphyseal growth plate (2); however, quantitative studies on their presence are lacking. In the present study, the data on the quality and quantity of growth factors present in the epiphyseal growth plate, and particularly in calcified cartilage (the site of initial bone formation) was assessed for the first time. These data may assist in identification of the growth factors most useful as a potential treatment for damaged bones postnatally.

The epiphyseal growth plate, serving as the template for bone formation, is composed of chondrocytes arranged in reserve, proliferative and hypertrophic zones (3). Ossification of cartilage is preceded by the deposition of a periosteal bone collar by osteoblasts differentiating it from the surrounding mesenchymal cells (4). Chondrocytes in the proliferative and hypertrophic zones form elongated columns separated from one another by longitudinal septa of the cartilage matrix, whereas chondrocytes within the columns are separated by transverse septa. In the hypertrophic zone, chondrocytes become enlarged and deposit calcium salts within longitudinal septa in the region adjacent to the metaphysis, known as the zone of provisional calcification. Approximately two-thirds of the longitudinal septa becomes partially or completely calcified (5). Transverse septa remain non-calcified. The metaphysis, in which osteoblasts deposit bone on the calcified longitudinal septa, and which represents the initial site of bone formation, is located just distal to the last intact transverse septum at the base of each cell column (6,7).

Calcification of the epiphyseal growth plate begins with the formation of matrix vesicles produced by chondrocytes in the proliferative and hypertrophic zones (8,9). Matrix vesicles contain enzymes that increase the local concentration of orthophosphate and lead to the formation of apatite-like deposits (7,10-12). Matrix vesicles also carry bone morphogenetic proteins (BMPs)-1-7 and vascular endothelial growth factor (VEGF), factors which serve an important morphogenetic role in endochondral bone formation (13). The presence of BMP1-7 was also demonstrated immunocytochemically in chondrocytes of the hypertrophic and calcifying zones during endochondral bone formation (14). In extracts from bovine articular cartilage, yet more growth factors were identified, including cartilage-derived morphogenetic...
The animals were killed by exsanguination after stunning in dairy breed and bought from a local butcher within 24 h of death.

Preparation of calcified cartilage.

Materials and methods

Role of growth factors during the early stages of bone formation could be important both for an improved understanding of the interaction of various growth factors that belong to the TGF-β superfamily. Currently, >20 types of BMPs have been identified (19). BMPs are involved in all stages of epiphyseal cartilage formation, including mesenchyme condensation, chondrocyte differentiation, hypertrophy, cartilage matrix calcification and subsequent resorption by chondroclasts, as well as the final bone deposition step (2,18-21). GDF-5 and NELL-1 participate in bone repair and regeneration (2,22). VEGF stimulates the formation of blood vessels required for epiphyseal cartilage nutrition (23).

An improved understanding of the interaction of various growth factors may emerge from studies on their signalling pathways. For example, growth factors from the TGF-β superfamily (BMPs/GDF-5) transduce signals by both the canonical SMAD-dependent signalling pathway and the non-canonical SMAD-independent signalling pathway. Both pathways converge at transcription factors, for example, Runx2, to promote osteoblast differentiation from mesenchymal precursor cells (24). NELL-1, which is not a member of the TGF-β superfamily, also acts through the Runx2 (18) and effectively induces the expansion of a bone marrow subset of mesenchymal progenitor cells (24,25).

The presence of growth factors within matrix vesicles (13) and in non-calcified cartilage (14) suggests that they may also be present within calcium deposits. Growth factors may be released during calcified cartilage resorption and stimulate bone formation. The quantitative profile of the growth factors that accumulate in calcified matrix and participate in endochondral bone formation remains to be fully elucidated. The primary obstacle is the paucity of calcified cartilage in epiphyseal cartilage. Even in large animals, obtaining a large amount of calcified cartilage from the epiphyseal cartilages of long bones is technically difficult. Based on the similarity in structure and function of epiphyseal cartilage and costochondral junctions (26-28), it was possible to harvest calcified cartilage from 24 ribs of one animal. The choice of calves was dictated by the local preference for calf meat assuring a regular supply of ribs and by the availability of ELISA tests for numerous bovine growth factors. Thus, costochondral junctions of calf ribs were collected, and growth factors were determined both in non-calcified and calcified matrices. The obtained data could be important both for an improved understanding of the role of growth factors during the early stages of bone formation and possibly, for assisting in the formulation of treatment regimens useful in the treatment of bone deficiencies.

Materials and methods

Preparation of calcified cartilage. The cartilage was dissected from the ribs of 6-20-week-old calves of the Holstein Friesian dairy breed and bought from a local butcher within 24 h of death. The animals were killed by exsanguination after stunning in accordance with the Polish norm (29). Costochondral junction (an equivalent of the cartilage growth plate of long bones) was cleared from the adhering tissues and hand broken at the level of the metaphysis, the provisional zone of calcification. Then, the calcified cartilage was scraped from the exposed surface with a knife, lyophilised and pulverised in liquid nitrogen. On average, 400 mg dried cartilage powder was obtained from one animal. Ribs were collected from 32 animals (768 ribs). In order to eliminate the possible individual differences between calves, cartilage powder was pooled in batches containing harvest from 10-12 animals.

Isolation of calcified cartilage. Cartilage powder (400 mg) was rehydrated in 40 ml dH$_2$O for 15 min at 4°C and centrifuged at 500 x g for 5 min at 4°C. The pellet was suspended in 10 ml dH$_2$O and further incubated for 5 min at 4°C. After a second centrifugation step (500 x g for 5 min at 4°C), the pellet was re-suspended in 10 ml dH$_2$O, placed atop a gradient consisting of 1.25 g and 1.0 g/ml of barium iodide in dH$_2$O and spun down at 500 x g for 10 min at 4°C. The pellet contained large fragments of calcified cartilage usually attached to some non-calcified substance, whereas in the material from the gradient interface, the latter predominated. Interface material was discarded, and the pellets were rinsed in dH$_2$O and lyophilised. The average weight of a lyophilised pellet was 300 mg.

Isolation of growth factors. The process of the isolation of growth factors involved: Hyaluronidase digestion; GuHCl extraction; enzymatic removal of non-calcified matrix remnants; HCl decalcification; GuHCl extraction of decalcified matrix (Fig. 1); and purification of growth factors using HiTrap heparin affinity columns.

Hyaluronidase digestion. Pellets obtained after gradient separation were digested in 10 ml 0.1% bovine testes hyaluronidase (Sigma-Aldrich; Merck KGaA) in PBS (Sigma-Aldrich; Merck KGaA) in order to obtain the fraction of growth factors (fraction 1) present in the matrix adjacent to calcified cartilage (30). The process of digestion was controlled by staining with 0.1% aqueous solution of toluidine blue at room temperature for 10 min (Sigma-Aldrich; Merck KGaA). Digestion lasting 4-5 h was found to be sufficient to remove all traces of stainable material. Fraction 1 was separated from the solid material (sediment 1) by centrifugation (500 x g for 10 min at 4°C), desalted in PD-10 columns (GE Healthcare Life Sciences) and lyophilised. Next, lyophilised material was dissolved in 4 M GuHCl in 50 mM TRIS (pH 8.0) with 0.15 M NaCl (all from Sigma-Aldrich; Merck KGaA). GuHCl served as the binding buffer during the separation of growth factors in heparin columns (GE Healthcare Life Sciences).

GuHCl extraction after hyaluronidase digestion. Sediment 1 was extracted with 4 M GuHCl (prepared as above). Extraction was performed twice for 2 h at 4°C each time. Extracts were pooled (fraction 2) and applied to 4 M GuHCl heparin columns.

Enzymatic removal of non-calcified matrix remnants. In order to remove the remnants of the organic material, the sediment (sediment 2) after extraction was digested with 0.25% collagenase and 0.05% DNase solution (all from Sigma-Aldrich; Merck KGaA) at 37°C for 2 h, in 0.25% trypsin (Sigma-Aldrich; Merck KGaA) at 37°C for 1 h and again in collagenase (under the same conditions). The enzymatic
solutions were discarded, and the calcified cartilage (sediment 3) was rinsed twice with 4 M GuHCl for 15 min, followed by two changes of dH2O.

**HCl decalcification.** Sediment 3 purified by enzymatic digestion was decalcified in 4 ml 0.6 M HCl (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. The progress of decalcification was controlled by microscopic observation. Then, the material was neutralised with 10 N NaOH (Sigma-Aldrich; Merck KGaA), and the supernatant (fraction 3) obtained by centrifugation (500 x g for 5 min at 4˚C), desalted in PD-10 columns (GE Healthcare Life Sciences), lyophilised, dissolved in 4 M GuHCl and applied to heparin columns.

**GuHCl extraction of decalcified cartilage.** After HCl decalcification (sediment 4), the pellet was extracted using GuHCl prepared as above, and the obtained fraction 4 was applied to heparin columns.

**Purification of growth factors by HiTrap heparin affinity columns.** Fractions were applied to HiTrap heparin affinity columns (GE Healthcare Life Sciences) in the binding buffer containing 20 mM Tris-HCl, 0.15 M NaCl, 4 M GuHCl (pH 8.0) (all from Sigma-Aldrich; Merck KGaA). The elution buffer contained 20 mM Tris-HCl with 2 M NaCl (all from Sigma-Aldrich; Merck KGaA). The eluate was desalted in PD-10 columns (GE Healthcare Life Sciences), lyophilised, and used for growth factor determination by ELISA and protein sequence analysis. All solutions used in this work contained 10 µl 100x concentrated protease inhibitors (Thermo Fisher Scientific, Inc.) per 1 ml.

**ELISA.** Growth factors were evaluated using immunoassay kits for BMP-1 (cat. no. GR106213), BMP-3 (cat. no. GR106215), BMP-5 (cat. no. GR106217) (all from Genorise Scientific, Inc.), BMP-2 (cat. no. E11B0810), BMP-4 (cat. no. E11B0542), BMP-6 (cat. no. E11B0395), BMP-7 (cat. no. E11B0390) and transforming growth factor-β1 (TGF-β1; cat. no. E11T0009) (all from BlueGene), VEGF (cat. no. MBS760448), mesencephalic astrocyte-derived neurotrophic factor (MANF; cat. no. MBS288818), NELL-like protein-1 (NELL-1; cat. no. MBS93668405), osteoclast-stimulating factor-1 (OSTF-1; cat. no. MBS741961), (all from MyBioSource, Inc.), bFGF (cat. no. orb403256) (Biorybt), connective tissue growth factor (CTGF; cat. no. RD-CTGF-Ra; RedDot), GDF-5 (cat. no. EK1504-BV-CAP; Boster Biological Technology) and insulin-like growth factor-1 (IGF-1; cat. no. MG100; Novateinbio) according to the manufacturers' protocols. Proteins present in calcified cartilage from calf costochondral junctions were sequenced.

**Protein sequence analysis.** Analysis and data processing was performed on a commercial basis at the Mass Spectrometry Laboratory of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, Poland according to the following procedure.

**Trypsin digestion.** Gel slices were subjected to the standard 'in-gel' digestion procedure during which proteins were reduced with 100 mM 1,4-dithiothreitol (Sigma-Aldrich; Merck KGaA) for 30 min at 56˚C, alkylated with iodoacetamide (45 min in a dark room at room temperature) and digested overnight with trypsin (sequencing Grade Modified Trypsin; cat. no. V5111; Promega Corporation). The resulting peptides were eluted from gels using 0.1% trifluoroacetic acid (TFA) and 2% acetonitrile (ACN) (both from Sigma-Aldrich; Merck KGaA).

**Mass spectrometry.** The material for sequencing was served as 300 mg pellets of enzymatically purified (hyaluronidase, collagenase, DNase and trypsin) calcified cartilage from the costochondral junction of calf ribs. A total of four samples were sequenced: A sample of proteins released during decalcification in 0.6 N HCl, and three samples of decalcified material. Peptide mixtures were separated by liquid chromatography prior to molecular mass measurements on the Orbitrap Velos mass Spectrometer (Thermo Electron Corp.). A peptide mixture was applied to RP-18 precolumn (nanoACQUITY Symmetry® C18; cat. no. 186003514; Waters UK) using water containing 0.1% TFA as a mobile phase and then transferred to a nano-HPLC RP-18 column (nanoACQUITY BEH C18; cat. no. 186003545; Waters UK) with an ACN gradient (0-60% ACN in 70 min) in the presence of 0.05% formic acid with a flow rate of 150 nl/min. The column outlet was directly coupled to the ion source of the spectrometer working in the regime of data-dependent MS to MS/MS switch. A blank run ensuring lack of cross-contamination from previous samples preceded each analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (31) partner repository with the dataset identifiers are PXD021781 and 10.6019/PXD021781.

**Data processing.** The acquired raw data were processed using Mascot Distiller followed by database searches within the Mascot program (Matrix Science, 8-processor on-site licence) against the National Centre for Biotechnology Information (version 20100203). Search parameters for precursors and product ions mass tolerance were 40 ppm and 0.8 Da respectively, with allowance made for one missed trypsin cleavage and the following fixed modifications: Cysteine carbamidomethylation and allowed variable modification - oxidation (M). Peptides with Mascot Score exceeding the threshold value

Figure 1. Process of growth factor isolation. GuHCl, guanidinium hydrochloride.

Calcified cartilage

- **Hyaluronidase digestion - fraction 1**
- **GuHCl extraction - fraction 2**
- **Enzymatic removal of non-calcified matrix remnants**
- **HCl decalcification - fraction 3**
- **GuHCl extraction of decalcified cartilage - fraction 4**
corresponding to <5% false positive rate, calculated by the Mascot procedure, were considered to be positively identified.

**Histological procedures.** Fragments of ribs were fixed in phosphate-buffered formalin, pH 7.2 or in 70% ethanol at room temperature for 24 h, decalcified in edetic acid (Sigma-Aldrich; Merck KGaA) at 37˚C for 6 days, and dehydrated and embedded in paraffin. Sections, 8 µM thick, were stained with haematoxylin‑eosin for 6 and 2 min respectively, at room temperature.

**Results**

The concentration of growth factors assessed in the calcified cartilage and detected in the material released during all steps of purification is shown in Table I. Pooled material was used in all determinations. BMP‑2, BMP‑5 and NELL‑1 accounted for the majority of growth factor content present compared with the remaining growth factors detected (VEGF, BMP‑2, BMP‑3, BMP‑4 and bFGF). TGF‑β1 was not released by hyaluronidase, but was present in small amounts in the remaining groups. BMP‑1, BMP‑5, BMP‑6, IGF‑1, MANF, CTGF and OSTF‑1 were not detected by assays with detection ranges of 31‑2,000 pg/ml for BMP‑1; 125‑8,000 pg/ml for BMP‑3; 50‑1,000 pg/ml for BMP‑4; 21.5‑2,000 pg/ml for IGF‑1; 6.25‑400 pg/ml for CTGF; 0.156‑10 ng/ml for MANF. Thus, from the 16 growth factors assessed in the calcified and non‑calcified material, bound to calcified cartilage matrix, 9 factors were determined to be present by ELISA.

Protein sequential analysis showed the presence of proteins characteristic of cartilage, such as collagen type II, cartilage oligomeric matrix protein, aggrecan core protein, hyaluronan, proteoglycan link protein 1, biglycan, chondroadherin (data are available via ProteomeXchange: ID, PXD021781). Sequenced material also contained >200 identifiable nuclear and cytoplasmic components. In decalcified cartilage, certain proteins, such as heterogeneous nuclear ribonucleoproteins, ADP‑ribosylation factor 4, α‑enolase, alkaline phosphatase, phosphoglycerate mutase 1 and Annexin A6 were detected in all three samples. Others, for example protein disulphide‑isomerase A3, histone H2B type 1‑K and peptidyl‑prolyl cis‑trans isomerase B were found in only two samples, and protein disulphide‑isomerase A4, glutaminyl‑peptide cyclotransferase and protein/nucleic acid deglycase DJ‑1 was only present in one sample. Approximately 50 proteins were also released from calcified cartilage during decalcification, but the profile did not differ from the previous group notably. Blood proteins in decalcified material were represented in all samples by serum albumin, α‑2‑HS‑glycoprotein, prothrombin, serotransferrin, haemoglobin foetal subunit β, complement component C9, haemoglobin subunit α, complement C3 and antithrombin‑III; however, others, such as apolipoprotein D or vitamin D‑binding protein, were identified in one sample only. Amongst the proteins released during decalcification, certain proteins were absent in the former group, for example angiogenin or leukocyte cell‑derived chemotaxin‑2. Their presence is not surprising, since core areas in large pieces of permanent cartilage are supplied via vascular canals, to ensure an adequate metabolic supply for the regions deep inside; reviewed by Gabner et al (32). Such canals were also present in the rib cartilage (Fig. 2). Among these proteins, several growth factors were detected, three of which had a Mascot score between 966 and 246; these were MANF, CTGF and OSTF‑1. None however, were detected by ELISA (Table II).
The structures and cell types forming the costochondral junction are shown in the haematoxylin-eosin stained histological sections (Figs. 3 and 4), prepared as an illustration for the processes elaborated in the following discussion.

**Discussion**

Since the cartilage matrix within calcium deposits contains growth factors important for bone cell differentiation and activity, it is intriguing to see how they are made accessible to osteoprogenitor cells. Resorption of non-calcified and calcified cartilage occurs through two different mechanisms (5,33,34). Non-calcified septa are invaded by capillary sprouts proceeding from the metaphysis at the bottom of the growth plate. Endothelial cells are accompanied by perivascular cells and pericytes, and by mononuclear cells expressing cathepsin B, termed septoclasts (5,33,35,36). Calcified septa are resorbed by multinuclear cells called osteoclasts or chondroclasts (33,37,38). The osteoclasts extend their cell processes into the cartilage matrix and entrap the calcified cartilage. They are followed by tube-forming endothelial cells migrating from the capillary sprouts, giving rise to the new capillaries (33,37,39,40). Stromal cells follow the vascular elements, differentiate into osteoblasts and deposit osteoid on the calcified longitudinal septa (3,7,39). Resorbing osteoclasts have different functionally separated zones. Within the peripheral fusion zone, osteoclasts secrete protons and proteolytic enzymes, dissolving the calcified matrix (41). In the uptake zone, osteoclasts collect the degraded bone material (42,43). Subsequently, this is transcytosed and discharged through the functional secretory domain localised at the opposite pole of the cell (44-47).

According to previously published data (13-17) and the results of the present study, it is evident that chondrocytes forming the epiphyseal growth plate produce growth factors and deposit them within the matrix, which subsequently undergoes mineralisation. These growth factors are accessible only to osteoclasts, which release them presumably at a rate optimal for the stimulation of various osteoprogenitor cells.

Schenk et al (5) reported that ~two-thirds of the longitudinal septa in the epiphyseal growth plate is partially or completely calcified, whereas the remainder is essentially non-calcified. This close contact of calcified and non-calcified matrices within longitudinal septa may explain why it was not possible to eliminate non-calcified cartilage during pulverisation in liquid nitrogen and gradient centrifugation.

Table II. Growth factors found by sequence analysis of proteins released during decalcification in 0.6 N HCl, and in the decalcified material.

| Factors                                      | Mascot score (number of matching peptides) |
|----------------------------------------------|--------------------------------------------|
| Mesencephalic astrocyte-derived neurotropic   | 966 (7)                                    |
| Connective tissue growth factor              | 739 (8)                                    |
| Osteoclast stimulating factor                | 243 (4)                                    |
| Hepatoma-derived growth factor               | 122 (3)                                    |

Growth factors present in the non-calcified matrix were released by hyaluronidase digestion followed by extraction with GuHCl. In vivo, the factors were liberated by septoclasts accompanying the capillary sprouts, accompanied by blood vessels, resorb the non-calcified transverse septa, whereas the calcified longitudinal septa are resorbed by osteoclasts. Osteoblasts deposit bone on the remnants of longitudinal septa. A few osteocytes, embedded in the bone matrix, are visible. Magnification, x400.
The present study was limited to only two determinations of each factor; however, as can be seen in Table I, the results were consistent in all steps of the isolation procedure (hyaluronidase digestion and GuHCl extractions). The quantities of the particular growth factors and general information regarding their function is summarized in Table III. Factors affecting osteoprogenitor cells are also presented in Fig. 5.

When planning the experiments, it was expected that the protein sequence analysis would assist in identifying proper ELISA tests and limit the amount of material needed for growth factors analysis.

Only three growth factors influenced osteoprogenitor cells: OSTF-1, CTGF and MANF. Thus, for further analysis, growth factors, which on the basis of published data were involved in cartilage and bone formation were chosen. These were BMP-1-7 (14,19) GDF-5 (15,48), NELL-1 (16), TGF-β1 (24), bFGF (49), VEGF (13) and IGF-1 (49).

It seems reasonable to assume that growth factors found in the non-calcified and calcified cartilage matrices (BMP-2, BMP-3, BMP-4, BMP-7, GDF-5, NELL-1, TGF-β1, bFGF and VEGF) directed the development of long bones by stimulating differentiation of mesenchymal cells into osteoblasts,

### Table III. Growth factors that were present or absent, based on ELISA, in the rib costochondral junctions, and their role in endochondral ossification.

| Growth factors | Functions | Concentration in calf costochondral junctions, pg/300 mg dry cartilage | Refs. |
|----------------|-----------|-----------------------------|-------|
| BMP-1          | Metalloprotease, cleaves procollagen, participates in bone repair. | Not detected | (17) |
| BMP-2          | Induces endochondral bone formation | 115 | (47,19,48) |
| BMP-4          | following in vivo implantation; participates in bone mineralization. | 208 | |
| BMP-7          | Differences in dose and time needed for reactions. Facilitates both chondrogenic and osteogenic differentiation of mesenchymal stem cells. | 1,182.5 | |
| BMP-3          | Induces endochondral bone formation following in vivo implantation, a negative modulator of bone formation, and an antagonist of BMP-2 signaling. | 23.5 | (19,48,50) |
| BMP-5          | Induces endochondral bone formation following in vivo implantation, and participates in bone mineralization. | Not detected | (19,47) |
| BMP-6          | Induces endochondral bone formation following in vivo implantation, and participates in bone mineralization. | Not detected | (19,47) |
| TGF-β1         | Regulates replication and differentiation of cartilage and bone forming cells. | 27 | (60) |
| bFGF           | Stimulates bone formation. | 16.75 | (76,77) |
| IGF-1          | Functions in coupling bone resorption and formation. | Not detected | (75) |
| VEGF           | Stimulates angiogenesis and endochondral bone formation. | 255.5 | (22) |
| GDF-5          | Closely related to BMP-5,6,7. Involved in skeletal repair and regeneration, inhibits bone formation stimulated by BMP-2 and induces angiogenesis. | 945.5 | (17,53,54) |
| NELL-1         | Specific to the osteochondral lineage, promotes orthotopic bone regeneration. | 2,190.50 | (16) |
| CTGF           | Involved in skeletogenesis. | Not detected | (78) |
| OSTF-1         | Stimulates formation and activity of osteoclasts. | Not detected | (80) |
| MANF           | Trophic factor for dopamine neurons. Involved in chondrocyte endoplasmic reticulum homeostasis. | Not detected | (79) |

*aMean of two determinations.*
the formation of osteoclasts, the formation of blood vessels and the deposition of osteoid on calcified longitudinal septa. Several questions remain: What are the specific roles of the factors? How is their action coordinated? And are they released simultaneously or consecutively? Considerable information on the action of particular factors alone, in pairs or in triplets, has already been accumulated in previous studies; however, elucidation of the role of the 9 factors at play (or more, as some other factors not identified in the present study may be present) is challenging.

In extracts from non-calcified and calcified cartilage, the quantity of BMP-7 and GDF-5 (BMP-14) was ~10x higher than that of BMP-2, and 5x higher than that of BMP-4; thus, the two former factors may serve a dominant role in osteoprogenitor stimulation. NELL-1, the third factor present in high concentrations, is highly specific to the osteochondral lineage and can promote orthotopic bone regeneration (18,25,50). The small quantity of BMP-2 in relation to BMP-7 was unexpected, as in the bone matrix, they appear to be present in comparable amounts. It has been demonstrated that there is ~2 µg protein with bone inductive activity (presumably primarily BMP-2) in 1 kg bovine bone (22), and 1 µg BMP-7 in 1 kg bone (51). Thus, in the present study, the content of BMP-7 in non-calcified and calcified cartilage is comparable or even higher than in bovine bone, whereas that of BMP-2 is much lower. BMP-2 not only stimulates bone formation, but significantly enhances osteoclastogenesis (52), the effect of which would probably not be beneficial during the early stages of bone deposition. BMP-4, which is less efficient than BMP-2 in promoting bone formation (1,22,53), was detected in the material at slightly higher concentrations than BMP-2. It has previously been shown that the combined use of BMP-2, BMP-5 and BMP-6 had an additive effect on matrix mineralisation (52), but the latter two factors were not detected in our samples, possibly due to the insufficient sensitivity of the ELISA kits used. The quantity of BMP-3 detected was negligible. BMP-3 is an inhibitor of osteogenesis in vitro and of bone formation in vivo, and may antagonise BMP-2 signalling (54).

GDF-5 (also referred to as CDMP-1/BMP-14) is closely related to BMP-5, BMP-6 and BMP-7. It is present within the cartilaginous cores of the developing long bones (15,16) corresponding to calcified cartilage in the present study. The expression of GDF-5 is required for proper skeletal patterning and joint development in vertebrate limbs (55,56). Of interest, GDF-5 was found to inhibit the BMP-2-induced increase in alkaline phosphatase expression in the promyoblast C2C12 cell line, and to inhibit bone formation stimulated by BMP-2 in vivo after simultaneous implantation of both factors into rat muscle. Thus, GDF-5 can act as an antagonist of BMP-2, which may have important implications in processes where both factors act simultaneously (57). In addition, GDF-5 induced angiogenesis in both chick chorioallantoic membrane and rabbit cornea, whereas BMP-2 did not (58). The results of the present study suggest that the interference of BMP-2 with GDF-5 during endochondral ossification is prevented or limited by the low levels of the former. Nevertheless, whereas BMP-7, GDF-5 and NELL-1 accounted for the majority of the growth factors present, the contribution of BMP-2 and BMP-4 to the deposition of bone in endochondral ossification should not be neglected. BMP-5, BMP-6 and BMP-7 show extensive sequence similarity to BMP-2, and the additive or synergistic contribution of these molecules to osteogenic activity has already been suggested (59). The lack of a detectable amount of BMP-6 is surprising, since amongst BMP-2, BMP-4, BMP-6

Figure 5. Growth factors affecting osteoprogenitor cells.
and BMP-7, BMP-6 is the most consistent and potent regulator of osteoblast differentiation (60).

TGF-β1 regulates the replication and differentiation of mesenchymal precursor cells, chondrocytes, osteoblasts and osteoclasts, chondrocyte hypertrophy, growth plate maturation and mineralisation (24,61-65). Latent TGF-β1 stored in the bone matrix is released and activated by osteoclasts (66). In the present study, TGF-β1 was detected only in low quantities in GuHCl extracts of non-calcified and calcified cartilage. Thus, in view of the numerous TGF-β1 properties, it is difficult to ascribe to it a specific function at the earliest stages of bone development.

VEGF is expressed by hypertrophic chondrocytes in the epihyseal growth plate. Members of the VEGF family are essential coordinators of chondrocyte death, chondroclast function, extracellular matrix remodelling, angiogenesis and endochondral ossification (23,67-69). BMPs, bFGF, TGF-β1, IGF-1 and vitamin D3 induce the expression of VEGF released from osteoblasts (70). Osteoblast-derived VEGF is critical for maintaining bone homeostasis by stimulating the differentiation of mesenchymal stem cells to osteoblasts and repressing their differentiation to adipocytes. VEGF also stimulates the differentiation of monocytes to osteoclasts via a paracrine mechanism (71-73). VEGF may synergistically enhance BMP-induced bone formation (74). VEGF and BMP-2 applied together induced the differentiation of mesenchymal cells to chondrocytes and osteoblasts, and these differentiated cells produced VEGF, creating a favourable environment for vascularisation in bony tissues (75,76). Dilling et al (76) found the immediate expansion of blood vessels in response to BMP-2, identified brown fat cells as the source of VEGF, and related it to the role of BMPs in the vascularisation of early embryos (77). In the present study, VEGF was detected both in non-calcified and calcified cartilage, and could stimulate angiogenesis as well as co-operate with other growth factors in the differentiation of osteoblasts and osteoclasts.

bFGF inhibits the anabolic activity of IGF-1 and BMP-7 in adult human articular chondrocytes (49). A low dose (0.1 mg/kg per day) of bFGF stimulates endosteal and endochondral bone formation and reduced periosteal bone formation in growing rats (78). A bFGF-loaded acellular dermal matrix membrane resulted in similar bone regeneration as that observed with BMP-2 through more efficient recruitment of mesenchymal stem cells. Moreover, bone marrow mesenchymal stem cells pre-treated with bFGF exhibited increased proliferation and osteogenic differentiation potential compared with BMP-2 pre-treatment (79).

CTGF (80), MANF (81) and OSTF-1 (48), although found by sequential analysis, were not detected by ELISA. A short summary of their role in cartilage physiology is presented in Table III.

BMP-2 and BMP-7 were used in clinics for the repair of bone defects and fractures (82,83) and for spine fusion (84); however, adverse effects of BMP-2 were observed in procedures aimed at cervical spine fusions soon afterwards (84). Moreover, BMP-2 is generally upregulated in several types of tumors and is associated with tumor cell proliferation, invasion, and at times a poor clinical prognosis. The basic biological importance of BMPs in different types of cancer raises potential concerns regarding their clinical use (85).

In calcified cartilage, BMP-7, GDF-5 and NELL-1 were present in high concentrations and seem to be principal actors in initial bone formation. The remaining factors could be characters of the second plan. This poses the question of whether a similar profile of factors serve a similar role in humans. This is a challenging topic to study, due to the lack of suitable material for study; however, sufficient amounts of calcified cartilage could be obtained from other large animals for comparative evaluation. An improved understanding of the function of growth factors during the early stages of bone formation may possibly assist in identifying effective regimens for bone regeneration.

In summary, in endochondral ossification, the septoclasts invading non-calcified matrices release factors required for the formation of osteoclasts. It may be the case, for example, that BMP-2 not only stimulates bone formation, but significantly enhances osteoclastogenesis, and that VEGF stimulates differentiation of monocytes to osteoclasts (71-73). Osteoclasts, once present, release growth factors stored in calcified matrix, which are required for initial bone deposition.

Acknowledgements

Not applicable.

Funding

This research was supported by the National Science Centre, Poland (grant no. 2016/21/B/NZ1/00289).

Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

SM designed the study. AI and AH performed the experiments. AI, SM and AH analysed the data. AI, SM and AH wrote the manuscript. All authors have read and approved the final manuscript. AI, SM and AH confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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