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RESEARCH

Gene expression profiles induced by growth factors in *in vitro* cultured osteoblasts

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Objectives

Effects of insulin-like growth factor 1 (IGF1), fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 2 (BMP2) on the expression of genes involved in the proliferation and differentiation of osteoblasts in culture were analysed. The best sequence of growth factor addition that induces expansion of cells before their differentiation was sought.

Methods

Primary human osteoblasts in *in vitro* culture were treated with IGF1, BMP2 or FGF2 (10 ng/ml) for 24 hours (IGF1) or 48 hours (BMP2 and FGF2). Experiments were performed during the exponential growth phase with approximately 1e7 cells per 75 cm^2 flask. mRNA was reverse transcribed directly and analysed using RT-PCR Taqman assays. Expression levels of key genes involved in cell growth and differentiation (CDH11, TNFRSF11B, RUNX2, POSTN, ALP, WNT5A, LEF1, HSPA5, FOS, p21) were monitored using RT-PCR with gene-specific Taqman probes.

Results

Autocrine expression of BMP2 is stimulated by FGF2 and BMP2 itself. BMP2 and FGF2 act as proliferative factors as indicated by reduced expression of ALP and POSTN, whereas IGF1 exhibits a more subtle picture: the Wingless und Int-1 (Wnt) signalling pathway and the Smad pathway, but not p38 mitogen-activated protein (MAP) kinase signalling, were shown to be activated by IGF1, leading to proliferation and differentiation of the cells.

Conclusions

For future use of autologous bone cells in the management of bony defects, new treatment options take advantage of growth factors and differentiation factors. Thus, our results might help to guide the timely application of these factors for the expansion and subsequent differentiation of osteoblastic cells in culture.

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Keywords: Osteoblasts, Tissue engineering, SMAD, Wnt, Alkaline phosphatase

Article focus

- The focus was on three major growth factors involved in bone development. The best sequence of growth factor addition that induces expansion of cells before their differentiation was sought.

Key messages

- Fibroblast growth factor 2 (FGF2) showed mainly stimulation of proliferation, bone morphogenetic protein 2 (BMP2) showed proliferation and some differentiation, and insulin-like growth factor 1 (IGF1) showed strong stimulation of both proliferation and differentiation of different fractions of the cells.

Strengths and limitations

- Strength: Tests were performed on primary human cells without transfection and artificial over-expression of any proteins.
- Limitation: No studies at the protein level were performed.

Introduction

Tissue engineering for the treatment of a large fracture gap using *in vitro* raised cells is a promising concept.[1] A coordinated action of various cell types is required for bone remodelling.[2] Various growth factors are involved in the proliferation and differentiation of these cells, of which BMPs or integrins play a vital role. Growth factors bind to

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specific cell surface receptors, which trigger cellular signalling pathways. The signals are transmitted to the nucleus and thereby regulate gene expression, many of which are involved in cartilage and bone formation. Through the use of *in vitro* models, the biology of the cells involved in bone remodelling has made important advances. Also, the favourable effect of culture expanded cells on healing fractures has been shown in animal models:[3] in particular, sufficient numbers of cells for transplantation can be obtained by culturing precursor cells that might find applications in wound healing after appropriate expansion and differentiation. Many different culture systems have been used[4] and a lot of biological processes need further clarification before cells from *in vitro* culture can be used routinely for transplantation.

Osteoblast differentiation is regulated by a number of hormones and factors that induce different signalling pathways in the cells. Major pathways that are known to modulate the number or activity of osteoblasts include the insulin-like growth factor (IGF) pathway, BMP pathway, and the Wingless und Int-1 (Wnt) pathway.[5] The main objective of this study was to analyse the effects of BMP2, IGF1 and FGF2 (also known as bFGF) on the expression of genes involved in the proliferation (increases in the expression levels of WNT, LEF1; Table I)[6-15] and differentiation (as measured by altered expression levels of CHD11, TNFRSF11B, ALP, RUNX2, POSTN; Table I) of osteoblasts in culture. Thus, cellular processes involved in chromatin organisation, transcription factors, adhesion molecules and differentiation were studied by analysing expression levels using quantitative real-time PCR of appropriate genes. Analysis of these genes allowed us to also differentiate between the Smad and the p38 mitogen-activated protein (MAP) kinase pathway. In this way, we aim to elucidate the addition of growth factors that would induce expansion of cells before their differentiation.

Table I. Genes in the current study

Gene	Comment
CDH11	Encodes a type II classical cadherin from the cadherin superfamily. Proteins from this family mediate calcium-dependent cell–cell adhesion[6]
TNFRSF11B	Osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF), or tumour necrosis factor receptor superfamily member 11B decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL). OPG can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors[7]
RUNX2	Runt-related transcription factor 2 (RUNX2), also known as core-binding factor subunit alpha-1 (CBF-alpha-1). Key transcription factor associated with osteoblast differentiation[8]
POSTN	Matricellular protein involved in adhesion, proliferation and differentiation of osteoblasts[9]
ALP	Alkaline phosphatase tissue-nonspecific, important for mineralisation of bone matrix and differentiation of osteoblasts[10]
WNT5A	Signal protein stimulating proliferation and differentiation of osteoblasts. Blocks apoptosis in osteoblasts[11]
LEF1	Transcription factor signalling via the canonical WNT/ß-catenin pathway. Stimulates proliferation, development, and regeneration of osteoblasts[12]
HSPA5	Heat shock protein induced by p38 mitogen-activated protein (MAP) kinase signalling[13]
FOS	Transcription factor induced by SMAD signalling[14]
p21	Also known as CDKN1a, is induced by SMAD signalling[15]

Materials and Methods

Primary human osteoblasts were isolated from femoral trabecular bone tissue from the knee or hip joint (obtained from PromoCell, Heidelberg, Germany; C-12760, no specific source information is available) and suspended in an osteoblast growth medium from PromoCell (C-27001; 50 000 cells per 25 cm^2 flask). Media were supplemented with Supplement Mix (PromoCell, C-39615), containing all supplements necessary for the optimal growth of human osteoblasts according to the manufacturer's protocol, Pen/Strep/Fungizone (PromoCell, C-42020), and 10% foetal bovine serum (PAA, A15-101). Cells were cultured at 37°C and 5% CO_2. Daily visual inspection of the cells during expansion indicated mostly undifferentiated cells. Cells were cryopreserved at passage 2 and passaged another time prior to the experiments. Growth factors were obtained from Sigma-Aldrich (St. Louis, Missouri). Experiments were performed during the exponential growth phase, with approximately 1e7 cells per 75 cm^2 flask. Osteoblasts were treated with IGF1, BMP2 or FGF2 (10 ng/ml) for 24 hours (IGF1),[16] or 48 hours (BMP2 and FGF2),[17] based on previous studies in the literature. Control cells were kept in a medium without added growth factors. After incubation, cell plates (two plates for all experiments) were rinsed with Trizol™ (Life Technologies, Darmstadt, Germany) for immediate mRNA extraction after the culture medium was removed and mRNA was purified according to the manufacturer's instructions. mRNA was reverse transcribed directly and the cDNA was stored at -70°C until the point of analysis. This protocol ensured the highest possible quality of mRNA, preserving the mRNA within seconds from nuclease digest. Random primers, desoxynucleotide triphosphates, protector RNase inhibitor and reverse transcriptase were obtained from Roche (Basel, Switzerland). RT-PCR was performed using Roche FastStart DNA Master HybProbe (Roche) and Taqman primers obtained from Life Technologies (CDH11: Hs00901475_m1; TNFRSF11B: Hs00900358_m1; RUNX2: Hs00231692_m1; POSTN: Hs01566734_m1; ALP: Hs01062534_m1; WNT5A: Hs00998537_m1; LEF1: Hs01547250_m1; HSPA5: Hs00607129_gH; FOS: Hs04194186_s1; p21: Hs00355782_m1; IGF1: Hs01547656_m1; FGF2: Hs00266645_m1; BMP2: Hs00154192_m1). RT-PCR was performed on an ABI Prims 7000 detection system (Life Technologies).

6-Carboxyfluorescein (FAM) fluorescence was used as a readout. The amplification blots were checked visually and the baseline was set manually. Every RT-PCR reaction was run in triplicate for every cDNA (Table I). Expression levels of the various genes are

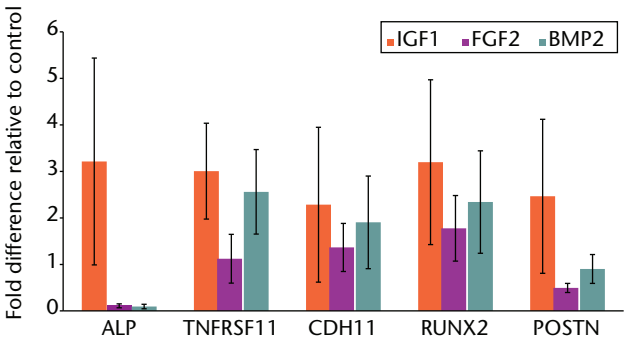

Fig. 1

Graph showing the regulation of differentiation and cell fate by growth factors osteoblasts in *in vitro* culture were treated with insulin-like growth factor 1 (IGF1), bone morphogenetic protein 2 (BMP2) or fibroblast growth factor (FGF). For the quantitative analysis, we compared expression levels of the genes shown with the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (comparative CT method - ΔCT). Error bars represent standard deviation of triplicate qPCR measurements. Expression levels in untreated control cells are normalised to 1 and are thus not shown.

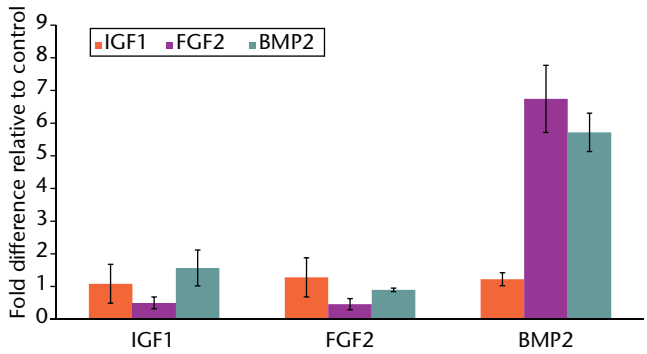

Fig. 3

Graph showing the influence of growth factors on the expression of the growth factors. Bone morphogenetic protein 2 (BMP2) is a strong inducer of its own gene (BMP2). Interestingly, BMP2 expression is also upregulated by fibroblast growth factor 2 (FGF2). This may explain the strong resemblance between our FGF2 and BMP2 results as insulin-like growth factor 1 (IGF1) does not show any influence on BMP2 expression. Expression levels in untreated control cells are normalised to 1 and are thus not shown.

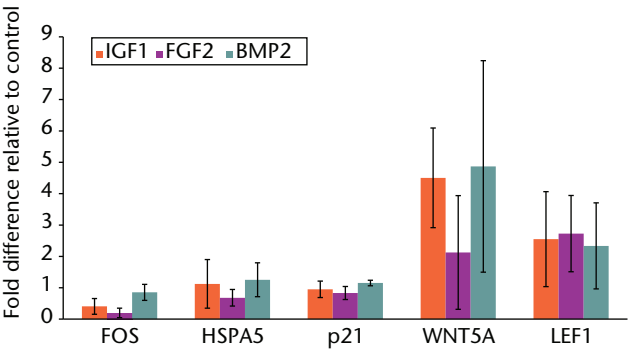

Fig. 2

Graph showing the expression of genes involved in cell signalling by growth factors. All growth factors tested increase expression of WNT5A and LEF1 which are involved in Wnt signalling. FOS, which is involved in Smad signalling, is attenuated by IGF1 and FGF2. No effect is seen in HSPA5 which is regulated by p38 MAP kinase signalling. Also, p21 levels remain constant under all conditions. Expression levels in untreated control cells are normalised to 1 and are thus not shown.

shown as means plus standard deviation of the triplicate qPCR measurements.

Results

For the quantitative analysis of mRNA expression levels, the amount of expression of target genes was compared with the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (in triplicates; comparative CT method - ΔCT). Different effects of the various growth factors on the expression of target genes were found:

IGF1 leads to increased expression of all genes analysed and, most especially, it shows a strong induction of ALP, whereas the other growth factors act to repress ALP.

Both BMP2 and FGF2 increase the expression levels of WNT5A and lymphoid enhancer-binding factor 1 (LEF1) as IGF1 does, and this upregulation is accompanied by a higher expression of CDH11, and RUNX2. TNFRSF11B expression is not increased markedly by FGF2, but is upregulated highly in cells treated with IGF1 and BMP2 (Fig. 1). FGF2 reduces the expression of POSTN, a regulator of osteoblast differentiation. For BMP2 this reduction is not significant (Fig. 1). Both growth factors decrease the expression of ALP, indicating that differentiation (as measured by expression of ALP) is suppressed by FGF2 and BMP2, but not by IGF1.

None of the growth factors seem to signal via the p38 MAP kinase pathway as HSPA5 expression is not markedly altered. However, genes involved in Wnt signalling (WNT5A, LEF1) are changed markedly by all three growth factors. The FOS gene, which is regulated by the Smad pathway, is repressed by IGF1 and FGF2. Surprisingly, expression of p21 remains totally constant under all the conditions that we applied, even though this gene has also been described as being regulated via the Smad pathway (Fig. 2).

Interestingly, the BMP2 gene is expressed strongly in the osteoblasts. This expression is further induced by FGF2 or BMP2 (Fig. 3).

Discussion

Since the first attempts of *in vitro* cultures of osteoblast cells from human bone,[18] a great improvement in the knowledge of osteoblastic cells has been achieved. Cell differentiation, cytokine and hormonal regulation and synthesis and excretion of matrix proteins among others have been deciphered.[4] Osteoblasts arise from osteoprogenitor cells located in the deeper layer of the periosteum and the bone marrow. These immature progenitor

cells express the master regulatory transcription factor RUNX2. Major pathways that are known to modulate the number or activity of osteoblasts include the IGF, BMP, and Wnt pathways. BMPs are members of the transforming growth factor-ß (TGF-ß) superfamily that play important roles in morphogenetic development.[19] IGF1 signalling is known to increase osteogenesis significantly and may be used for tissue engineering purposes. The combination of PDGF and IGF1 may be more beneficial than either alone.[20]

Thus, it was of interest to stimulate osteoblasts in *in vitro* cultures by various growth factors and highlight their effects on the cells by assessing the expression of key genes. Genes involved in proliferation, maintenance and differentiation of the cells were monitored before and after addition of three growth factors (Table I).

Clearly, IGF1, FGF2, and BMP2 trigger separate responses in the cells; IGF1 leads to increased expression of all genes analysed, however, the other two growth factors showed a much subtler and more differentiated response. Both BMP2 and FGF2 increase the expression levels of WNT5A and LEF1, developmental genes that regulate cell fate and patterning during embryogenesis.[21] This upregulation is accompanied by higher expression of CDH11 and RUNX2. RUNX2 and CDH11 are associated with osteoblast differentiation and both genes have specific functions in bone development and maintenance. RUNX2, especially, is a major transcription factor of osteoprogenitor cells. Consequently, its increased expression substantiates the proliferative response of the cells, and it can be assumed that the cell culture harbours both osteoblasts and osteoprogenitor cells. TNFRSF11B – which is critical for adequate bone metabolism via its surface-bound protein product that activates osteoclasts[22,23] – is not changed by FGF2 but is higher with BMP2. Both growth factors decrease the expression of POSTN – which codes for Periostin, a regulator of osteoblast differentiation and bone formation[24] – and ALP,[25] indicating that differentiation is suppressed by FGF2 and BMP2 but not by IGF1. This may seem paradoxical because it would imply that IGF1 induces proliferation and differentiation simultaneously, but it again indicates that proliferation or differentiation occur in different cell fractions.

In previous work, the inherent heterogeneity of bone cell cultures has been addressed in detail.[26] Interestingly, distinct patterns of gene expression were associated with the major signalling pathways (Wnt and p38 MAP kinase). Our results imply that FGF2 is best suited to expanding osteoblastic cells in culture, because this growth factor only stimulates the expression of WNT5 and LEF, whereas markers of differentiation (ALP and POSTN) are strongly down-regulated. IGF1 on the other side does stimulate proliferation, but most probably certain fractions of the cells also respond with heightened expressions of ALP and POSTN.

The mode of action of BMP2 stimulation on osteoblasts has been a matter of debate. BMP2 signals via two types of receptors, namely BMP receptor type I and type II (BR-I and BR-II). These receptors are expressed on the surface of the cells as homomeric as well as heteromeric complexes.[27] A total of two possible responses of the cells to BMP2 are under discussion: 1) binding of BMP2 to preformed receptor complexes activates the Smad pathway, whereas 2) BMP2-induced recruitment of receptors activates a different pathway, independent of Smad, resulting in the induction of alkaline phosphatase activity and possibly apoptosis[28] via p38 MAP kinase. We have investigated a cell's two possible responses to BMP2 stimulation by measuring the expression levels of genes implicated in the Smad pathway. Our results show that the strong downregulation of ALP by BMP2 is accompanied by unaltered expression of genes regulated by p38 MAP kinase. This suggests that the cells show preformed receptor complexities on their surface for the binding of BMP2 (= response 1). The ensuing signalling via the Smad pathway is thus accompanied by the downregulation of alkaline phosphatase.

Generally, the addition of BMP2 to osteoblastic cells is reported to stimulate differentiation of the cells by increased levels of ALP and COL I.[27] Interestingly, our study shows a strong downregulation of ALP by BMP2, whereas expression of RUNX2 and CDH11 is markedly increased. It can be speculated that this specific growth factor response is due to our cell system, because most cells used in previous studies are mouse osteoblasts from calvaria.[29-31] As mentioned above, BMP2-induced downregulation of ALP is mediated by the Smad pathway.

Clearly, there was mainly stimulation of proliferation of the cells in FGF2, proliferation and some differentiation in BMP2 and strong stimulation of both proliferation and differentiation in IGF1. Thus, our results might help to guide the timely application of these factors for the expansion and subsequent differentiation of osteoblastic cells in culture.

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Author contributions:
- A. Robubi: Cell culture, Molecular biology, Manuscript preparation
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- M. Schmid: Cell culture, Molecular biology
- K. R. Huber: Data collection, Data analysis, Manuscript preparation
- A. Engel: Data analysis, Manuscript preparation
- W. Krugluger: Study design, Data analysis, Manuscript preparation

ICMJE Conflict of Interest:
- None declared

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