Involvement of BMP-2, TGF-ß2 and TGF-ß3 Signaling in Initial and Early Stages of Heterotopic Ossification in a rat Experimental Model

by ***S Suutre[1,*], A Toom[2], A Arend[1], G Selstam[3]***

[1]University of Tartu, Department of Anatomy, Tartu, Estonia

[2]University of Tartu, Clinic of Traumatology and Orthopaedics, Tartu, Estonia

[3]University of Umeå , Department of Molecular Biology, Umeå, Sweden

Summary

This study focused on the localization and expression of bone morphogenetic protein 2 (BMP-2) and different isoforms of transforming growth factor ß (TGF-$ß_1$, TGF-$ß_2$ and TGF-$ß_3$) in the initial and early stages of heterotopic ossification (HO) employing an animal model mimicking the situation after total hip arthroplasty (THA). Bone growth was induced in rats using ß-tricalcium phosphate implants immersed either in osteoinductive rhBMP-2 solution or in saline and implanted at the site where the HO is usually expected to develop after THA. Implants were removed at 3 or 21 days after the operation and handled according to stereology principles. mRNA expression and protein staining of growth factors in different types of tissues was determined by *in situ* hybridization and immunohistochemistry, respectively.

After three days, TGF-$ß_3$ content in the undifferentiated mesenchymal-like cells in the rhBMP-2 treated implants was, as assessed by immunohistochemistry, 49.6% higher compared to the saline treated group (p=0.024). This was also supported by *in situ* hybridization of mRNA of TGF-$ß_3$, which showed stronger expression in rhBMP-2 treated group. Immunohistochemical investigation showed that after 21 days the connective tissue in the rhBMP-2 treated implants contained more TGF-$ß_1$, TGF-$ß_2$ and TGF-$ß_3$, compared to BMP-2 and osteoblasts contained significantly (27.2%) more TGF-$ß_3$ compared to TGF-$ß_1$ (p=0.045). In the formed HO the proportion of the TGF-$ß_2$ and TGF-$ß_3$ producing bone tissue was increased by 32.1% and 47.8% respectively, compared to the TGF-$ß_1$ producing bone tissue (p=0.007 and p=0.006) and although this difference was not so clear at mRNA level, this suggests that TGF-$ß_2$ and TGF-$ß_3$ signaling seem to play an important role during initial and early stages of HO formation.

Introduction

Heterotopic ossification (HO) is the formation of bone in tissues that are not normally ossified. HO may appear after trauma and after neurological injury. It is also a common complication after total hip arthroplasty (THA) occurring in a varying degree but typically affecting approximately a third of THA patients if no prophylactic treatment is applied *(Toom et al., 2001)*. In some patients it can cause pain and restricted movements of the joints depending on the location. The characteristics of HO are the presence of bone cells, hydroxyapatite crystals *(Nilsen, 1980)*, collagenous matrix and the absence of cellular atypism *(Nilsson and Persson, 1999)*. HO tissue is normally described as highly active , with high bone turnover and rapid bone formation *(Puzas et al., 1989)*.

The formation of HO after total hip arthroplasty (THA) is considered to be caused by surgical tissue damage at the operation site *(Hierton et al., 1983)*,

**Correspondence:* Siim Suutre, PhD
1University of Tartu, Department of Anatomy, University of Tartu, Ravila 19, Tartu 50411, Estonia
Tel + 372 7374255
Fax +372 737 4252
E-mail siim.suutre@ut.ee

which, in turn, can cause inflammation that apparently plays an important role in the pathogenesis of HO *(Thomas, 1992)*. Transforming growth factor ß (TGF-ß) isoforms are also involved in the development of the early inflammatory reaction by recruiting inflammatory cells, primarily neutrophils and macrophages to the site of inflammation, as they have been reported to be powerful chemoattractants *(Faler et al., 2006)*.

In the normal bone TGF-ß isoforms, as well as some bone morphogenetic proteins (BMPs), are important growth factors that affect bone growth, repair and regeneration. TGF-ß isoforms have been found in proliferating mesenchymal cells, in osteoblasts lining the forming bone, in young and mature chondrocytes and in the bone matrix *(Joyce et al., 1990)*. TGF-ß isoforms have been suggested to have bone stimulatory effects in early phases of osteoblast differentiation *(Bonewald, 2002)*. Five subtypes of TGF-ß isoforms have been discovered, three of them in mammals – TGF-$ß_1$, TGF-$ß_2$ and TGF-$ß_3$. Five isoforms share approximately 75% sequence identity, but exert different biological activities in normal bone formation although some overlapping occurs *(Mittl et al., 1996)*.

TGF-$ß_1$ is produced by bone cells and is the most abundant in bone. TGF-$ß_1$ is stored in complexes partially linked to the extracellular matrix. This isoform can stimulate bone growth and is believed to do so when osteoclasts are released after bone resorption. The release of TGF-$ß_1$ together with other growth factors such as insulin-like growth factor (IGF) stimulates bone formation and causes osteblasts to invade the lacuna *(Rodan, 1991)*. Knockout of TGF-$ß_1$ gene causes remarkable suppression of bone formation in mice: decrease in osteoblast proliferation, bone growth rate and mineralization *(Geiser et al., 1998)*. TGF-$ß_1$ is also present in plasma in physiologically significant levels but whether this reflects a high production, or a possible release from bone storage, is at present unclear.

Much less is known about TGF-$ß_2$ and TGF-$ß_3$ and results stem mainly from embryonic bone studies. These growth factors regulate osteogenesis in the postnatal period, and in the adult too, and together with TGF-$ß_1$ are upregulated during fracture healing *(Cho et al., 2002)*. TGF-$ß_2$ is mainly located in chondrocytes and in the cartilage *(Joyce et al., 1990)*, but is also present with TGF-$ß_1$ at sites of mineralization. TGF-$ß_2$ is highly expressed in preosteoblasts and osteoblasts in cambial layers indicating a role in bone formation *(Zhang et al., 1999)*. In TGF-$ß_2$ gene knockout mice a wide range of bone defects and defects in ossification have been recorded *(Erlebacher and Derynck, 1996)*.

TGF-$ß_3$ is more widely distributed in bone *(Horner et al., 1998)*, but is also present with TGF-$ß_1$ at sites of endochondral bone formation where TGF-$ß_3$ may have a direct influence on chondrogenesis *(Fan et al., 2008)*. The action of TGF-$ß^3$ is also in part related to changes in receptor availability for TGF-$ß^1$ and TGF-$ß^2$ (*Risbud et al., 2006*).

While TGF-ß isoforms stimulate bone formation in a very intricate way, they do not possess any osteoinductive properties in human or rodent cells (*Joyce et al., 1990; Matsaba et al., 2001*) in normal bone. The roles of TGF-ß isoforms in heterotopic ossification are far less known.

BMP-2 is a potent inducer of bone formation in ectopic sites and has been shown to play an important role in inducing osteoblastic differentiation of mesenchymal stem cells *(Cheng et al., 2003)*. BMP-2 has been evaluated in animal models and in human clinical trials and is available for clinical use – it has been used to enhance osteointegration of orthopedic implants and to help the incorporation of bone grafts *(Matthews, 2005)*. In the present study BMP-2 was used in half of the implants to ensure ossification.

In a previous study we reported that in human patients, immature HOs (younger than 17 months) express TGF-$ß_2$ and TGF-$ß_3$ more than mature ossificates (older than 17 months) after THA *(Toom et al., 2007)*. Therefore, we concluded that these two growth factors may have an effect on the early stages of heterotopic bone formation.

To study the very early stages of HO formation, which is not possible in human patients, a rat model

was introduced. TGF-ß isoforms were detected at the initial period, i.e. 3 days after the operation and also during a period of remodelling of the newly formed HO, i.e. 21 days after the operation.

The aim of this study was to determine if there are differences in the expression (detected by *in situ* hybridization) and localization (detected by immunohistochemistry) of different TGF-ß isoforms at the onset of HO in order to elucidate their different roles in the initial and early ossification processes.

Materials and Methods

Animals

Twenty 9-month old Bkl Wistar strain adult male rats weighing between 500–600 g were used in this study (purchased from Scanbur BK AB, Sweden). The rats were housed in polycarbonate cages (Tecniplast, Italy) (Eurostandard type III) and were maintained under SPF conditions – water, cages, lids and bedding were autoclaved. The room temperature was 20±2 ºC and relative humidity was 50±5 %. Food (Labfor R70, Lactamin, Sweden) and autoclaved water were available *ad libitum*. The rats were exposed to a 12 h: 12 h light/dark cycle. Lights were switched on from 08:00 to 20:00. Autoclaved aspen chips (chip size 4 × 4 × 1 mm, Estap, Estonia) were used as bedding. The cages were changed on each Monday.

This project received the approval of the Animal Ethics Committee at the University of Tartu (no of the approval – 2002/10; issued August 14, 2002).

Operative procedure and implantation technique

The animals were anaesthetized with isoflurane (Isoflurane Baxter®, Baxter Medical AB, Sweden) by inhalation. Analgesia was provided before the operation and for 72 hours post-operatively using morphine sulphate injections. No anti-inflammatory drugs were applied. The surgical procedure was as follows: A 12-14 mm incision was made dorsally over the greater trochanter. The transgluteal approach was used to reach the posterior part of the hip joint capsule. Gluteus maximus was retracted; the gluteus medius was pinched for 2 minutes with a standard vascular clamp with a width of 3 mm to produce muscular damage of approximately 9 mm^2. Bilateral femoral capsulotomy was performed. A cube-shaped implant of ß-tricalcium phosphate (ChronOS™ Block, Mathys Medical Ltd, Bettlach, Switzerland) with the size 3.3 x 3.3 x 3.3 mm and with the volume of 36 mm^3 and interconnected porosity of 70% was implanted in the capsulotomy wound. In half of the animals, the implant was immersed in osteoinductive recombinant human BMP-2 (rhBMP-2) solution, (Prof. Walter Sebald, Biozentrum der Universität Würzburg am Hubland, Germany). The estimated amount was 12.5 µg of BMP-2 in each implant. Control implants were immersed in vehicle (sterile phosphate-buffered isotonic saline). The surgical procedure has been described earlier *(Toom et al., 2006)*.

The differences of expression and localization of BMP-2 and three TGF-ß isoforms were studied in 4 groups of rats with 5 rats in each group. 2 implants were inserted into each rat, so each group consisted of 10 implants.

- Group I – implants were immersed in saline and the rats were killed 3 days after the operation and the implants removed.
- Group II – as in group I but implants were immersed in rhBMP-2 solution.
- Group III – as in group I, but rats were killed after 21 days.
- Group IV – as in group III but implants were immersed in rhBMP-2 solution.

In situ hybridization

The beta-tricalcium phosphate implants were removed after 3 or 21 days and systematic cryosections were made. The sections were bleached with 0.6% H_2O_2, then washed in PBT (phosphate buffered saline containing 0.1% Tween 20) and treated with proteinase K solution for 5 minutes. Proteinase K reaction was stopped by immersion in a glycine solution. Slides were refixed in 4% paraformaldehyde (PFA) containing 0.2% of glutaraldehyde. Commercial Digoxigenin (DIG)-labelled probes

(from GeneDetect, Auckland, New Zeland) were used to hybridize mRNAs of BMP-2; TGF-$ß_1$; TGF-$ß_2$ and TGF-$ß_3$. Hybridisation was carried out in RNA hybridisation buffer (50% formamide, 5x saline sodium citrate (SSC), 1% sodium dodecyl sulphate (SDS), at 70°C overnight, and post-hybridisation washes were performed at a final concentration of 50% formamide/2xSSC at 70°C for 2 x 30 min. Anti-DIG antibody (Roche 11 093 274 910) was used at a dilution of 1:5000, in blocking solution (1x 10x Tris-Buffered Saline Tween-20 (TBST) (0.25 M Tris-HCl pH 7.5, 1.4 M NaCl, 27 mM KCl, 1% Tween 20) + 9 X MQ filtered water) at 4°C overnight. Detection of antibody was carried out using NBT/BCIP stock solution (Roche (1 681 451)), which was diluted in a Tris-solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 2 mM levamisole). The washing steps were performed in PBT.

Immunohistochemical analyses

For immunohistochemistry, frozen sections were made according to the principle of systematic uniform and random selection. Sections with a thickness of 10 µm for surface analysis and cell counting according to Cavalieri's principle *(Gundersen et al., 1988)* were collected systematically after every 80 µm. The frozen sections were treated with 0.6% H_2O_2 and then with 1% bovine serum albumin (BSA) to block non-specific binding. After blocking, sections were incubated with mouse monoclonal antibody to TGF-$ß_1$ (ab27969), rabbit polyclonal antibodies to TGF-$ß_2$ (ab15539), TGF-$ß_3$ (ab15537) or BMP-2 (ab14933) overnight at 4ºC (all antibodies produced by Abcam Ltd., United Kingdom). Visualization of the primary antibodies was performed using the commercial kit "Strept ABComplex/HRP Duet Mouse/Rabbit system" (Dako Cytomation Denmark A/S, Denmark) and DAB+ Chromogen (Dako Cytomation, USA) for substrate. The washing steps in-between were done in phosphate buffered saline (PBS) which contained 0.07% of Tween 20 as a detergent. Thionine was used for background staining.

Stereological analysis of immunohistochemistry

Cell counting and evaluation of the growth factor distribution in different tissues were performed with the light microscope "Olympus BX51" (Olympus, Olympus Company Ltd., Japan) and analysis software "Cast 2" (Olympus, Olympus Company Ltd., Japan). At least 10 sections from each implant were assessed. The counting was done under a specific computer generated grid and the number of dots with positive staining per square area was counted. This counting method allowed us to distinguish between different tissue types during quantification. Osteoblasts were also identified on a morphological basis – the small round cells lining the newly formed bone were considered to be osteoblasts. Cavalieri's principle (which states that the volumes of two objects are equal if the areas of their corresponding cross-sections are in all cases equal) *(Gundersen et al., 1988)* was applied and relative as well as absolute volumes of different types of tissues in the implants were calculated. All the results are expressed as a percentage of growth factor producing tissue/whole volume of the specific tissue and the differences were compared using the paired t-test.

Semiquantitative evaluation of in situ hybridization

A subjective score system was adopted for visual interpretation of the *in situ* hybridization slides to allow semiquantitative analysis of the data. The scale ranged from 0 to +++ (0 - absence/faint staining; + - weak staining; ++ - moderate staining; +++ - strong staining) as described by Matsuzaki and co-authors for the evaluation of relative strengths of mRNA hybridization signals *(Matsuzaki et al., 1999)*. Differences between groups were tested by the Mann-Whitney U test.

Results

In rats of Group I and II the initial changes were studied at the site of expected HO formation on the third day after the inserting of beta-tricalcium phosphate implants into the capsulotomy wounds of the hip joint. In both groups inner pores of the implants were mostly empty and outward pores were filled

with connective tissue together with inflammatory and undifferentiated mesenchymal-like cells. Higher expression of TGF-ß$_2$ and TGF-ß$_3$ in group II, where rhBMP-2 was applied, was found by semi-quantitative evaluation of the *in situ* hybridization (see Table 1 and Figure 1). Immunohistochemical staining of proteins of all TGF-ß isoforms was observed in the connective tissue and mesenchymal-like cells. Since the amount of the connective tissue in the implants was limited, only the quantification of stained mesenchymal-like cells was possible. In group II the undifferentiated mesenchymal-like cells stained 49.6% more for TGF-ß$_3$, compared to the group I (p=0.024; see Table 1 and Figure 1). No other significant differences in the expression or production of growth factors at day three were found. Although immunohistochemical BMP-2 staining was increased in group II, it probably was due to remaining exogenous protein.

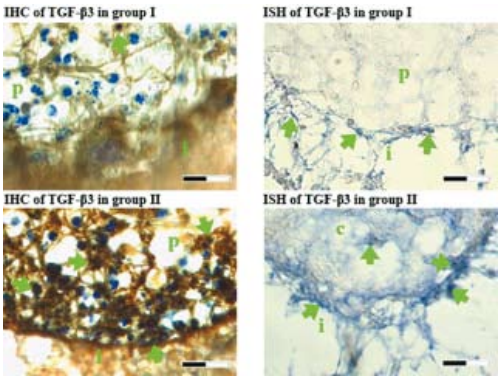

Figure 1. In group II (where the rhBMP-2 was applied) the undifferentiated mesenchymal-like cells produced more TGF-ß$_3$ compared to group I (brown staining marks the protein). Arrows show examples of the positive staining. Some differences could also be seen in the mRNA expression (the blue staining on the right). Legend: IHC – immunohistochemistry; ISH – *in situ* hybridization; i – remnants of the implant; p – pore of the implant; c – connective tissue. The scale bar represents 20 µm.

In the implants removed from the rats 21 days after the operation (Groups III and IV), early events in HO formation were studied. In group III, the surface of outer pores contained a limited amount of osteoid and cartilage. In group IV, where rhBMP-2 was applied, significant bone formation was found and cells penetrated the implants from all sides and widespread osteoid formation was observed. All studied growth factors were found both by *in situ* hybridization and immunohistochemical staining. Evaluation of *in situ* hybridization suggested that mRNA expression of TGF-ß$_3$ was stronger in group IV compared to group III, but this difference was not obvious in immunohistochemical staining (see Table 2). Immunohistochemistry showed, that in groups III and IV the growth factors were present in all types of the tissues, but in the connective tissue surrounding the heterotopic bone, all TGF-ß isoforms were generally more prevalent than BMP-2 (see Table 2). In group IV osteoblasts stained 27.2% more for TGF-ß$_3$ compared to TGF-ß$_1$ (p=0.045) and seemed to stain more for TGF-ß$_2$ compared to

Table 1. Percentage of immunohistochemically (IHC) stained mesenchymal-like cells (± standard deviation;n=10 in each group). [a] – p=0.024 in Group II vs Group I for TGF-ß$_3$. Total mRNA staining (*in situ* hybridization, ISH) in the implant graded as: 0 - absence/faint staining; + - weak staining; ++ - moderate staining; +++ - strong staining. [b] – p=0.0005 TGF-ß$_2$ in Group II vs TGF-ß$_2$ in Group I; [c] – p=0.001 TGF-ß$_3$ in Group II vs TGF-ß$_3$ in Group I.

	TGF-ß$_1$		TGF-ß$_2$		TGF-ß$_3$	
	Group I	Group II	Group I	Group II	Group I	Group II
IHC	55.67±16.96	63.81±22.28	41.61±28.12	52.31±19.84	47.11±21.68[a]	70.46±22.55[a]
ISH	+	+	0[b]	+[b]	0[c]	+[c]

Table 2. Percentage of immunohistochemically (IHC) stained cells/tissue (±standard deviation; n=10 in each group). NP – not present

[a] – p=0.045: TGF-ß$_3$ vs TGF-ß$_1$ in Group IV; [b] – p=0.016: TGF-ß$_1$ vs BMP-2 in Group III; [c] – p=0.012: TGF-ß$_2$ vs BMP-2 in Group III; [d] – p=0.031: TGF-ß$_3$ vs BMP-2 in Group III; [e] – p=0.007: TGF-ß$_2$ vs TGF-ß$_1$ in Group IV; [f] – p=0.006: TGF-ß$_3$ vs TGF-ß$_1$ in Group IV.

Total mRNA staining graded by *in situ* hybridization (ISH) as: 0 - absence/faint staining; + - weak staining; ++ - moderate staining; +++ - strong staining. [g] – p=0.04: TGF-ß$_3$ in Group IV vs TGF-ß$_3$ in Group III.

		BMP-2		TGF-ß$_1$		TGF-ß$_2$		TGF-ß$_3$	
		Gr III	Gr IV	Gr III	Gr IV	Gr III	Gr IV	Gr III	Gr IV
IHC	osteoblasts	NP	71.73± 15.88	NP	63.84± 26.53[a]	NP	77.87± 14.03	NP	81.20± 11.39[a]
	connective tissue	33.12± 18.62[b,c,d]	36.46± 28.64	51.99± 15.38[b]	55.58± 26.10	50.78± 10.16[c]	49.13± 11.67	50.62± 18.98[d]	55.03± 19.16
	bone tissue except osteoblasts	NP	52.92± 7.68	NP	46.76± 3.49[e,f]	NP	61.79± 6.92[e]	NP	69.12± 11.82[f]
ISH	Total mRNA staining in the implant	+	+	+	+	+	++	+[g]	++[g]

TGF-ß$_1$ (p=0.09) (see Table 2). As seen in Table 2, in group IV, the bone tissue contained 32.1% and 47.8% more of TGF-ß$_2$ and TGF-ß$_3$ respectively, compared to TGF-ß$_1$ (p=0.007 and p=0.006, respectively, for representative pictures see Figure 2). In group IV the implants were largely reabsorbed and replaced by bone, characterizing the good osteoconductive properties of beta-tricalcium phosphate.

Discussion

This study shows that the growth factors (BMP-2 and different TGF-ß isoforms) undergo specific changes during the initial and early ossification periods. In this model, undifferentiated mesenchymal-like cells and connective tissue cells invaded the outer parts of the implant after three days. No cartilage, osteoid or bone was formed at that time. A proportion of TGF-ß$_3$ was increased in the mesenchymal-like cells, if rhBMP-2 was added; however, no overall changes in histological appearance were noted. From this we conclude that an addition of rhBMP upregulates the production of TGF-ß$_3$ and that more TGF-ß$_3$ is produced in the area, where the HO formation is expected. This was also supported by *in situ* hybridization of mRNA of TGF-ß$_3$, which showed stronger expression in group II compared to group I (see Table 1).

The second finding was that total expression of mRNA of TGF-ß$_3$ in the implant was stronger in group IV compared to group III (see Table 2). This difference was not obvious in the estimations of immunohistochemical staining of TGF-ß$_3$ in the connective tissue in the implant, while staining of bone tissue was not possible to compare as there was almost no bone formation in group III. However, TGF-ß$_3$ is produced more in the osteoblasts lining the newly formed heterotopic bone in group IV, compared to TGF-ß$_1$, which was not noted in group III (see Table 2). These results indicate a likely regulating role for this growth factor for the process of HO formation, since it has been described to participate in the differentiation of mesenchymal stem cells *(Barry et al., 2001)*. This also shows the diversity of the actions of TGF-ß$_3$, since depending on the location exogenous TGF-ß$_3$ seems to inhibit the osteoblastic differentiation of the mesenchymal cells and the osteocalcin expression *(Moioli et al., 2007)* and also induce ectopic ossification and in-

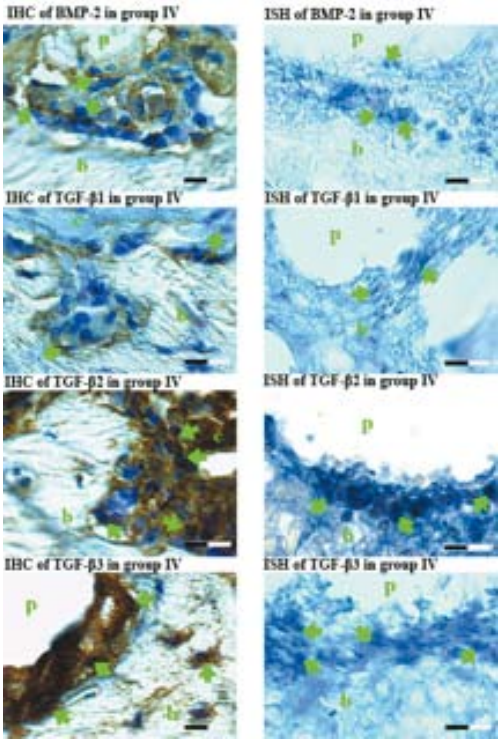

Figure 2. In group IV the cells lining the newly formed bone stained more for TGF-$ß_3$ compared to TGF-$ß_1$ (see Table 2). After the application of rhBMP-2, the newly formed bone tissue produced more TGF-$ß_2$ and TGF-$ß_3$, compared to TGF-$ß_1$ (see Table 2) (brown staining marks the protein). Some differences could also be seen in mRNA expression (the blue staining on the right). Legend: IHC – immunohistochemistry; ISH – *in situ* hybridization; p – pore of the implant; b – bone tissue; c – connective tissue. The scale bar represents 20 µm.

crease the levels of osteocalcin in the cells *(Huojia et al., 2005)*.

The third finding was that there is more immunohistochemically detectable TGF-$ß_2$ and TGF-$ß_3$ in the newly formed heterotopic bone compared to TGF-$ß_1$ (see Table 2). Although this difference was not clear at mRNA level, this suggests that these isoforms are produced more during the remodelling of newly formed HO. Since the level of these two isoforms in bone was elevated in the early stages of HO, it may be that these isoforms take part in both formation and remodelling of HO. In induced HOs the expression of TGF-$ß_1$ has been noted to increase by the day 30 *(Ripamonti, 2005)*. Our results indicate that during induced HO formation changes in the production of the other isoforms (TGF-$ß_2$ and TGF-$ß_3$) take place earlier. Since isoforms TGF-$ß_2$ and TGF-$ß_3$, rather than TGF-$ß_1$ have been shown to be expressed during the course of fracture healing in murine bone *(Cho et al., 2002)*, these results allow us to say that the pattern of changes of tissue content of TGF-$ß_2$ and TGF-$ß_3$ during HO formation is similar to the one noted at fracture healing. Although TGF-$ß_1$ is considered to be the most abundant isoform of the three, with 200 µg/kg of TGF-$ß_1$ being present in the bone (*Seyedin et al., 1985*), it seems that this accumulation does not occur within 21 days of induction but may occur later during bone remodelling.

It should be noted that very little cartilage formation was found in groups III and IV. Increased expression of TGF-ß isoforms has been found during endochondral ossification *(Thorp and Jakowlew, 1994)*, but they have also been detected at sites of intramembranous ossification *(Horner et al., 1998)*. It has also been shown that TGF-$ß_1$ is more related to endochondral heterotopic ossification *(Maroulakou et al., 1999)* while TGF-$ß_2$ and TGF-$ß_3$ are more involved in the direct ossification of soft tissues, e.g. ossification and calcification of atherosclerotic lesions *(Jeziorska, 2001)*. It is also known that TGF-$ß_3$ is more expressed during membranous bone healing *(Bouletreau et al., 2000)*. Taken together, it suggests that intramembranous ossification plays an important part in heterotopic ossification and the shift of the mesenchymal-like cell and osteoblast content of TGF-$ß_3$ may be involved in intramembranous ossification. However since TGF-$ß_3$ has been noted to induce chondrogenesis *(Moioli et al., 2007)*, we may say that it affects both endochondral and intramembranous ossification and these two forms of ossifi-

cation are both involved in the formation of HO.

In groups II and IV, rhBMP-2 was added to the implants to ensure bone growth. We took into consideration that some of the immunohistochemical BMP-2 signal after three days in group II might have been caused by the applied rhBMP-2, since it usually takes two weeks for an organism to remove exogenous BMP-2 from the site *(Seeherman and Wozney, 2005)*. The calculation of the quantity of BMP-2 in the implants at day 3 was therefore excluded from the results. Although application of rhBMP-2 seems to make it difficult to properly evaluate the early changes, it is essential to ensure bone growth in the operation area. Nevertheless the formation of HO after the operation appears to be initiated by inductive agents secreted by the cells *(Toom et al., 2007)*, and standardized randomized and double controlled stereological analysis allowed us to evaluate the exact changes in the growth factor content in different tissues. Although the results are mostly descriptive, they provide thorough data about the TGF-ß distribution in early HO due to the standardized stereological analyses.

Since TGF-ß isoforms have tended to affect cell proliferation and differentiation *(Jenner et al., 2007; Fromigué et al., 1998)* and also bone and cartilage development *(Joyce et al., 1990)*, we believe that these growth factors (isoforms TGF-$ß_2$ and TGF-$ß_3$ in particular) have an effect on initial and early stages of heterotopic bone formation.

Acknowledgments

David Gunnarsson, Regina Ulfsparre and Christina Sandström from the Department of Molecular Biology, University of Umeå, Sweden, and Merly Malva and Mall Kure from the Institute of Zoology and Hydrobiology and dr. Elle Põldoja, from the Department of Anatomy, University of Tartu are thanked for their assistance in the molecular biology field. This project was supported by targeted financed projects No. 0182688s05 and 0180030s07 from the Estonian Ministry of Education and Research and supported by grants from the Estonian Science Foundation (5445, 7284 and 7770), Medical Faculty of the University of Umeå, Sweden, and a stipend from Kristjan Jaagu Stipendiumid, Estonia.

References

Barry F, RE Boynton, B Liu & JM Murphy: Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 2001, *268(2)*, 189-200.

Bonewald LF: Transforming growth factor-ß. Principles of bone biology (second edition) volume 1. Bilezikian JP, LG Raisz and GA Rodan (eds). Academic press 2002, pp 903-918. *Bouletreau PJ, D Steinbrech, JA Spector, SM Warren, JA Greenwald, BJ Mehrara, RC Detch & MT Longaker:* Gene expression of transforming growth factor-beta 3 and tissue inhibitor of metalloproteinase type 1 during membranous bone healing in rats. J Craniofac Surg 2000, *11(6)*, 521-526.

Cheng H, W Jiang, FM Phillips, RC Haydon, Y Peng, L Zhou, HH Luu, N An, B Breyer, P Vanichakarn, JP Szatkowski, JY Park & TC He: Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 2003, *85(8)*, 1544-1552.

Cho TJ, LC Gerstenfeld & TA Einhorn. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res 2002, *17*, 513-520.

Erlebacher A & R Derynck. Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. J Cell Biol 1996, *132(1-2)*, 195-210.

Faler BJ, RA Macsata, D Plummer, L Mishra & AN Sidawy: Transforming growth factor-beta and wound healing. Perspect Vasc Surg Endovasc Ther 2006, *18(1)*, 55-62.

Fan H, C Zhang, J Li, L Bi, L Qin, H Wu & Y Hu: Gelatin microspheres containing TGF-beta3 enhance the chondrogenesis of mesenchymal stem cells in modified pellet culture. Biomacromolecules 2008, *9(3)*, 927-934.

Fromigué O, PJ Marie & A Lomri: Bone morphoge-

netic protein-2 and transforming growth factor-beta2 interact to modulate human bone marrow stromal cell proliferation and differentiation. J Cell Biochem 1998, *68(4)*, 411-426.

Geiser AG, QQ Zeng, M Sato, LM Helvering, T Hirano & CH Turner: Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene. Bone 1998, *23(2)*, 87-93.

Gundersen HJ, P Bagger, TF Bendtsen, SM Evans, L Korbo, N Marcussen, A Møller, K Nielsen, JR Nyengaard & B Pakkenberg: The new stereological tools: dissector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 1988, *96(10)*, 857-881.

Hierton C, G Blomgren & U Lindgren: Factors associated with heterotopic bone formation in cemented total hip prostheses. Acta Orthop Scand 1983, *54(5)*, 698-702.

Horner A, P Kemp, C Summers, S Bord, NJ Bishop, AW Kelsall, N Coleman & JE Compston: Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. Bone 1998, *23(2)*, 95-102.

Huojia M, N Muraoka, K Yoshizaki, S Fukumoto, M Nakashima, A Akamine, K Nonaka & M Ohishi: TGF-beta3 induces ectopic mineralization in fetal mouse dental pulp during tooth germ development. Dev Growth Differ 2005, *47(3)*, 141-152.

Jenner JM, F van Eijk, DB Saris, WJ Willems, WJ Dhert & LB Creemers: Effect of transforming growth factor-beta and growth differentiation factor-5 on proliferation and matrix production by human bone marrow stromal cells cultured on braided poly lactic-co-glycolic acid scaffolds for ligament tissue engineering. Tissue Eng 2007, *13(7)*, 1573-1582.

Jeziorska M. Transforming growth factor-betas and CD105 expression in alcification and bone formation in human atherosclerotic lesions. Z Kardiol 2001, *90 Suppl 3*, 23-26.

Joyce ME, S Jinguishi & ME Bolander: Transforming growth factor-beta in the regulation of fracture repair. Orthop Clin North Am 1990, *21(1)*, 199-209.

Joyce ME, AB Roberts, MB Sporn & ME Bolander: Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J Cell Biol 1990, *110(6)*, 2195-2207.

Maroulakou IG, MA Shibata, M Anver, CL Jorcyk, M Liu, N Roche, AB Roberts, I Tsarfaty, J Reseau, J Ward & JE Green: Heterotopic endochondrial ossification with mixed tumor formation in C3(1)/Tag transgenic mice is associated with elevated TGF-beta1 and BMP-2 expression. Oncogene 1999, *18(39)*, 5435-5447.

Matsaba T, LN Ramoshebi, J Crooks & U Ripamonti: Transforming growth factor-beta1 supports the rapid morphogenesis of heterotopic endochondral bone initiated by human osteogenic protein-1 via the synergistic upregulation of molecular markers. Growth Factors 2001, *19(2)*, 73-86.

Matsuzaki S, T Fukaya, T Suzuki, T Murakami, H Sasano & A Yajima: Oestrogen receptor alpha and beta mRNA expression in human endometrium throughout the menstrual cycle. Mol Hum Reprod 1999, *5(6)*, 559-564.

Matthews SJ: Biological activity of bone morphogenetic proteins (BMP's). Injury 2005, *36 Suppl 3*, 34-37.

Mittl PR, JP Priestle, DA Cox, G McMaster, N Cerletti & MG Grütter: The crystal structure of TGF-beta 3 and comparison to TGF-beta 2: implications for receptor binding. Protein Sci 1996, *5(7)*, 1261-1271.

Moioli EK, L Hong & JJ Mao: Inhibition of osteogenic differentiation of human mesenchymal stem cells. Wound Repair Regen 2007, *15(3)*, 413-421.

Nilsen R. Electron microscopic study of mineralization in induced heterotopic bone formation in guinea pigs. Scand J Dent Res 1980, *88(4)*, 340-347.

Nilsson OS & PE Persson: Heterotopic bone forma-

tion after joint replacement. Curr Opin Rheumatol 1999, *11(2)*, 127-131.

Puzas JE, MD Miller & RN Rosier. Pathologic bone formation. Clin Orthop 1989, *245*, 269-281.

Ripamonti U: Bone induction by recombinant human osteogenic protein-1 (hOP-1, BMP-7) in the primate *Papio ursinus* with expression of mRNA of gene products of the TGF-beta superfamily. J Cell Mol Med 2005, *9(4)*, 911-928.

Risbud MV, A Di Martino, A Guttapalli, R Seghatoleslami, V Denaro, AR Vaccaro, TJ Albert & IM Shapiro: Toward an optimum system for intervertebral disc organ culture: TGF-beta 3 enhances nucleus pulposus and anulus fibrosus survival and function through modulation of TGF-beta-R expression and ERK signaling. Spine 2006, *31(8)*, 884-890.

Rodan GA: Mechanical loading, estrogen deficiency, and the coupling of bone formation to bone resorption. J Bone Miner Res 1991, *6(6)*, 527-530.

Seeherman H & JM Wozney: Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. Cytokine Growth Factor Rev 2005, *16(3)*, 329-345.

Seyedin SM, TC Thomas, AY Thompson, DM Rosen & KA Piez: Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proc Natl Acad Sci 1985, *82(8)*, 2267-2271.

Thomas BJ: Heterotopic bone formation after total hip arthroplasty. Orthop Clin North Am 1992, *23(2)*, 347-358.

Thorp BH & SB Jakowlew: Altered localisation of transforming growth factor-beta 3 during endochondral ossification in rachitic chicks. Bone 1994, *15(1)*, 59-64.

Toom A, A Arend, D Gunnarsson, R Ulfsparre, S Suutre, T Haviko & G Selstam: Bone formation zones in heterotopic ossifications: histologic findings and increased expression of bone morphogenetic protein 2 and transforming growth factors beta2 and beta3. Calcif Tissue Int 2007, *80(4)*, 259-267.

Toom A, T Haviko & L Rips: Heterotopic ossification after hip arthroplasty. Orthop 2001, *24(6)*, 323-326.

Toom A, S Suutre, A Arend & A Märtson: Cellular dynamics in bone formation. A biomaterial based model. 5th World Congress of Biomechanics; Munich, Germany 2006 July Liepsch D, Monduzzi Editore 261-265.

Zhang R, SC Supowit, X Hou & DJ Simmons: Transforming growth factor-beta2 mRNA level in unloaded bone analyzed by quantitative *in situ* hybridization. Calcif Tissue Int 1999, *64(6)*, 522-526.