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An immunohistochemical analysis of the temporal and spatial expression of growth factors FGF 1, 2 and 18, IGF 1 and 2, and TGFß1 during distraction osteogenesis

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Summary. Distraction osteogenesis (DO) is a well established surgical technique that generates new bone by gradual distraction of two bony segments. In this study, we investigated the temporal and spatial profile of FGF 1, 2 and 18, IGF 1 and 2, and TGFß1 during distraction osteogenesis using immunohistochemistry. An osteotomy was performed on the right tibia of 13 white male New Zealand rabbits. After a delay of 7 days, distraction was started at a rate of 0.25mm/12hrs for 3 weeks which was followed by a 3 week period of consolidation. Immunohistochemical analysis was performed on a weekly interval to determine the expression of the growth factors. Staining of all growth factors was apparent at various levels in the centre and callus region in fibroblasts and chondrocyte cells. FGF2 however, showed continued high expression in osteoblasts. Within two weeks after the end of distraction all growth factors showed a reduction in expression except for FGF18 which maintained high levels of expression (up to 100% staining) throughout the distraction and consolidation phases. The study suggests that in comparison to the other investigated growth factors, FGF18 may play in important role throughout the entire process of distraction osteogenesis.

Key words: Distraction osteogenesis, Immunohistochemistry, Growth factors, FGF, IGF, TGFß1

Introduction

Distraction osteogenesis is a widely used surgical technique for the management of several orthopaedic conditions secondary to trauma, infection or post-resection for malignant tumors (Ilizarov, 1989a,b). The process involves performing an osteotomy and subjecting the two bone ends to slow controlled distraction using an external or internal fixation device. Osteogenesis is induced in the distracted gap and when distraction is stopped, the newly formed bone in the gap gradually consolidates. One of the limitations of this technique is the long period of time required for the newly formed bone to consolidate. The external fixator needs to be kept on during this prolonged process which may in turn lead to or exacerbate social, psychological and medical complications (Paley, 1990).

The mechanical forces applied in DO, initiate a biological response involving numerous growth factors capable of enhancing and accelerating bone formation (Weiss et al., 2002). These include BMPs, TGFß (Transforming Growth Factor), IGFs (Insulin Growth Factors) and FGFs (Fibroblast Growth Factors).

FGFs are a family of polypeptides which have been extensively studied for skeletal development and are involved in cell growth, differentiation, and both embryonic and skeletal development (Liu et al., 2002; Dailey et al., 2005). Of the 23 FGFs identified so far, FGF 1, 2 and 18 seem to be the most important ones in bone development and repair (Hurley et al., 2001; Marie, 2003). The significance of the FGF signaling pathway became apparent after the finding that several human skeletal dysplasias including achondraplasia occur as a result of specific mutations in FGFR 1, 2 and 3 (Chen et al., 2005; Ornitz, 2005). Several studies have reported the potential therapeutic uses of FGFs in stimulating bone formation (Mayahara et al., 1993; Nakamura et al., 1995, 1998; Kato et al., 1998; Kawaguchi et al., 2001; Chen et al., 2005).

TGFß enhances bone cell differentiation and proliferation and modulates bone resorption. Its role in accelerating bone formation post fracture has been previously shown in rabbits (Lind et al., 1993; Critchlow et al., 1995) and rats (Nielsen et al., 1994). Similarly, both IGF 1 and 2 are known to stimulate preosteoblasic cell replication and synthesize bone matrix (Solheim, 1998).

All of these growth factors have been studied for embryonic development (Ornitz et al., 2002; Chen et al.,

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2005) and to a lesser extent in fracture healing (Bolander, 1992; Andrew et al., 1993; Bourque et al., 1993), and distraction osteogenesis (Eingartner et al., 1999; Yates et al., 2002; Aronson, 2004). However, to the best of our knowledge, studies on FGF18 have not been previously reported in distraction osteogenesis or fracture healing. In the following study, we investigated the temporal and spatial expression of FGF 1, 2 and 18, as well as related growth factors IGF 1 and 2 and TGFß1 in a rabbit model of distraction osteogenesis.

Materials and methods

Thirteen skeletally mature (9 month old) male New Zealand rabbits, weighing 3.5-4.5 kg, were used. The housing, care, and experimental protocol were approved by McGill University Animal Care and Ethics Committee (protocol # 3571).

Operative protocol

The rabbits were anesthetized by intramuscular administration of ketamine (30 mg/kg) and xylazine (6 mg/kg). Anesthesia was maintained with halothane, oxygen, and nitric oxide after endotracheal intubation. An Orthofix uniplanar fixator (M-100 series, Orthofix, Inc., Verona, Italy) was applied to the medial aspect of the right tibia under sterile conditions (Fig. 1). Four self-tapped half-pins were inserted, two above and two below the osteotomy site. The tibia was exposed subperiosteally, and the osteotomy was performed with an oscillating saw just below the fusion site between the tibia and fibula. One week following the osteotomy, distraction was started at a rate of 0.25 mm/12h for 3 weeks. After 3 weeks of distraction, the fixator was held in place for 3 more weeks (consolidation phase).

The rabbits were examined daily for signs of infection, weight loss and pain. None of the animals had these manifestations and all of the animals survived the surgery as well as the entire duration of the experiment. Antero-posterior and lateral X-ray views of the lengthened tibiae were taken weekly where week 1 corresponded to 1 week after surgery when distraction was to be started, week 2-4 corresponded to the distraction phase and week 5-7 corresponded to the consolidation phase. Every week following the osteotomy, one (at week 1) or two (after starting distraction from weeks 2-7) rabbits were sacrificed by intravenous injections of Euthanyl (MTC pharmaceutical, Cambridge, Ontario). At each time point, material from one animal was used for immunohistochemistry. The samples from the other rabbits were used for standard histology.

Sample preparation

After the rabbits were euthanized, the external fixator was removed and the right tibia was resected. Specimen from rabbits assigned to histology were undecalcified and 6 μm sections were obtained and stained with Goldner Trichrome. Specimen harvested for immunohistochemical analysis were fixed in 4% paraformaldehyde overnight, decalcified in 20 % EDTA for 3 weeks and embedded in paraffin. 7 μm sections were cut. Parallel sections were taken so that the temporal and the spatial expressions of FGF 1,2 and 18, IGF 1 and 2 and TGFß1 were evaluated and compared with each other.

Immunohistochemistry

After deparaffinization and hydration, endogenous peroxidase was blocked with 1% hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubation in 10% Normal Horse Serum (NHS) (Vector Labs, Burlingame, CA) for 10 minutes in a humidified chamber. For immunostaining, the following antibodies were tested: FGF 1, 2, 18, IGF 1,2 and TGFß1 (Santa Cruz Biotechnologies, Santa Cruz, CA). Sections were incubated with these primary antibodies in 1% NHS using a 1 in 40 dilution, for 1 hour in a humidified chamber. A biotinylated antigoat antibody was used as a secondary antibody in a 1:100 dilution for 1 hour (Vector Labs., Burlingame, CA). Sections were then stained using the avidin-biotin complex method (Vector Labs, Burlingame, CA) and 3,3'-dimaminobenzidine tetrachloride. Finally, the sections were counterstained with hematoxylin and mounted with Permount (an adhesive slide mounting media purchased from Fisher Scientific., Ontario, Canada). For negative controls, the same procedure was followed except the primary antibodies were omitted.

According to data provided by the manufacturer (Santa Cruz Biotechnologies, Santa Cruz, CA), the primary antibodies used in the present study recognize mouse, rat, and human FGF, IGF and TGFß1 proteins. Therefore, it was necessary to determine if these antibodies recognized specific rabbit FGF, IGF and TGFß1 proteins. Blocking peptides are available for all Santa Cruz Biotechnology Inc. affinity-purified rabbit and goat polyclonal antibodies and monoclonal antibodies raised against peptide antigens. Antibodies binding to antigens may be blocked/competed by pre-absorption with the blocking peptide. To perform a blocking/competition process, we combined 1μl of the primary antibody (concentration of 200mg/ml) with a five-fold (by weight) excess of its respective blocking peptide (concentration of 200 μg/ml) in a small volume of Phosphate Buffered Saline totalling 500 ml. This was then incubated overnight at 4°C. The following morning, the immunohistochemistry protocol was followed using the same 1 in 40 dilution in NHS and following the same protocol described above. Treated samples showed no evidence of staining thus confirming that the antibodies used in the present study were specific to the rabbit protein of interest. This technique has been previously published by us (Campisi et al., 2003; Hamdy et al., 2003; Haque et al., 2005).

Quantification

Chondroctyes, osteoblastic and fibroblastic cells were identified morphologically, and the number of cells expressing FGF 1, 2, 18, IGF 1, 2 and TGFß1 was assessed by cell counting in each cell type. This semi-quantitative method used for analyzing immuno-histochemistry images has been previously described by us (Haque et al., 2005). Briefly, the semi-quantitative data obtained was based on the percentage of cells showing positive staining. Each of the immunostained sections was graded blinded, and percentage of cells expressing the specified growth factors was graded as follows: - represents no staining in the majority of cells; + represents staining in less than 25% of cells; ++ represents staining in 25–50% of cells; +++ represents staining in 50-75% of cells; ++++ represents staining in more than 75% of cells. The number of cells showing expression was assessed by cell counting. These analyses were performed separately for the callus region and the central region containing the fibrous interzone.

Results

Radiological findings

Radiology images revealed that new bony callus could be observed in the centre of the distraction zone two weeks after the start of the distraction. Three weeks after the end of distraction the distracted site was bridged with new bone. Fig. 1 shows an image of the rabbit tibia at 2, 5 and 7 weeks post start of distraction.

Histological findings

During the distraction phase, a fibrous interzone was produced between the osteotomy ends, and afterwards a large amount of fibrillar matrix was produced and numerous cells which morphologically represented a continuum between fibroblast and chondrocytes appeared next to the fibrillar matrix region. Osteoblastic cells were found around those cells. During the consolidation phase, the fibrous inter-zone was rapidly replaced by mineralized bone and the consolidation was completed by the end of 7 weeks post surgery. Fig. 2 shows a stained histology image of the bone at week 5 (first week of consolidation) .

Immunohistochemistry

A quantitative evaluation of growth factor expression is given in Table 1 where week 1 describes results taken 1 week after the surgery before starting distraction, weeks 2-4 describe the distraction phase and weeks 5-7 describe the consolidation phase. Figs. 3, 4 and 5 provide representative examples of immunostained

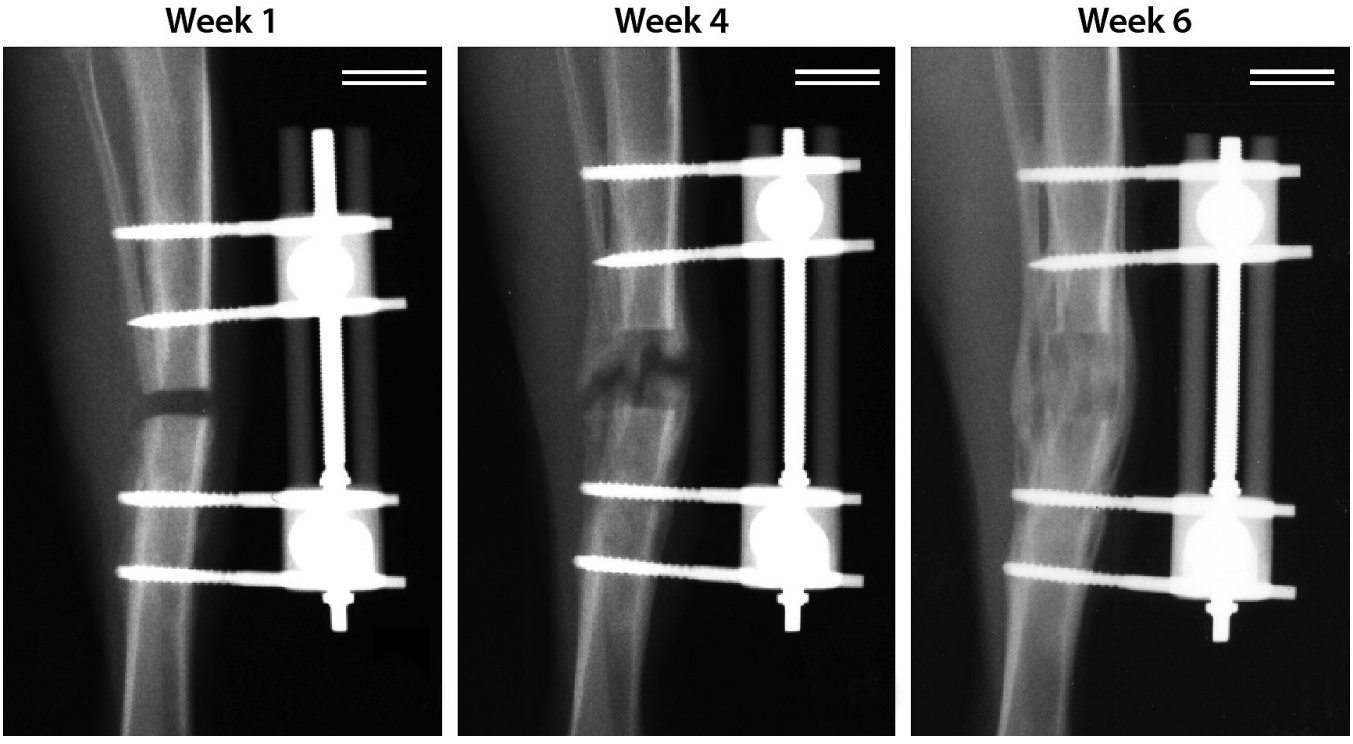

Fig. 1. Radiological images of rabbit tibia at 1, 4 and 6 weeks post start of distraction. At the end of the distraction phase, bony callus is present at the centre of the distraction zone and at the end of the consolidation phase, the distraction gap is completely filled with new bone.

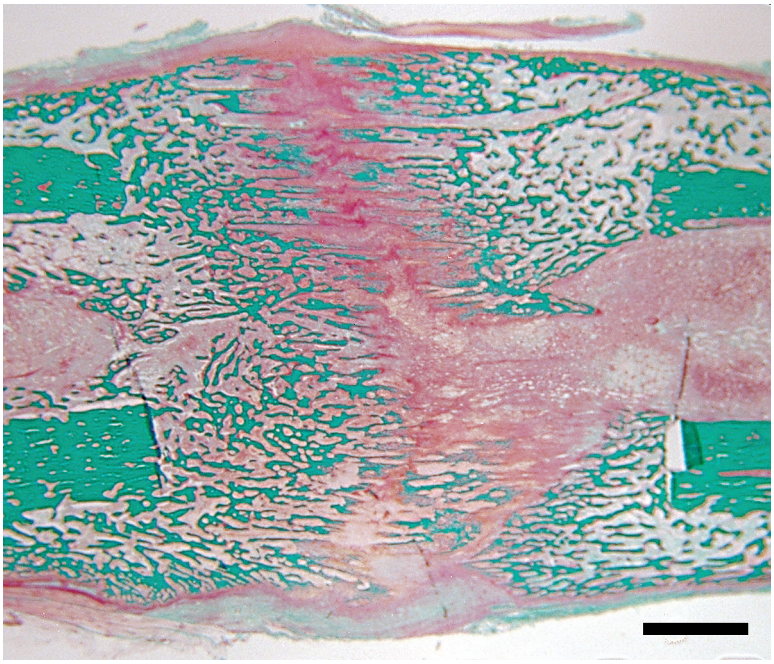

Fig. 2. Histological image of bone using Goldner trichrome staining at 5 weeks after surgery. Fibrous interzone is beginning to be replaced by mineralized tissues. Mineralized bone is shown in green. Magnification bar: 2 μm.

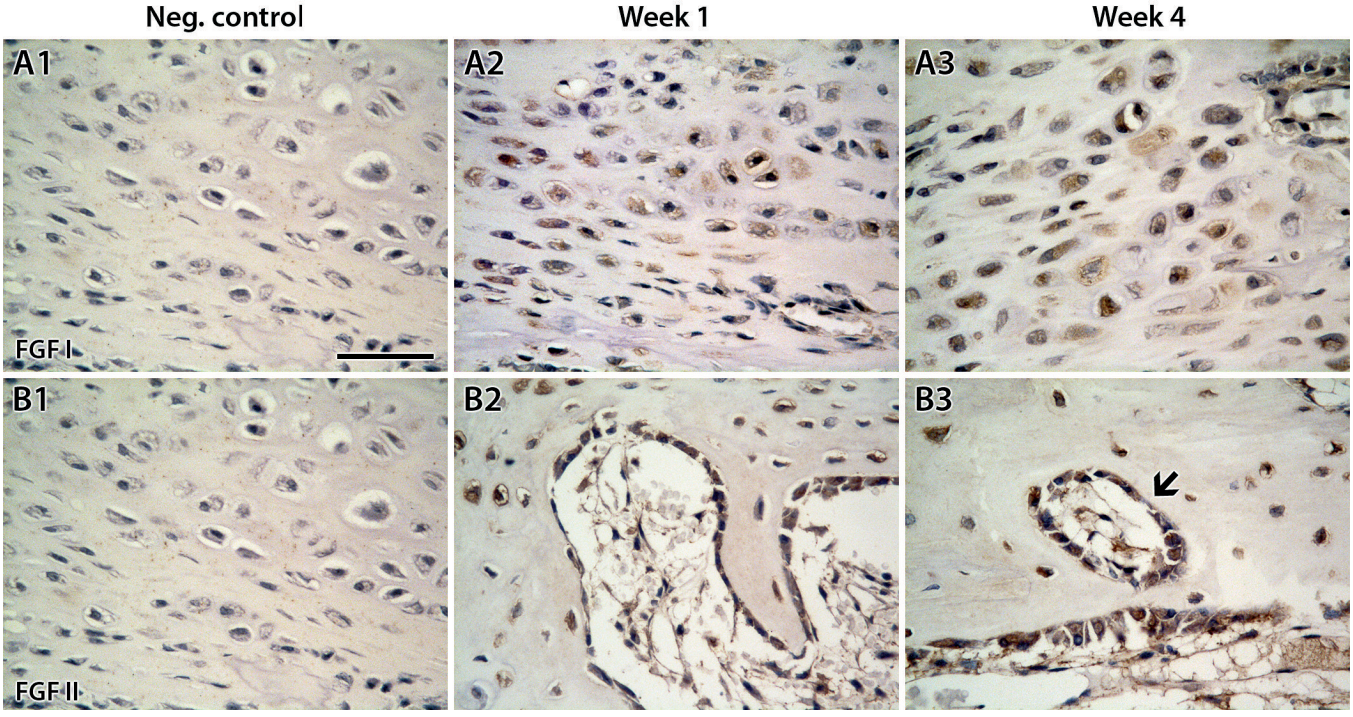

Fig. 3. Immunostaining of FGF1 (A1-A3) and FGF2 (B1-B3). Figures A1 and B1 represent the negative controls, A2 and B2 show staining at 1 week after the start of distraction and A3 and B3 show staining 4 weeks after the start of distraction. Staining was present throughout the distraction phase up to the beginning of the consolidation phase. The arrow in figure B3 indicates expression of FGF2 in osteoblasts. Scale bar: 50 μm.

sections at week 2 (1 week after starting distraction) and week 5 (1 week after starting consolidation) for all growth factors evaluated.

FGF 1, 2 and 18

Staining for FGF1 was evident during the distraction phase, and the signal was mainly localized in chondrocytes both in the callus and in the fibrous interzone, at the center of the distraction zone. There was no staining in osteoblasts, but a mild staining was apparent in fibroblastic cells during distraction. The signal in chondroctyes was reduced at the end of distraction and during consolidation. On the other hand, a marked expression of FGF2 was observed during the distraction phase among the osteoblasts, chondrocytes and fibroblastic cells in both the center and callus region. The signal in chondroctyes and fibroblastic cells disappeared during the consolidation phase, however, the osteoblasts continued to show positive staining until the end of consolidation (Fig. 3). FGF18, revealed significantly stronger staining compared to FGF1 and FGF2. Results showed high expression during the end of the distraction phase and this expression remained strong throughout the consolidation phase in chondrocytes and fibroblastic cells in the center and callus region (Fig. 4). FGF18 expression was only mildly visible in osteoblasts in the central region during distraction.

IGF1, 2 and TGFß1

IGF1 and IGF2 appeared in chondrocytes and

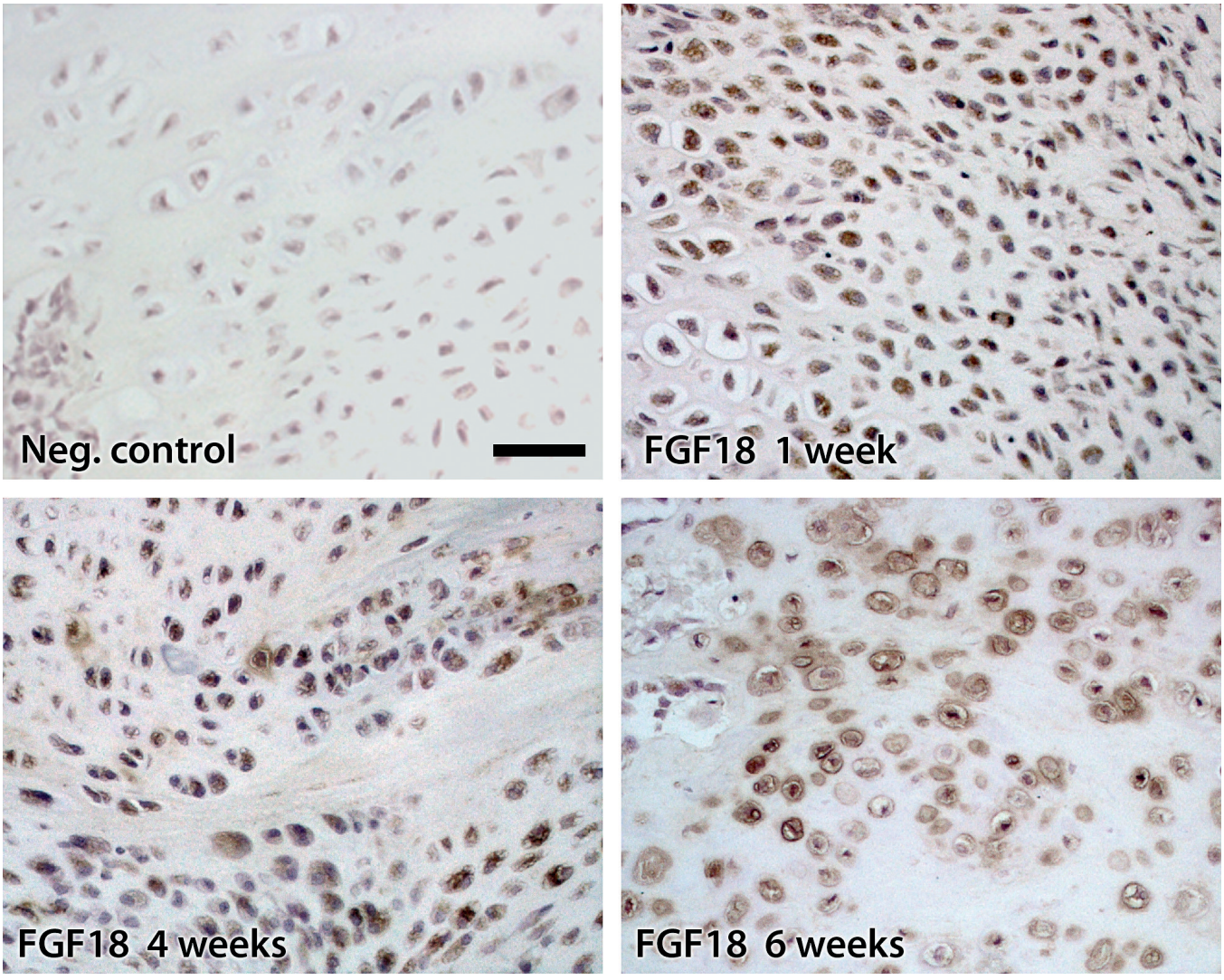

Fig. 4. Immunostaining of FGF18 at 1, 4 and 6 weeks, post start of distraction. Figures show staining present throughout the distraction phase and sustained at the end of the consolidation phase (6 weeks). Scale bar: 50 μm.

fibroblastic cells during the distraction phase, but they were not apparent in osteoblasts, IGF2 expression was greater than IGF1. However, the expression of both IGFs decreased after the end of distraction and was not expressed during late consolidation.

TGFß1 expression was not as intense compared to the expression of FGFs and IGFs, however, all type of cells (chondrocytes, osteoblasts and fibroblasts) were positively stained. Its expression diminished during consolidation. Expression at the centre region ceased at

Table 1. FGF, IGF and TGF ,1 expression during distraction osteogenesis in osteoblasts (osteo), chondorctyes (chond.) and fibroblasts (fibro.).

Protein	Stage	Week	Center			Callus		
			Osteo	Chond	Fibro	Osteo	Chond	Fibro
FGF 1	A	1	-	-	-	-	-	-
	B	2	-	+	+	-	+	+
		3	-	++	+	-	++	+
		4	-	++	+	-	++	+
	C	5	-	++	+	-	+++	+
		6	-	-	-	-	-	-
		7	-	-	-	-	++	-
FGF 2	A	1	-	-	-	-	-	-
	B	2	+++	+	+	+++	+	+
		3	+++	+++	+++	+++	+++	+++
		4	+++	+	++	+++	+++	+++
	C	5	+++	+++	++	+++	++	++
		6	++	-	-	++	-	+
		7	++	-	-	++	+	-
FGF 18	A	1	-	-	-	-	-	-
	B	2	+	+	+++	-	+	++
		3	+	+++	+++	-	++++	++++
		4	+	++++	++++	-	++++	++++
	C	5	-	+++	++++	-	++++	++++
		6	-	+++	+++	-	++++	++++
		7	-	+++	-	-	++++	_
IGF I	A	1	-	-	-	-	-	-
	B	2	-	+	+	-	+	-
		3	-	+	-	-	+	+
		4	-	+	+	-	+	+
	C	5	-	+++	+	-	+++	+
		6	-	-	-	-	-	-
		7	-	-	-	-	+	-
IGF II	A	1	-	-	-	-	-	-
	B	2	-	+	+	-	+	+
		3	-	+++	+++	-	+++	+++
		4	-	++	+++	-	++	+++
	C	5	-	+++	+++	-	++	++
		6	-	-	-	-	-	-
		7	-	-	-	-	+	-
TGF _1	A	1	-	-	-	-	-	-
	B	2	-	+	+	-	++	+
		3	+	++	+	+	++	+
		4	+	++	++	+	++	++
	C	5	+	+	++	+	+	++
		6	-	-	-	-	-	-
		7	-	-	-	-	+	-

Semi-Quantitative analysis where + represents staining in less than 25% of cells; ++ represents staining in 25–50% of cells; +++ represents staining in 50-75% of cells; ++++ represents staining in more than 75% of cells. Stage A (week 1): 1 week after surgery, Stage B (weeks 2-4): distraction phase, stage C (week 5-7): consolidation phase.

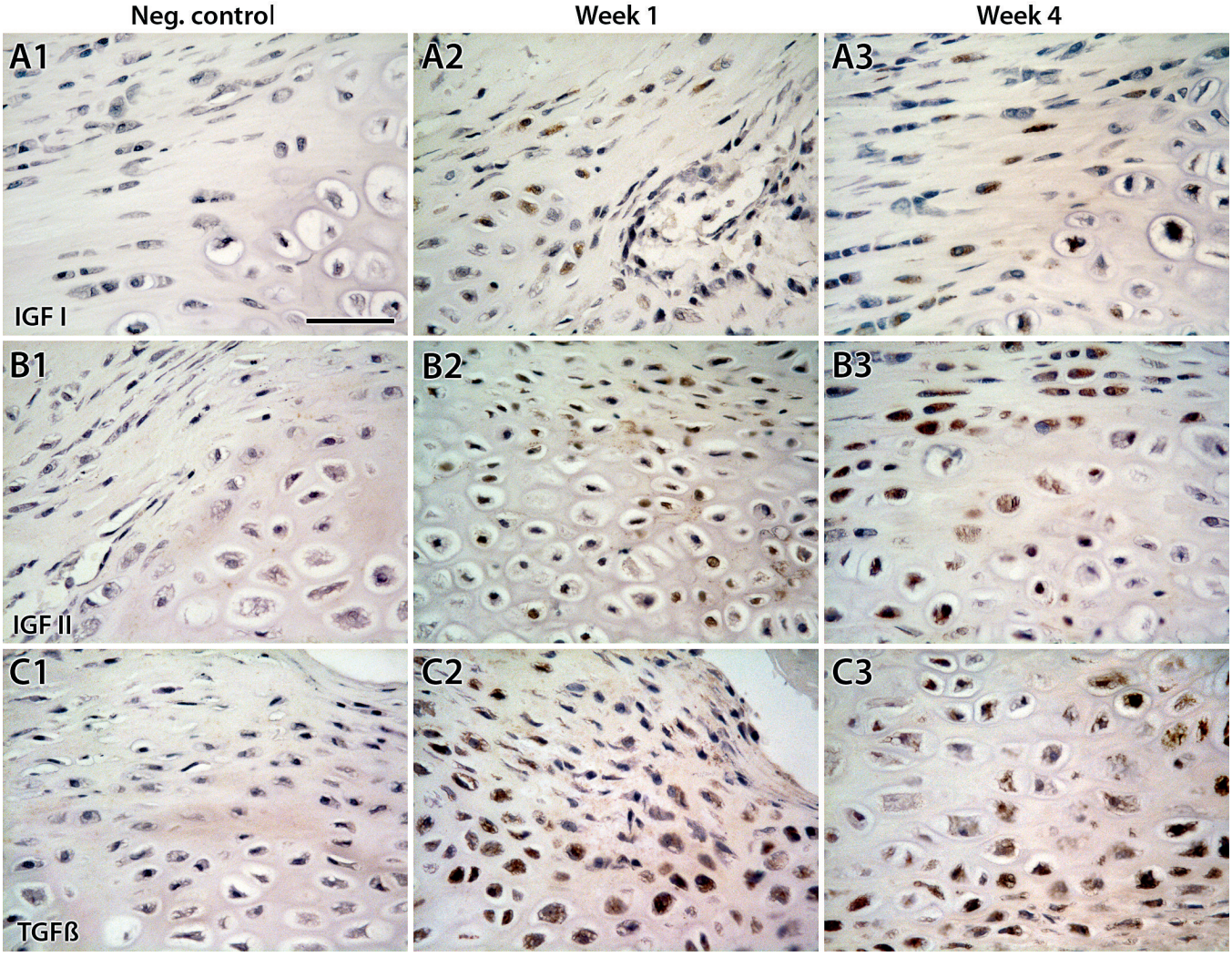

Fig. 5. Immunostaining of IGF1 (A1-A3), IGF2 (B1-B3) and TGF,1 (C1-C3). Figures A1, B1, C1 represent negative controls for IGF1, IGF2 and TGF,1 respectively, Figures A2, B2, C2 show staining at 1 week post the start of distraction and Figure A3, B3 and C3 show staining at 4 weeks post the start of distraction. Staining was present throughout the distraction phase up to the beginning of the consolidation phase. Scale bar: 50 μm.

5 and 6 weeks post distraction (Fig. 5).

Discussion

We investigated the temporal and spatial expression of FGF 1, 2, 18, IGF 1, 2 and TGFß1 in a rabbit model of distraction osteogenesis during the distraction and consolidation phases using immunohistochemistry. There was no expression observed post osteotomy, however, as soon as distraction was applied, all of these growth factors were expressed primarily in chondrocytes and fibroblastic cells, and when distraction was discontinued, the expression of these growth factors appeared to be diminishing with the exception of FGF18. Several studies have reported that mechanical forces could stimulate the expression of growth factors (Cillo et al., 2000; Yeung et al., 2001). It is therefore possible that the expression of FGF 1, 2 and 18, IGF 1 and 2, and TGFß1 could be a direct consequence of the distraction process, however, all of these growth factors did show expression also during the first week of consolidation. In addition, FGF2 continued to be expressed mildly in osteoblasts and FGF18 continued to show high expression throughout the three week consolidation phase.

Although the expression of growth factors during the distraction phase may be attributed to mechanical forces, what induces their presence during the consolidation phase still remains unclear. It is possible that it is the lack of growth factors FGF1, IGF 1 and 2 or TGFß1, rather than the high expression of FGF18 that plays an important role during the consolidation phase.

Furthermore, the expression of FGF1, IGF 1 and 2 and TGFß1 during the beginning of consolidation may be the result of residual effects from the mechanical forces during distraction. It may also be that other cytokines (which are not yet identified) could play a role during the consolidation phase. Although, our results showed that the only growth factors expressed during the late consolidation phase were FGF2 (mildly in osteoblasts) and FGF18 (high expression in chondorcytes and fibroblasts), further studies are required in order to explain these observations and clarify the role of these growth factors in DO.

The process of bone formation in distraction osteogenesis closely resembles and recapitulates both embryonic limb development and fracture healing (Li et al., 1998). Growth factors IGF, FGF and TGFß were chosen in this study based on previous reports which have revealed that several growth factors including BMPs, TGFß, IGFs and FGFs are involved in regulating bone regeneration and remodeling during bone growth and repair (Canalis et al., 1991; Li et al., 1998; Barnes et al., 1999; Eingartner et al., 1999). We have previously investigated and reported the expression profile of BMPs (Hamdy et al., 2003). Although all of these growth factors were expressed during the process of distraction osteogenesis, the timing and intensity of the various growth factors are important in determining their specific roles.

FGF signaling modulates cell proliferation and differentiation during both osteogenesis and chondrogenesis (Ohbayashi et al., 2002). However, whether it promotes or inhibits these processes is dependent on the stage of development (Ellsworth et al., 2002; Ornitz et al., 2002). The expression profiles and time of expression for FGF 1, 2 and 18 were different and most likely corresponded to their respective functions. FGF 2 and 18 showed the highest intensity of staining and widest temporal and spatial expression compared to the other studied growth factors. Previous studies in fetal rat cultures have reported that FGF1 is involved in the growth of fetal and neonatal osteoblasts (Tang et al., 1996). The observation that FGF1 was concentrated in chondrocytes is in concordance with other findings which indicated that FGF1 is expressed during chondrogenesis (Barnes et al., 1999) and is synthesized by chondroctyes during fracture repair (Bolander, 1992). Several research have shown that FGF1 administration can increase new bone formation (Dunstan et al., 1999; Mackenzie et al., 2001; Kelpke et al., 2004), however, our study revealed that FGF1 expression in DO is significantly less than FGF2 and 18. It is well known that FGF2 is a more potent inducer of bone formation than FGF1 (Canalis et al., 1991; Barnes et al., 1999)

FGF2 was expressed in all cell types after the start of distraction. FGF2 has been shown in numerous studies to be involved in fracture healing (Radomsky et al., 1998; Okazaki et al., 1999; Kawaguchi et al., 2001; Aronson, 2004). Our results showed high expression of FGF2 in osteoblasts throughout the distraction and consolidation phase. This observation is consistent with previous findings in a goat model of DO (Yeung et al., 2001), as well as previous reports which revealed that mice lacking FGF2 result in decreased osteoblast replication and bone formation (Montero et al., 2000; Ellsworth et al., 2002).

Interestingly, of all the growth factors investigated, FGF18 showed the highest expression (up to 100% staining) which was sustained throughout the consolidation phase. To the best of our knowledge, this is the first report on the expression of FGF18 in DO. It has previously been reported that FGF18 may be involved in stimulating cartilage repair and cell proliferation (Ellsworth et al., 2002; Moore et al., 2005). It has also been suggested that the role of FGF18 may be more important in bone development than FGF2 (Shimoaka et al., 2002). Clearly, the different expression pattern observed for FGF2 and 18 emphasizes that the two growth factors most likely function in different ways.

The mechanism of action of FGF18 functions is still under investigation. A recent study indicated that FGF18 may potentially function to induce bone formation by suppressing the activity of BMP antagonist noggin (Reinhold et al., 2004). Mukherjee reported that the effects of TGFß could potentially be mediated by FGF signaling suggesting that growth factors function in an intertwined relationship for regulating bone formation (Mukherjee et al., 2005).

TGFß1 and both IGF 1 and 2 expression were found to be concentrated primarily during the distraction phase. It has previously been shown that IGF and TGFß are located at the site of regeneration during distraction (Eingartner et al., 1999; Weiss et al., 2002). Both IGFs and TGFß1 show nearly no expression during weeks 5 and 6 (consolidation phase).

The expression of TGFß1 during distraction osteogenesis has been previously reported (Canalis et al., 1991; Lammens et al., 1998; Eingartner et al., 1999; Cillo et al., 2000). The finding that TGFß1 expression reaches a peak at the beginning of distraction in osteoblasts, chondrocytes and fibroblasts can be explained by reports that relate the expression of TGFß to the proliferation stage of bone regeneration (Lammens et al., 1998; Weiss et al., 2002).

On the contrary, studies on serum levels of growth factors during DO, suggested that IGF1 could be involved in the maturation period (Weiss et al., 2002). This may help explain our observation that IGF1 had low expression throughout the distraction and consolidation phase except at week 4 where cells showed up to 75% staining in chondrocytes. It has been reported that IGF1 plays a more significant role in bone synthesis than IGF2 which is most likely due to its high affinity for IGF1 receptors (Canalis et al., 1991). Contrary to this report, our data revealed that IGF2 was more intensely expressed than IGF1. We were unable to find any reports on IGF2 expression during distraction

osteogenesis to explain this discrepancy, however, previous studies on IGF 1 and 2 expression in human fracture healing has shown that although both IGFs were expressed, IGF2 was expressed for a longer period of time and was also evident in osteoclasts during bone remodeling (Andrew et al., 1993). Our results showed no expression of either IGF 1 or 2 in osteoblasts, however the intensity of IGF2 expression in chondroctyes and fibroblasts was greater than IGF1. These observations suggest that compared to IGF1, IGF2 may be a more important factor in bone formation during distraction osteogenesis.

Conclusion

In conclusion, this study revealed the expression profiles of growth factors FGF 1, 2 and 18, IGF 1 and 2, and TGFß1 during distraction osteogenesis. Even though all the growth factors showed expression from the first week of distraction to the first week of consolidation, FGF2 was the only growth factor showing expression in osteoblasts throughout the entire process. This suggests that FGF2 may play a significant role in osteoblast proliferation during DO. FGF18 was the only factor that showed intense staining in chondrocytes and fibroblasts throughout the three week consolidation phase. This is the first report of FGF18 expression during distraction osteogenesis, however, further investigations on its mechanism of action will need to be performed in order to clarify the exact role of FGF18 during distraction osteogenesis.

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