Bone 45 (2009) 862–869

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Low-intensity pulsed ultrasound affects RUNX2 immunopositive osteogenic cells in delayed clinical fracture healing ☆

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ARTICLE INFO

Article history:
Received 6 May 2009
Revised 15 July 2009
Accepted 15 July 2009
Available online 23 July 2009

Edited by: T. Einhorn

Keywords:
Fracture healing
Delayed union
Low-intensity pulsed ultrasound
RUNX2
Immunolocalization
Bone progenitor cells

ABSTRACT

Introduction: Osteogenic cell proliferation and differentiation play an important role in adequate fracture healing, and is target for osteoinductive therapies in delayed fracture healing. The aim of this study was to investigate whether low-intensity pulsed ultrasound enhances fracture healing at the tissue level in patients with a delayed union of the osteotomized fibula through an effect on the presence of RUNX2 immunopositive osteogenic cells. The effect was studied in both atrophic and hypertrophic delayed unions.
Materials and methods: Biopsies were obtained from 6 female and 1 male patient (age 43–63) with a delayed union of the osteotomized fibula after a high tibial osteotomy treated for 2–4 months with or without low-intensity pulsed ultrasound in a randomized prospective double-blind placebo-controlled trial. Immunolocalization of RUNX2 protein was performed to identify osteogenic cells. Histomorphometrical analysis was performed to determine the number of cells expressing RUNX2 located within and around the newly formed woven bone at the fracture end (area of new bone formation), and up to 3 mm distant from the fracture end.
Results: Cells expressing RUNX2 were present in all histological sections of control and low-intensity pulsed ultrasound-treated bone evaluated. Within the area of new bone formation, RUNX2 immunopositive cells were found in the undifferentiated soft connective tissue, at the bone surface (presumably osteoblasts), and within the newly formed woven bone. Low-intensity pulsed ultrasound treatment of fibula delayed unions significantly reduced the number of RUNX2 immunopositive cells within the soft connective tissue at the fracture ends, whereas the number of RUNX2 immunopositive cells at the bone surface was not affected. The number of RUNX2 immunopositive cells was similar for the atrophic and hypertrophic delayed unions.
Conclusions: Immunolocalization of RUNX2 positive cells in delayed unions of the fibula reveals that delayed clinical fracture healing does not result in impairment of osteogenic cell proliferation and/or differentiation at the tissue level, even if delayed unions are clinically regarded as atrophic. Reduced number of osteogenic RUNX2 immunopositive cells within the soft connective tissue, and unchanged number of RUNX2 immunopositive cells at the bone surface, implicate that low-intensity pulsed ultrasound does not increase osteogenic cell presence, but likely affects osteogenic cell differentiation.

Introduction

Fracture healing is a complex process requiring the recruitment of the appropriate cells and expression of the appropriate genes at the right time in the right place [1]. Osteogenic cell proliferation and differentiation play a central role in adequate fracture healing to increase extracellular bone matrix production [2]. The majority of clinical fractures heal spontaneously, i.e. only 5–10% of the fractures show impaired healing [1]. Impairment of fracture healing leads to a delay in union or may even result in nonunion. If fracture healing is impaired, it can be initiated or enhanced by surgical and/or nonsurgical means [1].

Fracture healing is modulated in response to external stimuli, such as growth factors, hormones, and mechanical forces [1,3]. Insight in the molecular biological mechanisms involved in fracture healing has resulted in the development of new treatment modalities for impaired fracture healing, such as bone morphogenic proteins, extracorporeal shock wave treatment, electro-stimulation, and low-intensity pulsed ultrasound (LIPUS) [4–8]. LIPUS and electro-stimulation have the advantage over surgery that they are noninvasive, do not cause any side effects, and can be used in an outpatient setting making them less expensive [9,10]. LIPUS is a form of mechanical energy transmitted transcutaneously by high frequency acoustic pressure waves [11]. The

☆ Clinical trial approval: approval was obtained from the Medical Ethical Review Board of theVU University Medical Center, 3 May 2004, registration number 2004-005.

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doi:10.1016/j.bone.2009.07.012

intensity of LIPUS (30 mW/cm2) is within the range of ultrasound intensities used for diagnostic purposes (1–50 mW/cm^2). Diagnostic ultrasound is regarded as nonthermal and nondestructive [9]. LIPUS stimulation may result in a fractional temperature rise, however its mechanism of action is likely of nonthermal origin [12].

Bone cells are sensitive to strains caused by physical loading [13,14]. Mechanoreceptors convert biophysical stimuli into biochemical responses that alter gene expression and cellular adaptation [15]. Mechanical adaptive modeling can promote bone tissue formation by a proliferative response or by a direct anabolic effect on bone cells [16]. The micro-mechanical stress produced by LIPUS may provide a surrogate for the forces normally applied on bone by physical loading according to Wolff's law [17,18]. Although the strain induced by LIPUS at the tissue level is several orders of magnitude lower than the peak strains generated by functional load bearing [13], high frequency low-magnitude strains can result in strong regulatory signals to bone tissue [14,19,20].

LIPUS increases prostaglandin E_2 production via the induction of cyclooxygenase-2 in MC3T3-E1 osteoblastic cells *in vitro* [21]. PGE_2 is a potent inflammation mediator that may help migration and proliferation of mesenchymal stem cells [21]. Outcomes of LIPUS treatment of fetal mouse metatarsal rudiments *in vitro* suggested a direct effect of LIPUS on osteoblasts and ossifying cartilage by stimulation of cell activity and/or differentiation, but not proliferation [22,23]. In clinical fracture healing, LIPUS has been shown to increase bone volume, osteoid thickness and mineral apposition rate in the area of fracture healing, indicating increased osteoblast activity [24]. Mechanical forces can promote bone tissue formation by a proliferative response or by a direct anabolic effect on bone cells [2]. The positive effect of LIPUS on clinical fracture healing may be caused by a stimulation of the different processes involved in fracture repair and bone formation.

Runx2 is regarded as the master gene of osteogenic cell differentiation and bone matrix production. RUNX2 is a transcription factor, required for the activation of osteoblast differentiation and is crucial for the regulation of genes responsible for the production of bone specific proteins (collagen type 1, osteocalcin, osteopontin and bone sialoprotein) [25,26]. Without RUNX2 expression no bone will be formed. RUNX2 is also thought to be the target of a mechanical signal by which physical stimulation dictates the cellular and metabolic activities of osteoblasts [27]. Stimulation of rat bone marrow stromal cells and human osteoblasts with LIPUS resulted in a time-dependant increase in cellular RUNX2 expression [28]. It is therefore possible that LIPUS affects fracture healing by affecting RUNX2 expressing cells.

The aim of this study was to investigate whether LIPUS enhances fracture healing at the tissue level in patients with a delayed union of the osteotomized fibula through an effect on the presence of RUNX2 immunopositive osteogenic cells. We studied the effect in both atrophic and hypertrophic nonunions.

Materials and methods

Clinical study procedures

The present study utilizes a clinical delayed union model of the fibula, which has been reported previously [24]. The clinical study procedures also have been described in detail [24]. In short, biopsies of delayed unions of the human osteotomized fibula after a high tibial osteotomy were obtained from 6 female patients and 1 male patient (ages 43–63 years) treated with or without LIPUS (3 LIPUS-treated, 4 untreated controls) in a randomized prospective double-blind placebo-controlled clinical trial (Table 1). The patients enrolled in the clinical trial used the EXOGEN 2000+® low-intensity pulsed ultrasound device (Smith & Nephew Inc., Memphis, TN, USA) at home for a daily 20-minute treatment. Active and placebo devices were identical in every way (they had the same visual, tactile, and auditory signals) except for the ultrasound signal emitted. The active LIPUS device produced a 200 μs burst of 1.5 MHz acoustic sine waves, that repeated at a modulation frequency of 1 kHz, and provided a peak pressure of 30 mW/cm^2. Mean fracture age at inclusion was 195 days (range 180–214, median 193 days) for the sham-treated controls, and 233 days (range 180–331, median 187 days) for the LIPUS-treated patients. Delayed unions were classified as atrophic when clear callus formation on the clinical radiographs was absent, and no healing progression was seen on X-rays taken before inclusion in the trial [6]. Two to 4 months after the start of LIPUS or sham treatment, a biopsy was taken from the delayed union of the fibula. The standardized biopsy procedure was performed under general or spinal anesthesia, with the use of a hollow trephine burr (2.5 mm inner diameter, 3.5 mm outer diameter, ITI-Straumann, Basel, Switzerland), which resulted in cylindrical biopsies of 2.5 mm in diameter. The direction of drilling was in a straight angle to the fibula axis, from the lateral to medial cortex, crossing the fracture plane at an angle of approximately 45°. The biopsy specimen contained (pre-existing) cortical bone, (pre-existing) cancellous bone, and newly formed fracture callus at the fracture end with the adjacent fibrous/cartilaginous tissue of the fracture gap. The histological biopsies showed endosteal callus formation, but not periostal/cortical fracture bridging. Similar heterogeneous tissue samples were obtained, as was verified by post-biopsy X-rays. Comparison among biopsies was based on distinctive histological landmarks identifying the areas of interests, as has been described previously [24]. Patients received LIPUS treatment for 89 days (range 61–115, median 90 days) or sham treatment for 81 days (range 72–89, median 82 days). Two of the three LIPUS-treated patients, and two of the four sham-treated controls included in this study showed healing at 5 months of treatment (Table 1). Trial approval was obtained from the Medical Ethical Review Board of the VU University Medical Center, registration number 2004–005.

Table 1
Patient and fracture characteristics at trial inclusion, duration of LIPUS treatment of delayed unions at the time of the biopsy procedure, histomorphometric data, and clinical outcome of delayed unions of the osteotomized fibula treated with or without LIPUS.

Treatment modality	Sex	Age	Smoking	Fracture age (days)	Delayed union type	Treatment time (days)	N.RUNX2+ /SCT.Ar (0.01 mm^2)	N.RUNX2+ /BS (0.1 mm)	BV/TV (%)	Clinical outcome at 5 months
LIPUS	F	43	No	187	Hypertrophic	61	9.8	1.9	52.9	Failed
"	M	63	Yes	331	Hypertrophic	115	0.9	0.4	49.7	Healed
"	F	57	No	180	Atrophic	90	3.0	1.3	41.7	Healed
Control	F	48	No	183	Atrophic	72	12.5	1.7	36.7	Failed
"	F	54	No	180	Hypertrophic	83	20.0	3.3	35.7	Healed
"	F	57	No	202	Hypertrophic	81	14.3	1.6	44.1	Failed
"	F	44	No	214	Hypertrophic	89	10.8	1.1	28.8	Healed

Histomorphometric data is presented for the area of new bone formation, representing the newly formed bony callus within 0.6 mm distance from the fracture ends. N.RUNX2+/SCT.Ar, number of cells immunopositive for RUNX2 per area of soft connective tissue; N.RUNX2+/B.Pm, number of cells immunopositive for RUNX2 per bone perimeter; BV/TV, bone volume of total tissue volume.

Fixation and embedding

All biopsies of the delayed unions of the fibula were immediately fixed in 4% formaldehyde solution in 0.1 M phosphate buffer, pH 7.3, at 4 °C for 24 h. The biopsies were rinsed with 0.1 M phosphate buffer, and then decalcified with 5% EDTA,1% formaldehyde at pH 7.3 at 4 °C for 8 weeks. After rinsing with 0.1 M phosphate buffer, these biopsies were dehydrated through ethanol 90% and 100% and cleared twice in xylene, and finally embedded in molten paraffin at 56 °C. Sections of 6–7 μm thickness were cut with a microtome, mounted on polylysine-coated glass slides, and dried overnight in a 37 °C stove to prepare for immunohistochemistry as described below.

Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated in successively decreasing grades of aqueous ethanol solution [29,30]. After three repeated washes with phosphate buffered saline (PBS), sections were subjected to antigen retrieval by placing them in citrate buffer at boiling temperature for 20 min. Endogenous peroxidase was quenched by incubation in 3% H_2O_2 in PBS for 3 min, followed by 3 repeated washes with PBS. Sections were blocked with 30% normal horse serum for 30 min at room temperature. Incubation with the primary antibody to RUNX2 (mouse recombinant monoclonal antibody Pebp2αA, kindly provided by K. Sasaguri, Kanagawa Dental School, Japan) at 1:800 dilution, was performed overnight in a humidified chamber at 4 °C. After rinsing the sections were allowed to react with biotinylated horse anti-mouse IgG. After rinsing again, sections were incubated with ABC peroxidase (Elite kit, Vector Labs, Burlingame, CA, USA) according to the manufacturer's instruction. Development was carried out with a DAB peroxidase substrate kit (Vector Labs, Burlingame, CA, USA). Sections were counterstained with methyl green for 5 min, mounted in 50% glycerol, and covered with a glass cover slip. Positive cells stained brown, while negative cells only showed blue/green nuclei and no brown cytoplasm. Negative control sections were incubated with normal mouse IgG replacing the primary antibody.

Histomorphometric analysis

All analyses were performed using a Leica DMRA microscope connected to a computer using an electronic stage table, and a Leica DC 200 digital camera [24]. All measurements were done using the Leica QWin© computer program (Leica Microsystems Image Solutions, Rijswijk, the Netherlands). Histomorphometric analysis was performed to determine the number of cells expressing RUNX2 located within and around the newly formed woven bone at the fracture end, and up to 3 mm distant from the fracture end. The fracture end was used as a distinct landmark and guideline to indicate the beginning of the area of new bone formation [24]. The areas of interest were the area of new bone formation, representing the newly formed bony callus within 0.6 mm distant from the fracture ends, and the consecutive measurement fields of 0.6 mm in length, up to 3 mm distant from the fracture ends. Nomenclature, symbols, and units used are as recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research [31]. The total number of RUNX2 immunopositive cells (N.RUNX2+) was expressed per area of soft connective tissue (0.01 mm^2). Number of RUNX2 immunopositive cells at the bone surface (presumably osteoblasts) was expressed per bone perimeter (0.1 mm). Number of RUNX2 positive osteocytes (N.Ot(RUNX2+)) was expressed as percentage of the total number of osteocytes (N.Ot(RUNX2+) / N.Ot × 100%).

The mouse recombinant monoclonal antibody Pebp2αA is a specific antibody to RUNX2. It has been well characterized as previously reported [32], and was also used on clinically derived human tissue [33]. Optimizing cellular staining quality and background reduction resulted in a staining procedure which involved antigen retrieval. It should be noted that our antibody detects all RUNX2 isotypes. Type II (til-1 isoform) expression has been found exclusively in osseous tissues, and type I (Pebp2αA isoform) expression has been detected in both osteoblast progenitor cells and in nonosseous murine tissues, including lung, liver, muscle, and skin [34]. Accordingly, it is conceivable that in our study the immunostaining observed in blood vessels and other nonosseous tissues represent the nonosseous isoform I [32]. Increase in staining intensity with osteogenic differentiation is due to staining with type II. Endothelial cells were identified based on their histological appearance, i.e. lining blood vessel lumina. The endothelial cells lining blood vessels were not incorporated in the quantitative analysis on RUNX2 positive cells. The osteoblastic nature of the cells identified as RUNX2 immunopositive cells at the bone surface was made based on morphological and histological criteria [35].

Statistical analysis

Statistical analysis of the data was performed using a Student's independent *t*-test (two-tail). The values of the histomorphometric parameters are expressed as mean ± SEM. A *p*-value of <0.05 is considered significant.

Results

Cells expressing RUNX2 were present in all histological sections from untreated and LIPUS-treated bone evaluated. In some biopsies, the soft connective and/or cartilaginous tissue within the fracture gap was lost, and only a small area of soft connective and/or cartilaginous tissue adjacent to the newly formed bony callus remained. In this soft connective and/or cartilaginous tissue adjacent to the fracture ends

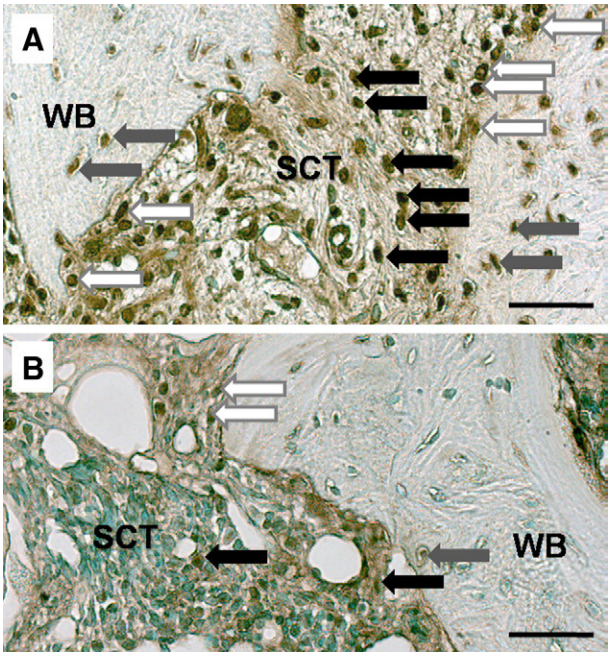

Fig. 1. Immunolocalization of cells expressing RUNX2 in newly formed bony callus at the fracture ends of fibula delayed unions in patients treated with or without LIPUS. (A) Control section. Many cells localized within the soft connective tissue (SCT) of newly formed bone show immunopositive staining for RUNX2 (brown nuclei/cytoplasm, black arrows). Strong expression of RUNX2 is found in cells at the woven bone surface (presumably osteoblasts, white arrows). Within the newly formed woven bone (WB), (young) osteocytes (grey arrows) also display immunopositive staining for RUNX2. (B) Section of a LIPUS-treated delayed union, showing less RUNX2 immunopositive cells within the soft connective tissue and newly formed woven bone in comparison with controls. Original magnification, ×200; scale bar, 50 μm.

only few RUNX2 immunopositive cells were observed, which were all located within the vicinity of the fracture ends (data not shown). At the fracture ends, endosteal callus formation was seen in all 7 biopsies (4 control, 3 LIPUS-treated). All controls showed endochondral ossification, whereas biopsies of LIPUS-treated delayed unions showed direct bone formation as well. Within the area of new bone formation, RUNX2 immunopositive cells were found in the undifferentiated soft connective tissue, at the bone surface, and within the newly formed woven bone (Figs. 1A, B).

Strong expression of RUNX2 was found in cells located at the woven bone surface (presumably osteoblasts) and in cells within the proximity of the osteoblastic layer (presumably pre-osteoblasts) (Fig. 2A). The presence of cuboidal-shaped osteoblasts lining seams of osteoid suggested active bone formation. Many of the young osteocytes embedded in osteoid or still located close to the bone surface also showed RUNX2 expression (Fig. 2A). Apart from RUNX2 immunopositive osteogenic cells, RUNX2 immunopositive staining was also detected in the endothelial lining of young blood vessels (Fig. 2A). Some of the osteocytes located in the newly formed woven bone had a round shape with abundant cytoplasm. The newly formed woven bone containing these large "hypertrophic" osteocytes, seems to be the subject of remodeling activities as it attracts numerous osteoclasts (Fig. 2B). Dendrite-like cytoplasmic projections were seen in young osteocytes located in the vicinity of the bone surface (Fig. 2C). This type of woven bone, containing large hypertrophic osteocytes, might be transchondric bone, which is an intermediate between bone and cartilage, as we also reported previously [24]. One of the controls was an atrophic delayed union, but this did not result in an arrest of proliferation and/or recruitment of osteogenic cells. The atrophic control showed active bone formation as well as remodeling activities (Fig. 2C). With increasing distance from the fracture end, the number of RUNX2 immunopositive cells as well as the intensity of staining decreased (Fig. 2D). Only few hypertrophic chondrocytes located within the vicinity of the fracture end stained positive for RUNX2 (Fig. 2E). Control sections in which the primary RUNX2 antibodies had been replaced by nonimmune IgG were negative (Fig. 2F).

In the area of new bone formation, the number of cells expressing RUNX2 within the soft connective tissue was 69% lower ($p<0.05$) as a result of LIPUS stimulation (Fig. 3A). The number of RUNX2 immunopositive cells at the bone surface (presumably osteoblasts, Fig. 3B), and the percentage of RUNX2 immunopositive osteocytes showed no significant difference between LIPUS-treated bone and untreated controls (Fig. 3C). In both treatment and control groups, there was a wide range with regard to percentage of RUNX2 expressing osteocytes (control, range 5–72%; LIPUS, range 1–27%). When referring to collected data of our previously reported histomorphometrical study [24], and correcting for delayed unions not evaluable in the present study, in the select specimens LIPUS significantly increased mineralized bone volume by 41% (control, $31.0 \pm 2.7\%$; LIPUS $43.8 \pm 2.7\%$; mean $\pm$ SEM; $p = 0.021$). The increased mineralized bone volume was accompanied by a 33% borderline significant increase of total bone volume ($p = 0.053$, Fig. 3D).

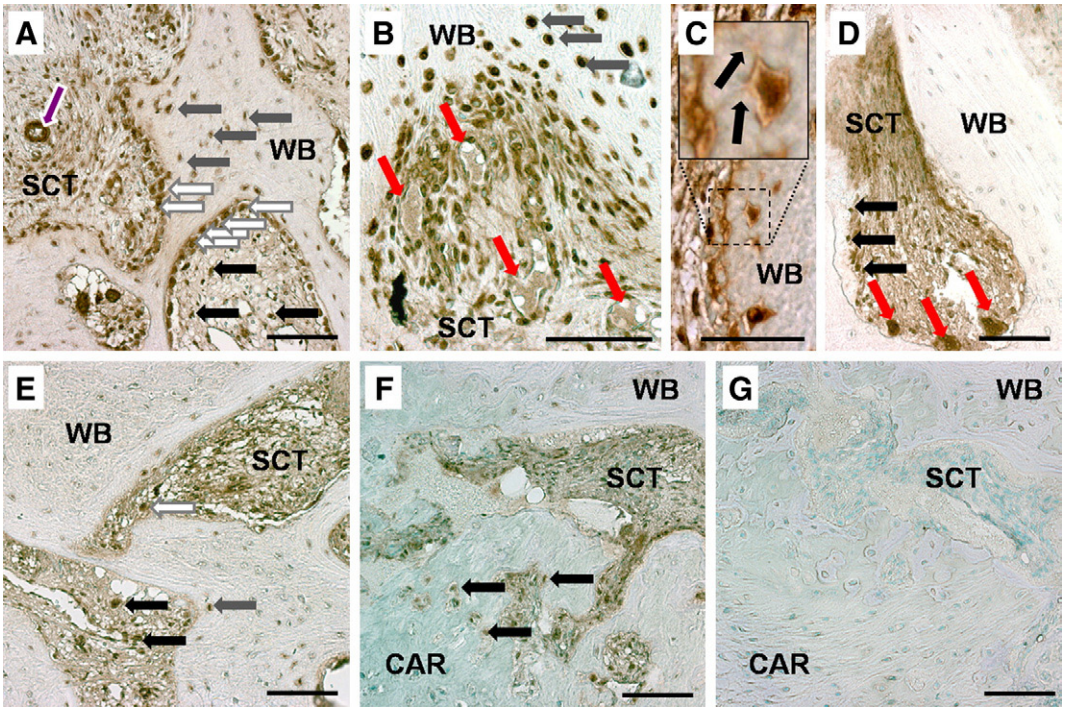

Fig. 2. Immunolocalization of cells expressing RUNX2 in newly formed bony callus at the fracture ends of fibula delayed unions. (A) Many of the cells localized within the soft connective tissue (SCT) show immunopositive staining for RUNX2 (brown nuclei/cytoplasm, black arrows). Strong expression of RUNX2 is found in cuboidal-shaped cells (presumably osteoblasts, white arrows) lining seams of osteoid, suggesting that active bone formation is present. Numerous young osteocytes embedded in osteoid or located in the woven bone (WB) still close to the bone surface, also show RUNX2 expression (grey arrows). Apart from staining osteogenic cells, RUNX2 immunopositive staining is also detected in the endothelial (purple arrow) lining of young blood vessels. (B) Some of the osteocytes located in the newly formed woven bone have a round shape with abundant cytoplasm (grey arrows). The newly formed woven bone containing these large "hypertrophic" osteocytes, seems to be the subject of remodeling activities as it attracts numerous of osteoclasts (red arrows). (C) Some of these large osteocytes with abundant cytoplasm showed dendrite-like cytoplasmic projections. Rectangle (dashed line), osteocyte; rectangle (solid line), high magnification osteocyte. Arrows indicate dendrite-like cytoplasmic projections towards the bone surface. (D) One of the controls was an atrophic delayed union, but this did not result in an arrest of proliferation and/or recruitment of osteogenic cells, as illustrated by the bone formation and/or remodeling activities present in the form of a cutting cone. (E) With increasing distance from the fracture end (4.2 mm), the number of RUNX2 immunopositive staining cells, as well as the intensity of staining decreases. (F) Within the cartilage matrix (CAR) adjacent newly formed bony callus, only few hypertrophic chondrocytes (black arrows) are immunopositive. (G) Negative control stained with a control IgG, note the absence of RUNX2 immunopositive staining. (A, B, D–G) Original magnification ×200; scale bar, 100 μm. (C) Original magnification ×400; scale bar, 50 μm.

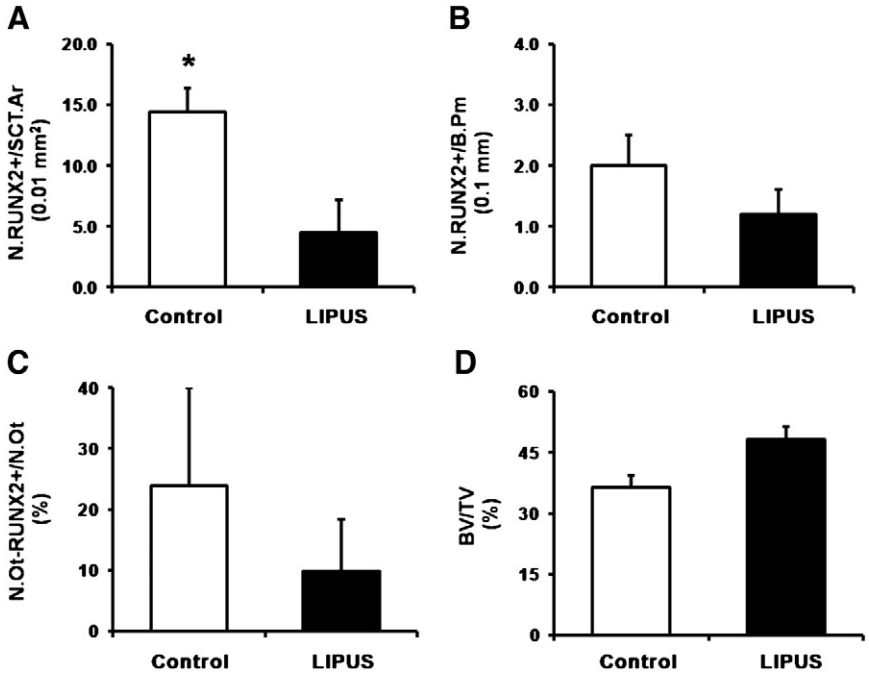

Fig. 3. Histomorphometric data of the localization of RUNX2 immunopositive cells within the area of new bone formation in delayed unions of the fibula treated with or without LIPUS. (A) Number of cells immunopositive for RUNX2 per area of soft connective tissue (N.RUNX2+/SCT.Ar). (B) Number of cells immunopositive for RUNX2 per bone perimeter (N.RUNX2+/B.Pm). (C) Number of RUNX2 immunopositive osteocytes as percentage of total number of osteocytes (N.Ot-RUNX2+/N.Ot×100). (D) Bone volume as percentage of total tissue volume (BV/TV×100), data refers to collected data of a previously performed histomorphometrical study [24]. Values are mean±SEM. Statistics: Student's independent *t*-test, two–tailed. *$p<0.05$.

Among the controls, no difference was seen in the number of RUNX2 immunopositive cells within the soft connective tissue of the atrophic delayed union when compared to the other hypertrophic delayed unions (atrophic, $12.5\pm5.7/0.01$ mm^2; hypertrophic, $15.0\pm2.7/0.01$ mm^2; mean±SEM; $p=0.74$). Furthermore the number of RUNX2 immunopositive cells at the bone surface was similar for the atrophic and hypertrophic delayed unions (atrophic, $1.7\pm0.1/0.1$ mm^2; hypertrophic, $2.0\pm0.7/0.1$ mm^2; mean±SEM; $p=0.52$). Among the LIPUS-treated delayed unions, the delayed union type did also not affect the number of RUNX2 immunopositive cells per soft connective tissue area (atrophic, $3.0\pm0.90.01$ mm^2; hypertrophic, $5.3\pm3.1/0.01$ mm^2; mean±SEM; $p=0.51$), and bone surface perimeter (atrophic, $1.3\pm0.9/0.1$ mm^2; hypertrophic, $1.1\pm0.7/0.4$ mm^2; mean±SEM; $p=0.86$).

In the LIPUS-treated as well as in the control group, the total number of RUNX2 immunopositive cells declined with regard to increasing distance from the fracture ends (Figs. 4A–B). Within 3 mm of the fracture ends the number of RUNX2 expressing cells within the soft connective tissue showed a decrease of 87% in biopsies from patients treated with LIPUS, whereas in the untreated controls only a decrease of 47% was seen (Fig. 4A). At 3 mm distant from the fracture end, the number of RUNX2 expressing cells within the soft connective tissue was 92% lower ($p<0.05$) in the LIPUS-treated delayed unions. Within 3 mm of the fracture end, the number of RUNX2 immunopositive cells at the bone surface showed a decrease of 75%, whereas in the controls a decrease of only 25% was seen (Fig. 4B). At 3 mm distant from the fracture end, the data suggest a trend toward decreased number of RUNX2 immunopositive cells at the bone surface for the LIPUS-treated patients (decrease of 80%, $p=0.11$) when compared to the controls. Only one LIPUS-treated delayed union showed some RUNX2 staining at 3 mm distant from the fracture end. No immunopositive staining was seen in osteocytes after 1.2 mm distant from the fracture end in LIPUS-treated delayed unions, whereas in the control group immunopositive staining of osteocytes remained up to 3 mm distant from the fracture ends.

Discussion

In this study immunolocalization of RUNX2 as an early bone cell marker was performed to determine the presence of osteogenic cells

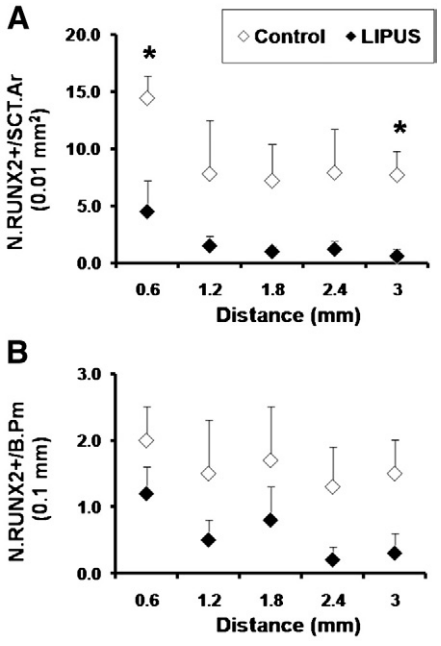

Fig. 4. Histomorphometric data of the localization of RUNX2 immunopositive cells up to 3 mm distance from the fracture ends, in delayed unions of the fibula treated with or without LIPUS. (A) Number of cells immunopositive for RUNX2 per area of soft connective tissue (N.RUNX2+/SCT.Ar). (B) Number of cells immunopositive for RUNX2 per bone perimeter (N.RUNX2+/B.Pm). Values are mean±SEM. Statistics: Student's independent *t*-test, two-tailed. *$p<0.05$.

in LIPUS-treated delayed unions of the osteotomized fibula and sham-treated controls in a double-blind clinical trial. The clinical model for delayed fracture healing, which we reported previously [24], allows us to report on bone healing and osteogenic potential present at the tissue level in fractures of over 8 months of age. Secondly this delayed fracture healing model offers us the unique possibility to investigate the effect of LIPUS, as a single treatment option, on clinical bone healing at the tissue level. Histomorphometrical analysis was performed to determine the number of cells expressing RUNX2 located within and around the newly formed woven bone at the fracture end (area of new bone formation), and up to 3 mm distant from the fracture end. Although clinical fracture healing was impaired in the delayed unions of the osteotomized fibula, cells expressing RUNX2 were present in all histological sections evaluated. Impaired clinical fracture healing therefore does not result in impairment of osteogenic cell proliferation and/or osteoblast differentiation at the tissue level. Two of the three LIPUS-treated patients, and two of the four sham-treated controls included in this study showed healing at 5 months of treatment. Biopsies were taken in an early treatment phase, while the clinical outcomes refer to a later date/phase. The present study cannot conclude that LIPUS is effective in augmenting the delayed healing response as compared to the sham-treated controls. Our findings show that LIPUS does not enhance fracture healing through increased osteogenic cell presence, but likely enhances osteoblast differentiation.

LIPUS treatment resulted in a significant lower number of cells expressing RUNX2 within the soft connective tissue (69%, $p<0.05$) of the newly formed woven bone at the fracture ends. LIPUS stimulation can induce an inflammatory and proliferative response by an increase in prostaglandin E_2 production in mouse MC3T3-E1 osteoblastic cells *in vitro* [21]. Our current findings however show that the previously reported increased bone formation in delayed unions of the human fibula by LIPUS treatment [24], is not associated with an increase in osteogenic cell population. Although osteogenic cell proliferation plays an important role in the fracture healing response [1,2], the enhancement of cell proliferation may however be limited to the early phase of fracture healing when cell proliferation is most active. The clinical study design did not allow us to take biopsies at different time points, i.e. not at the start of the treatment phase or during the treatment phase, nor at the end of the treatment phase. Differences in the number of RUNX2 immunopositive cells as a result of LIPUS treatment as found in the present study may also relate to a more advanced or later phase in fracture healing as result of LIPUS treatment. Prolonged stimulation with LIPUS (2–3 months) of cell cultures of a mouse osteoblastic cell line and human periosteal cells, does not result in a proliferative response [36,37]. LIPUS treatment of fetal mouse metatarsal rudiments *in vitro* has been shown to stimulate endochondral ossification, which resulted from a direct effect of LIPUS on osteoblasts and ossifying cartilage by stimulation of cell activity and/or differentiation, but not proliferation [22,23]. Our findings therefore suggest that in the impaired clinical fracture healing, the mechanism of action by which LIPUS enhances fracture healing does not involve a proliferative response.

The number of RUNX2 immunopositive cells at the bone surface (presumably osteoblasts) showed no significant difference between LIPUS-treated bone and untreated controls, whereas the number of cells expressing RUNX2 within the soft connective tissue at the fracture ends was significantly lower by 69% in the LIPUS-treated delayed unions. These findings suggest that LIPUS enhances osteogenic cell differentiation in impaired clinical fracture healing. LIPUS is capable to enhance osteogenic cell differentiation in animal and human cell cultures studies [36–38]. Furthermore as a result of enhanced osteogenic cell differentiation by LIPUS, a proliferative response to increase osteogenic cell number and subsequent osteoblast number at the bone surface as seen in the controls may therefore be absent in the LIPUS-treated delayed unions. Our findings therefore suggest that LIPUS enhances osteoblast differentiation but not proliferation in clinical fracture healing.

RUNX2 is regarded as the master gene of osteogenic cell differentiation and bone matrix production. Activation of RUNX2 increases osteocalcin and collagen I gene expression [39,40]. With increasing distance from the fracture end, the number of RUNX2 immunopositive cells, as well as the intensity of staining decreased in LIPUS-treated delayed unions and untreated controls. Decreased cellular staining intensity suggests a decrease of cellular RUNX2 expression. Proliferation and differentiation of osteogenic cells play an important role in the recruitment of new osteoblastic cells at and near the fracture end, as was also seen in our findings. With increasing distance from the fracture end, an osteogenic cell population is already present, and osteoid formation is restricted to remodeling activities. This explains our findings that with increasing distance from the fracture end, the number of RUNX2 immunopositive cells, as well as the intensity of staining decreased.

RUNX2 is thought to be the target of a mechanical signal by which physical stimulation dictates the cellular and metabolic activities of osteoblasts [27]. Chen et al. suggested that a specific pathway through RUNX2 expression is involved in the promotion of osteogenic transcription by LIPUS [15]. Cell culture studies show that LIPUS is capable of influencing cellular RUNX2 expression [15,28,36]. Stimulation of rat bone marrow stromal cells and human osteoblasts with LIPUS resulted in a time-dependant increase in cellular RUNX2 expression. Increased bone formation as found in our previously reported work, may suggest an increased cellular RUNX2 expression as a result of LIPUS stimulation, since it is essential for the production of bone specific proteins [24–26]. The outcome of a decreased number of RUNX2 positive cells in combination with an increase in bone volume and mineralized volume as a result of LIPUS treatment may appear contradicting. RUNX2 immunolocalization however can only identify cells expressing RUNX2, but cannot quantify cellular RUNX2 expression. Although LIPUS does not increase the number of cells expressing RUNX2, we cannot exclude that these cells indeed have increased RUNX2 expression, which may result in increased bone formation.

No RUNX2 immunopositive staining was seen in osteocytes beyond 1.2 mm distant from the fracture end in LIPUS-treated delayed unions, whereas in the control group immunopositive osteocytes remained up to 3 mm distant from the fracture ends. At 3 mm of the fracture ends, a significantly higher number of RUNX2 positive cells was seen in the soft connective tissue of controls when compared to the LIPUS-treated delayed unions. Our findings suggest a relationship with regard to RUNX2 immunopositive osteocytes and osteogenic cell proliferatation. RUNX2 is a transcription factor for osteoblast differentiation and plays a role in the maintenance of their differentiated state [25,26]. In the control group relatively young osteocytes are embedded, but still no terminal differentiation has occurred as shown by the RUNX2 expression, which may be related to an active signaling process which stimulates osteogenic cell proliferation. Conditioned medium from the osteocytic MLO-Y4-cell line added to mouse bone marrow cultures, in which mesenchymal stem cells were induced to osteoblasts, showed increased mesenchymal stem cell proliferation [41]. Another study showed that mechanical stimulation of osteocytes resulted in an opposite reaction, i.e. osteocytes subjected to pulsating fluid flow inhibit proliferation but stimulate differentiation of osteoblasts *in vitro* via soluble factors [42]. LIPUS may therefore have a direct effect on osteogenic cells by stimulating osteoblastic differentiation but may also indirectly affect osteogenic cell proliferation and differentiation through affecting osteocytes. Osteocytes are believed to perceive the stress put on the bone tissue leading to deformation (strain) and to transfer signals through their canalicular network to the effector cells at the bone surface, the bone forming osteoblasts and bone degrading osteoclasts [43]. Our findings therefore may illustrate an effect of LIPUS on

osteocytes, resulting in terminal differentiation of osteocytes and inhibiting a proliferative response.

The etiology of atrophic nonunion is not well understood; they are often thought to be nonreactive and metabolically inactive. Among the controls, no difference was seen in number of cells expressing RUNX2 within the soft connective tissue of the atrophic delayed union when compared to the other hypertrophic delayed unions. Our findings show that impaired fracture healing does not lead to a decline in number of osteogenic cells, even if the fracture is radiographical regarded as atrophic. Furthermore an increase in osteogenic cell population does not necessary relate to increased bone formation, as may be concluded when comparing data from the present study with our previous report on the stimulating effect of LIPUS on bone volume, osteoid thickness and mineral apposition rate [24]. Inducing fracture healing in atrophic nonunions by osteoinductive therapies [1], should be carefully considered since the number of osteogenic precursor cells in delayed union does not seem to correlate with increased bone formation and number of osteoblasts.

In conclusion, immunolocalization of RUNX2 positive cells in delayed unions of the fibula reveals that delayed clinical fracture healing does not result in impairment of osteogenic cell proliferation and/or osteoblast differentiation at the tissue level, even if delayed unions are clinically regarded as atrophic. Reduced number of osteogenic RUNX2 immunopositive cells within the soft connective tissue, and unchanged number of RUNX2 immunopositive cells at the bone surface, implicate that low-intensity pulsed ultrasound does not increase osteogenic cell presence, but likely affects osteogenic cell differentiation.

Acknowledgments

The authors would like to thank Dr. A.L.J.J. Bronckers (Dept. Oral Cell Biology, ACTA, University of Amsterdam and VU University Amsterdam, the Netherlands) for his help and expertise with RUNX2 immunostaining. Furthermore the authors wish to thank J.J. Schrik, D.A. Vergroesen (Spaarne Ziekenhuis, Hoofddorp, the Netherlands), G.H.R. Albers, A.J.W. Marsman, R.A.W. Verhagen, and H.M. van der Vis (Tergooiziekenhuizen, Hilversum, the Netherlands) for performing the surgical procedure enabling the biopsy procedure. The work of S. Rutten is performed under auspices of the Skeletal Tissue Engineering Group Amsterdam (STEGA), which has received financial support from Smith & Nephew (Memphis, TN, USA). S. Rutten received salary from the Foundation Orthopaedic Research Spaarne Hospital (Hoofddorp, the Netherlands), and the STEGA Foundation (Amsterdam, the Netherlands).

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