Cysteine-rich matricellular protein improves callus regenerate in a rabbit trauma model

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Abstract

Purpose Open fractures with severe soft-tissue trauma are predisposed to poor bone healing. The vital coupling between osteo- and angiogenesis is disturbed. Cysteine-rich protein 61 (CYR61) is an angiogenic inducer promoting vascularisation. However, little is known about the effect of CYR61 on the callus regenerate after acute musculoskeletal trauma. Therefore, our aim was to determine whether local administration of CYR61: (1) has an influence on callus formation and remodelling, (2) increases bone volume and (3) partially restores callus stability.

Methods A musculoskeletal trauma was created in 20 rabbits. To simulate fracture-site debridement, the limb was shortened. In the test group, a CYR61-coated collagen matrix was locally applied around the osteotomy. After ten days, gradual distraction was commenced (0.5 mm/12 h) to restore the original length. New bone formation was evaluated histomorphometrically, radiographically and biomechanically.

Results Osseous consolidation occurred in all animals. Average maximum callus diameter was higher in the test group [1.39 mm; standard deviation (SD)=0.078 vs 1.26 mm

(SD=0.14); $p=0.096$]. In addition, bone volume was higher ($p=0.11$) in the test group, with a mean value of 49.73 % (SD=13.68) compared with 37.6 % (SD=5.91). Torsional strength was significantly higher ($p=0.005$) in the test group [105.43 % (SD=31.68 %) vs. 52.57 % (SD=24.39)]. Instead, stiffness of the newly reconstructed callus decreased (64.21 % (SD=11.52) vs. 71.30 % (SD=32.25) ($p=0.81$)). **Conclusions** CYR61 positively influences callus regenerate after acute trauma, not only histologically and radiographically but also biomechanically, most probably by a CYR61-associated pathway.

Introduction

Trauma and fracture repair occur as a complex process that involves inflammation, angiogenesis, chondrogenesis, enchondral ossification and bone remodelling. This process is disturbed in high-energy injuries with open fractures of the lower limb associated with soft-tissue damage, bone defects and impaired local perfusion. Initial treatment of this fracture type includes radical soft-tissue and bone debridement, acute shortening to close bone defects and stabilisation with an external fixator [1]. Elevated compartment pressure impairs bone and soft-tissue perfusion, which improves when the limb is shortened about 10 % [2]. One treatment option for restoring the original length is a distraction procedure, normally using a second osteotomy near the metaphyseal region. Alternatively, distraction osteogenesis at the fracture site can gain bone regeneration in a shorter period of time without the necessity for further operations [1, 3–5].

Microvascularisation and microcirculation are crucial for fracture repair and bone regeneration [6]. Soft-tissue trauma and elevated compartment pressure are two of the main reasons for regional hypoxia. Also, initial fracture haematoma,

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eliminated by surgery, consists of a variety of physiological growth factors important for angio- and vasculogenesis and fracture repair [7, 8], which are then missing at the fracture site. Previously, we demonstrated that acute soft-tissue trauma significantly compromises callus formation in a rabbit tibia after primary acute limb shortening and secondary distraction [9]. In animals with soft-tissue trauma and elevated compartment pressure, normalised mechanical values of the newly reconstructed tibia and average normalised callus diameters were smaller than in animals without soft-tissue trauma [9].

The cysteine-rich protein 61 (CYR61, CCN1) is an extracellular-matrix-associated angiogenic regulator [10] supporting cell adhesion, stimulating endothelial cell migration and enhancing growth-factor-induced cell proliferation in culture [11]. Direct proliferative action of CYR61 was described on mesenchymal stem cells, osteoblasts and endothelial cells [12], with evidence of angiogenic activity, including in rabbit ischemic hindlimbs [13]. As a proangiogenic regulator, it is transcriptionally induced under hypoxia, a condition that favours blood-vessel growth by inducing several angiogenic factors, including vascular endothelial growth factor (VEGF), through the action of hypoxia inducible factor-1 α (HIF-1 α). Furthermore, CCN proteins can also modulate activities of several growth factors and cytokines, including transforming growth factor beta (TGF- β), tumour necrosis factor alpha (TNF- α), VEGF, bone morphologic proteins (BMPs) and Wnt proteins, and it may thereby regulate a broad array of biological processes [14].

The purpose of this study was to investigate the influence of locally applied CYR61 on musculoskeletal trauma. We investigated the effect of CYR61 on osteogenesis after acute soft-tissue trauma and following a limb-shortening distraction procedure in a rabbit tibia. We postulated that local administration of CYR61 compensates callus deficiency after acute soft-tissue trauma. Therefore, we aimed to determine whether local administration of CYR61: (1) has an influence on callus formation and remodelling, (2) increases the bone volume and (3) partially restores callus stability.

Methods

Principles of laboratory animal care were followed and the study protocol was approved by the Institutional Animal Care and Use Committee and the Government Animal Control.

Injury and limb shortening

Twenty skeletally mature New Zealand white rabbits with an average weight of 3.8 kg were placed under general anesthesia with ketamine–xylazine and Isoflurane. The animals underwent a standardised musculoskeletal trauma

simulating a muscle contusion injury of one lower leg, with the untreated limb serving as a paired control [9]. A unilateral external fixator–distractor (Orthofix® M-103; Orthofix SRL, Verona, Italy) was applied to the anteromedial aspect of the injured tibia, a ten millimetre diaphyseal bone block including periosteum was resected and the limb was shortened until compression-free contact of the proximal and distal bone fragments was attained.

CYR61 Application and distraction procedure

Twenty evaluable rabbits were split into two equal groups. In the test group, a 1-mm $\times$ 0.5-mm $\times$ 0.5-mm collagen matrix (Resorba) coated with 25 μ g CYR61 was applied locally around the osteotomy site. In the control group, no CYR61 and no collagen matrix was applied. In both groups, bone was covered completely with local soft tissue, and a tension-free skin closure followed. After surgery, radiographs were taken in two planes. After ten days of compression-free contact between proximal and distal fragments, a gradual distraction of 0.5 mm/12 hours for ten days was performed using an external fixation device (Orthofix® M-103; Orthofix SRL, Verona, Italy) until the original length was restored (ten days).

Soft-tissue trauma, compartment pressure and blood pressure monitoring

Compartment pressure was gauged using a piezoelectric transducer (KODIAG; Braun-Dexon GmbH, Spangenberg, Germany). Bilateral pressure measurements confirmed the onset of a critically elevated compartment pressure (>30 mmHg) on the traumatised side in the test group. Arterial blood pressure was documented continuously after cannulating the medial auricular artery (PowerLab 4/35, Scope v.4.1.4 for Mac, ADInstruments GmbH, Spechbach, Germany).

Radiological analysis

Radiographs were taken after a ten day period of compression-free contact and weekly during the distraction and follow-up periods. At day 40, maximum callus diameters were measured in digital radiographs, and values were averaged in two planes and normalised to the proximal and distal ends of the diaphysis (callus–diaphysis index). Additionally, a high-resolution computed tomograph analysis (FHG HR-CT 150/3, Fraunhofer Institute, Bayreuth, Germany) was performed, and the animals were euthanised. The radiological analyses were performed under anesthesia with ketamine and xylazine.

Biomechanical testing

Both injured and uninjured tibias were dissected from soft tissues and deep frozen at -18°C until testing. Bone

specimens were embedded distally and proximally in a metal alloy (Woods Metal MCP70; HEK-Medizintechnik GmbH, Lübeck, Germany), exposing 32 mm of diaphysis, including the new bone formation, for torsion testing. Testing was performed on a motor-driven hydraulic universal material testing machine (Zwick/Roell Z020, Zwick GmbH & Co.KG, Ulm, Germany) by applying load to failure in external rotation of 108°/min. Ultimate strength was defined as the maximum applied torque. Values were normalised to the contralateral untreated side; results are presented as means $\pm$ standard deviation (SD).

Histomorphometric analysis

After mechanical testing, a 30-mm-long specimen from each tested tibia containing the new bone formation was cut longitudinally in the sagittal plane with a rotating diamond saw (EXAKT; Fa. PSI-Grünwald GmbH & Co.KG, Laudenbach, Germany). One side was decalcified for histomorphometry, and the other side was stored for further radiographic analysis of the undecalcified tissue. Specimens for decalcification were fixed in 4 % formalin, decalcified with ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin (Reichert-Jung; Leica Microsystems Nussloch GmbH, Wetzlar, Germany). For histology and histomorphometry, 7- μ m sections were stained with haematoxylin and eosin (H&E) and analysed with a standard light microscope (Olympus BX61, Japan) connected to a digital camera (Olympus) using its specific software program. Each callus section was photographed at 4 $\times$ magnification to obtain an individual overview. Digital pictures containing 50–70 fields with a 40 $\times$ magnification were stored. The final area calculation was determined by Image Pro Plus, Version 7.0 (Media Cybernetics, Bethesda, MD, USA).

Statistical analyses

Power analysis with $\alpha = 0.05$, and a power of 80 % revealed for torsional strength (with the expected values mean test = 100 %, mean control = 50 %, SD=30 %), revealed a minimum sample size of seven animals. Descriptive analysis, boxplots and normal probability plots were performed for callus diaphysis index, bone volume, torsional strength and stiffness for both groups (control and test). The probability plots showed approximately normal distribution with few outliers. Due to these outliers and to low sample size, non-parametric methods for inferential statistics were done. Wilcoxon test for independent samples was used to investigate the difference in torsional strength and callus diaphysis index between test and control groups. Scatterplot and Spearman correlation were performed for analysing the relationship between torsional strength and callus diaphysis index for the complete data and for the two groups separately. For statistical

analysis, we used SPSS 19.0 (SPSS Inc, Chicago, IL, USA) and R 2.13.1 [R Development Core Team (2011); R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria).

Results

No animals had soft-tissue problems or infections. Two animals suffered of pin loosening and had to be sacrificed. One animal died due to perioperative anaesthetic problems. The overall dropout rate was 15 %. In all other animals, osseous consolidation occurred, with correct axes and length in both planes. Compartment-pressure measurement at the tibialis anterior muscle showed a pretrauma pressure of 5.7 ± 1.4 mmHg, an increase to 33.8 ± 4.0 mmHg post-trauma, and a decrease postsurgery down to physiological levels of 12.4 ± 2.1 mmHg. After eight, 24 and 48 hours, compartment pressure was constant at physiological levels. Diastolic blood pressure was measured at 80.2 ± 13.9 mmHg before trauma, at 68.9 ± 15.4 mmHg posttrauma and during surgery and at 68.4 ± 14.2 mmHg postsurgery. Postsurgery, there was no critical compartment pressure, thus no fasciotomy was necessary.

Osseous consolidation was evident in both planes in all animals. Furthermore, straight callus formation and mineralisation in the lengthening gap were observed in both groups. In the test group, average maximum callus diameter was higher [1.39 mm (SD=0.078, $n=7$) ($p=0.096$)] than in the control group [1.26 mm (SD=0.14, $n=10$)] (Table 1, Fig. 1a). Furthermore, bone volume was higher ($p=0.11$) in the test group, with a mean value of 49.73 % (SD=13.68, $n=7$) compared with 37.6 % (SD=5.91, $n=10$) in the control group (Figs. 1b and 2). There was a significant difference ($p=0.005$) between groups in torsional strength, with a mean value of 105.43 % (SD=31.7 %, $n=7$) in the test group and 52.6 % (SD=24.39, $n=10$) ($p<0.005$) in the control group (Fig. 3a). Difference in torsional strength is defined as the strength ratio of the newly reconstructed limb after trauma to the contralateral side. There was a difference in stiffness of the newly reconstructed callus between groups, which was lower in the test group, with a mean value of 64.2 % (SD=11.52, $n=7$) compared with 71.3 % (SD=32.25, $n=10$) ($p=0.81$) in the control group (Fig. 3b). Scatterplot and Spearman correlation showed no correlation (Spearman-Rho 0.68, $p=0.11$) between the callus diaphysis index and the torsional strength.

Discussion

High-energy injuries causing open lower-limb fractures associated with soft-tissue damage, bone defects and impaired

Table 1 Callus–diaphysis indices and torsional strength in the test versus control group

Specimen	Torsional strength (nm)		
	Treated	Contralateral	Percent ^a
Test group			
1	1.75	2.33	75.27
2	2.33	3.30	70.45
3	2.83	3.70	76.35
4	1.29	2.47	52.02
5	2.33	2.47	94.32
6	3.55	2.89	81.27
7	1.91	2.09	91.39
Mean	2.28	2.75	77.30
SD	0.74	0.57	14.11
Control group			
1	0.65	2.90	22.57
2	0.72	2.71	26.70
3	2.40	2.40	100.00
4	0.86	2.70	31.94
5	1.35	4.04	33.34
6	1.62	2.99	54.10
7	1.67	2.87	58.21
8	2.01	3.24	61.95
9	1.67	2.63	63.46
10	1.72	2.34	73.59
Mean	1.47	2.88	52.59
SD	0.57	0.49	24.26

SD standard deviation

^a Segment strength in relation to the nonoperated side (100 %; normalised values)

local perfusion disturb the natural bone healing process. Primary treatment includes radical soft-tissue and bone debridement, acute shortening to close bone defects and

stabilisation with an external fixator [2]. This process is a complex mechanistic cascade composed of the interplay of inflammation, angiogenesis, endochondral ossification and bone remodelling. Derived from the clinical situation, we simulated a musculoskeletal injury, which leads to reduced bone quality, as previously shown [9]. Based on this trauma, the natural coupling between angio- and osteogenesis, which is a basic prerequisite for fracture healing, is diminished. Hypoxia and necrosis of the soft tissues are risk factors for poor fracture healing. Therefore, we assessed whether local administration of proangiogenic growth factor CYR61: (1) influences callus formation and remodelling, (2) increases bone volume and (3) partially restores callus stability.

There was a difference in callus bridging and competence between groups. In particular, there was a higher callus diaphysis index and at the same time a higher bone volume in the test group, which was treated with CYR61. As an angiogenic inducer, CYR61 promotes vascularisation [15]. According to the literature, increased bone volume—as seen in our experiment—seems to correlate with blood-vessel volume, which is vital for bone healing [16]. Endothelial proliferation and neovascularisation are enhanced by stimulation of integrin-dependent recruitment of CD34⁺ progenitor cells to endothelial cells [17]. In particular, proangiogenic activities of CYR61 are mediated through integrins $\alpha v \beta 3$ and $\alpha 6 \beta 1$ in endothelial cells [18, 19]. Recently, angiogenic activity of CYR61 was demonstrated in a rabbit ischemic hindlimb assay [13], which underlines our findings that CYR61 plays a vital role in ischemic tissues, rearranging angiogenic processes and therefore increasing bone volume. Moreover, CCNs regulate expression and activity of other angiogenic factors, such as VEGF-A and C [20–23]. Apart from hypoxia, CYR61 expression in osteoblasts is enhanced by many chemical mediators, including growth factors such as VEGF and TGF- β , which

Fig. 1 In the test group, average maximum callus diameter (a) and bone volume ($p=0.11$) (b) were higher in the test group

Fig. 2 Radiological (sagittal and coronal plane; high-resolution computed tomography) and histomorphometrical aspects of callus formation in the control (a–c) and test (d–f) at day 40 showing new bone

formation with higher bone volume and at the same time a higher callus diaphysis index at the distraction area in the test (CYR61) group (hematoxylin and eosin stain, magnification $\times 40$, c, f)

are present in the callus and are known to directly enhance bone formation [14]. Moreover, the experimentally induced soft-tissue trauma in our experiment and with it the collapse of vascular volumes can lead to local hypoxia [24], resulting

in a callus deficiency [9], which explains the smaller callus diaphysis index in the control group but a higher one after local CYR61 treatment. In turn, hypoxia stabilises and activates the transcription factor HIF-1 α , one of the key

Fig. 3 Significantly higher torsional strength in the test group (a). Less stiffness of newly reconstructed callus in the test group (b)

regulators of CYR61 next to TGF- β 3 [25]. The HIF and CYR61 pathway is likely to be critically important for the interplay of angio- and osteogenesis and thus for fracture healing and therefore for bone volume. In general, CCN protein expression is associated with injury repair and inflammation [14], which emphasises the natural availability of CYR61 in regenerative processes. Beyond that, local administration seems to enhance the endogenous physiological regeneration by slightly increasing callus diaphysis and bone volume, as seen in our experiment.

Biomechanical analysis showed that torsional strength of the newly reconstructed callus was significantly higher in the CYR61 group. At the same time, stiffness was lower. Preservation of a higher density of blood vessels in the consolidation period can lead to better mineralisation of the distracted callus [16], which might increase the stability of the newly reconstructed bone, as in our experiment. Lately, a potential loop consisting of VEGF-mediated up-regulation of CCN1 in osteoblasts that attracts endothelial cells and promotes angiogenesis has been discussed [26]. Aside from that, using an axial-shortening model of the rabbit tibial callus, HIF-1 α and VEGF levels increased and were associated with decreases in vascularity around the front of enhanced membranous bone formation [24]. Moreover, it has been postulated that CYR61 contributes to bone healing through its angiogenic potential [27]. It stimulates osteoblast differentiation but inhibits osteoclastogenesis, suggesting a role as a bifunctional regulator that promotes osteogenesis [28] that, on the other hand, might lead to enhanced bone formation. Increased callus resistance is often associated with enhanced bone and tissue volume [29], which confirms our results in respect of torsional strength. Aside from that, findings in the literature also show that a rigid fracture fixation can lead to a stiffer and smaller callus but with better mineralised tissue in the entire callus, compared with a flexible or dynamised fixation [30]. Respectively, we used the same standardised fixation techniques assuming that the significant increase in stability and enlargement of bone volume might result from a faster mineralised callus reconstruct after CYR61 treatment. Coinciding with our findings, another study in a murine fracture model showed that mice receiving sildenafil, which also influences angiogenic growth factor expression and bone formation during fracture healing, had a significantly increased osseous fracture bridging [31]. In contrast, they found an increased biomechanical stiffness and a smaller callus area with a slightly reduced amount of cartilaginous tissue. Herein, Western blot analysis showed a significantly higher expression of proangiogenic and osteogenic CYR61 [31]. Corresponding to our results, this might indicate an accelerated healing process underlining the benefit of angiogenic growth factors such as CYR61. In accordance with our results and findings in the literature, callus quality with

regard to stability depends on bone volume and torsional strength, among other factors, which was increased in our experiment. Conversely, the size of the newly formed callus must not be associated with stability. Local administration of CYR61 seems to increase callus stability while increasing bone volume and callus diaphysis index as an osteogenic regulator.

We recognise limitations to our study. First, experimental studies like ours generally must be cautiously transferred to human clinical practice. There are no clinical data on the use of CYR61 in human musculoskeletal trauma. Second, the high demands of production and the costs of CYR61 might be a limiting factor. Third, we showed a positive effect of CYR61 in rabbit limbs, but it must be emphasised that these tissues are not identical to those in human limbs. The differences by nature in anatomy and physiology in the rabbit bone can mean that the dynamics related to the injury may vary from those in humans. Translation of clinical parameters between models have to be carefully examined, which does not negate the potential clinical importance of our results. Forth, it cannot be excluded that some effects observed in this study were induced by the collagen matrix. Nevertheless, these effects might be negligible, as the matrix is supposed to be inert, biocompatible and biodegradable.

In comparison with other growth factors, such as BMPs, which are well known and in clinical use, not much is known about CYR61. There are no comparable studies pointing out the applicability or feasibility of CYR61 application after musculoskeletal trauma. To elicit the potential of CYR61 and its use, more studies are needed. Our results suggest that CYR61 might promote fracture healing after acute soft-tissue trauma by increasing newly reconstructed bone stability. Thus, CYR61 used locally may prospectively be an effective therapeutic weapon for orthopaedic trauma surgeons dealing with large bone defects and extensive vasculature or soft-tissue damage predisposed to nonunions. We show for the first time that CYR61 positively influences callus regenerate after acute soft-tissue trauma, not only histologically but biomechanically, most probably by a CYR61-associated pathway.

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