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Mechanical Stability Affects Angiogenesis during Early Fracture Healing

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Abstract

Objectives—The goal of this study was to determine to what extent mechanical stability affects vascular repair during fracture healing.

Methods—Stabilized and non-stabilized tibia fractures were created in adult mice. Fracture tissues were collected at multiple time points during early fracture healing. Vasculature in fractured limbs was visualized by immunohistochemistry with an anti-PECAM-1 antibody on tissue sections and then quantified with stereology. Oxygen tension, vascular endothelial growth factor (VEGF) expression, and lactate accumulation at the fracture site were measured. Gene expression was compared between stabilized and non-stabilized fractures by micro-array analysis.

Results—We found that new blood vessel formation was robust by 3 days after fracture. Quantitative analysis showed that non-stabilized fractures had higher length density and surface density than stabilized fractures at 3 days after injury, suggesting that non-stabilized fractures were more vascularized. Oximetry analysis did not detect significant difference in oxygen tension at the fracture site between stabilized and non-stabilized fractures during the first 3 days after injury. Further micro-array analysis was performed to determine the effects of mechanical stability on the expression of angiogenic factors. No significant difference in the expression of VEGFs and other angiogenic factors was detected between stabilized and non-stabilized fractures.

Conclusions—Mechanical instability promotes angiogenesis during early fracture healing and further research is required to determine the underlying mechanisms.

Keywords

mechanical stability; fracture; angiogenesis; microarray; VEGF

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Conflicts of Interest For the remaining authors none were declared.

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Introduction

Mechanical stability plays an important role in fracture healing. Clinically, both inadequate fixation and immobilization that is too rigid may impair bone repair. Mechanical stability also regulates the mode of repair. In a mouse model, non-stabilized fractures heal through endochondral ossification, during which a cartilage template forms and is subsequently replaced by bone. In contrast, rigidly stabilized fractures heal through intramembranous ossification where bone forms directly without a cartilage intermediate [1]. Mechanical stimuli are involved in stem cell differentiation during fracture healing. Mechanical instability induces chondrocyte differentiation and mechanical stability leads to osteoblast differentiation. *In vitro*, cyclic, hydrostatic or static compression enhances formation of the cartilaginous matrix produced by bone marrow-derived mesenchymal cells [2]. Additionally, angiogenesis is required for normal bone formation and fracture healing and is regulated by mechanical signals. For example, shear stress was found to promote the formation and expansion of microvessel networks in collagen gel [3].

Stem cell differentiation and angiogenesis occur concurrently during early fracture healing. In our murine fracture model, early chondrocyte and osteoblast differentiation at the fracture site can be detected by 4 days after injury [4], and we have observed an increase in vascularity at day 3. However, the relationship between stem cell differentiation and angiogenesis has not been well determined. In the current study, we compared early tissue vascularization, tissue oxygenation, and expression of angiogenesis-related genes in stabilized and non-stabilized tibia fractures in a mouse model.

Materials and Methods

Creation of tibia fractures

All procedures in this work have been approved by the Institutional Animal Care and Use Committee (IACUC) at Dartmouth Medical School, Hanover, NH and at the University of California at San Francisco, San Francisco, CA. Non-stabilized fractures were created in the midshaft of tibiae of adult male 129J/B6 mice by three-point bending and were not stabilized. Stabilized fractures were created by applying an external fixator after induction of tibia fracture and manual examination confirmed that the fractures were rigidly stabilized [1]. All animals were allowed to ambulate freely after recovery.

Measuring oxygen tension in fracture callus

Stabilized (n=4) and non-stabilized (n=5) tibia fractures were created in adult male mice. Immediately after fracture, oxygen-sensitive lithium phthalocyanine (LiPc) crystals were implanted using a 25 gauge needle into the facture site. Oxygen levels at the fracture site were analyzed by Electron Paramagnetic Resonance (EPR) on the day of fracture, at days 1 and 3 after injury. This work was done at the EPR Center, Dartmouth Medical School (Hanover, NH). The mice were anesthetized with isoflurane (1.2–1.5%) in 21% oxygen and placed in the magnet. EPR oximetry was performed using a low frequency L-band (1.2 GHz) spectrometer that was developed specifically for in vivo studies [5, 6]. Spectra were detected using a surface loop resonator, which was positioned over the region of interest (fracture area). Body temperature was monitored by a rectal probe and maintained within the normal range (37-38 °C) using a heating pad. One-way ANOVA and LSD Post Hoc test were used to determine if there is significant difference in pO_2 between stabilized and non-stabilized tibia fractures at each time point. Significance level is p=0.05.

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Assessment of tissue vascularization

A second set of animals with stabilized (n=4) or non-stabilized (n=5) fractures were euthanized at 3 days after injury, and fractured tibiae with surrounding soft tissue were collected, fixed, decalcified, and prepared for frozen sectioning. Un-fractured normal tibiae (n=5) were collected as control. Vertical uniform random (VUR) sections (10μm) were prepared through each whole tissue block. Ten-15 VUR sections were analyzed using systemic random selection from each sample. Endothelium was labeled by immunohistochemistry with an anti-PECAM antibody [7]. Quantification of tissue vascularization using stereology was performed as described [8]. The length and the area of the outer surface of blood vessels within calluses were estimated by analyzing the length density (the length of blood vessels per unit volume of the reference space, Lv) and surface density (the area of the outer surface of blood vessels per unit volume of the reference space, Sv). One way ANOVA and LSD Post Hoc test were performed to determine the effect of stability on tissue vascularization at each time point. Significance level is p=0.05.

Measuring tissue lactate and VEGF levels

To quantify tissue lactate and VEGF levels, a third set of fracture samples were collected at 3 and 10 days after injury (n=5-6/group/time). Normal un-fractured legs (n=6) served as controls. Tissues were snap-frozen on dry ice, weighed, and homogenized in 6 volumes of distilled water plus 1/100 volume of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) [9]. Tissue homogenates were centrifuged at 10,000 for 5 minutes. Supernatant was collected and kept at -20°C. Levels of lactate were measured using an YSI 1500 Sport Lactate Analyzer (YSI Life Sciences, Yellow Springs, Ohio). VEGF levels were determined using a Quantikine Mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN), following manufacturer's instruction. Levels of lactate and VEGF were normalized with the weight of the tissue. One way ANOVA and LSD Post Hoc test were performed to determine the effect of stability on tissue lactate or VEGF levels at each time point. Significance level is p=0.05.

Microarray analysis

To determine the effect of mechanical stability on the expression of genes that are related to angiogenesis, cDNA micro-array was performed on fracture tissues collected from a new set of un-fractured legs and non-stabilized or stabilized fractures at 2 and 7 days after injury (n=4/group/time). The rationale to choose these two time points for micro-array analysis and later time points (days 3 and 10) for histology and protein analyses was that changes in gene expression occurs earlier than proteins and histology. Total RNA was prepared from the entire lower leg from the knee joint to the ankle after removing the skin. This reduces confounding results due to the different modes of healing that occur in each mechanical environment and allows us to examine gene expression in the entire limb in response to the different mechanical environments. For gene expression analysis, Agilent whole mouse genome 4x44K Ink-jet array was used. Arrays were scanned using the Agilent microarray scanner and raw signal intensities were extracted with Feature Extraction v9.1 software (Agilent). This data set was normalized using quantile normalization to make the distribution of probe intensities to be the same across all arrays [10]. Differentially expressed genes are identified by fitting a 2X2 ANOVA model to the array data within each day using the *limma* package in Bioconductor. Genes that have 2-fold up- or down-regulation and B value > 0 were selected for functional annotation clustering using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/). In addition, the differential expression of more than 30 important angiogenesis-related genes was examined individually.

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Results

Mechanical environment does not affect tissue pO_2 at the fracture site

Our first goal was to assess the effect that mechanical stability had on tissue oxygen levels during early stages of facture healing. Our previous work has shown that the oxygen tension in uninjured tibia is at about 45.5 ± 15.3mmHg[11]. In the current study, tissue oxygen levels dropped to hypoxic levels immediately after fracture. By one day after injury oxygen levels increased in both stabilized and non-stabilized fractures. At three days after injury the oxygen level appeared the same as at day 1. We did not observe a statistically significant difference in oxygen levels between stabilized and non-stabilized fractures at any time point examined (Table 1).

Non-stabilized fractures are more vascularized

Our second goal was to assess the extent to which the mechanical environment influenced vascularity at the fracture site during the early stages of repair. At 3 days after injury, visual inspection of PECAM staining suggested that there were more blood vessels at the fracture site in non-stabilized fractures compared to stabilized fractures (Fig. 1A,B). This observation was confirmed quantitatively by stereology. Compared to stabilized fractures, non-stabilized fractures had significantly higher length density, and surface density (Fig. 1C,D), suggesting that mechanical instability could lead to a more robust angiogenic response after fracture. In addition, stereology analysis also demonstrated that fracture induces a robust angiogenic response. Compared to normal tibiae, stabilized fractures exhibited a significant higher surface density of vasculature (Fig. 1D). Non-stabilized fractures also showed significantly higher length density and surface density compared to normal tibiae (Fig. 1C, D).

Effect of mechanical environment on tissue VEGF and lactate levels

Next, we wanted to assess whether VEGF protein or lactate were significantly different in non-stabilized and stabilized fractures. Both of these molecules promote angiogenesis. Lactate acts as an intrinsic mediator of oxidant signaling by stabilizing Hif1a which leads directly to upregulation of VEGF [9, 12, 13]. VEGF and lactate were measured at 3 and 10 days after injury (Fig. 2). Non-stabilized fractures had higher VEGF levels at 10 days after injury compared to levels at day 3 and in control unfractured legs. We did not detect significant differences in VEGF expression between the two types of fractures. Stabilized fractures showed higher lactate levels at 3 days after injury compared to non-stabilized fractures. At 10 days, lactate levels in both stabilized and non-stabilized fractures were higher compared to controls. However, no difference was detected between the two types of fractures.

Mechanical environment regulates gene expression

Finally, we expanded our analysis of the effect that the mechanical environment has on angiogenic gene expression by examining a microarray data set that we have generated. We first compared overall expression patterns of genes in fracture limbs to uninjured limbs. Compared to uninjured limbs, non-stabilized fractures had 972 genes that were down-regulated by at least two folds, and 1166 genes that were up-regulated at least two folds at 2 days after injury (B>0). At 7 days after injury, non-stabilized fractures had 1717 down-regulated genes and 1597 up-regulated genes (DE>2X, B>0). Similar patterns were observed in stabilized fractures, which had 1435 down-regulated and 1351 up-regulated genes at 2 days compared to uninjured limbs (DE>2X, B>0). At 7 days, stabilized fractures had 1597 down-regulated and 1663 up-regulated genes (DE>2X, B>0). DAVID analysis showed enrichment of genes associated with angiogenesis or vasculature development.

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Fewer genes were differentially expressed between non-stabilized and stabilized fractures. At day 2, non-stabilized fractures had 127 genes that were down-regulated two folds and 136 genes that were up-regulated two folds compared to stabilized fractures (B>0). At day 7, 166 genes were down-regulated and 273 genes were up-regulated in non-stabilized fractures compared to stabilized fractures (B>0). Among these genes of differential expression, few were associated with angiogenesis or vascular development and there was a low enrichment score.

To better understand the biological significance of the microarray data, a list of more than 30 angiogenesis-related genes was selected and their fold change was examined (Table 2). We found that both non-stabilized and stabilized fractures had increased expression of VEGF-d, hif-1a, npn-2, and a set of MMPs by 1-2 folds and slightly decreased the expression of VEGFa and VEGFb at the fracture site at both 2 and 7 days after fracture. However, the expression levels of these genes were not significantly different between non-stabilized and stabilized fractures.

Discussion

Mechanical environment affects angiogenesis

Our previous studies have shown that non-stabilized fractures heal through endochondral ossification and stabilized fractures heal via direct bone formation [1, 14]. Here, we found that non-stabilized fractures had more vascularization at 3 days after injury compared to stabilized fractures, suggesting mechanical instability may promote angiogenesis during early stages of fracture healing. This finding is consistent with what has been reported in the literature. In an ovine tibia osteotomy model, larger interfragmentary movement is associated with increased cortico-medullary blood supply at 2 weeks after injury [15]. In a sheep osteotomy model, larger degrees of instability increases tissue vascularization at 2 weeks after injury [16]. However, at later stages of fracture healing mechanical instability may have different effects on tissue vascularization. Lienau et al. showed that there were fewer blood vessels in the less stable fractures at 6 weeks after osteotomy[16]. In another study, Claes et al. found that larger interfragmentary movement in a sheep metatarsal osteotomy model led to more fibrocartilage formation and fewer blood vessels at 9 weeks after injury [17]. The decreased vasculature at these later time points may be the result of increased amounts of avascular cartilage in the callus.

What is the role of angiogenesis in tissue differentiation during early fracture healing? Differentiation of mesenchymal stem cells into chondrocytes or osteoblasts at the fracture site occurs early after bone injury. In mice, chondrocyte/osteoblast differentiation in fracture callus is detected by 4 days after injury [4]. In the current study, we observed robust blood vessel formation in non-stabilized fractures by 3 days after injury. These findings show that mechanical instability favors chondrocyte differentiation, while at the same time promotes angiogenesis. We may speculate that increased angiogenesis leads to chondrocyte differentiation or vice versa, but there is lack of experimental evidence demonstrating this point. In fact, published results in the literature regarding the relationship between angiogenesis and chondrogenesis remain controversial. On one hand, inhibiting angiogenesis [18] or disrupting the blood supply [19] leads to decreased chondrogenesis in fracture healing. On the other hand, treatments that improve tissue vascularization are also associated with less cartilage formation. For example, a mutation in the *thrombospondin-2* (*Tsp2*) gene in mice leads to a two-fold increase in tissue vascularization and an accompanying decrease in chondrogenesis during early fracture healing [20]. As another example, VEGF treatment improves tissue vascularization and promotes bone formation during early fracture healing [21]. Therefore, an alternative hypothesis is that mechanical instability increases angiogenesis but may constrain the blood supply thus the function of

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blood vessels due to constant disruption of new vasculature. Our EPR oximetry data may support this hypothesis, which showed that oxygen tension in non-stabilized fractures was not significantly improved compared to stabilized fractures during the first 3 days after injury. However, our data on lactate levels at the fracture site is not consistent. At 3 days after fracture, non-stabilized fractures had less lactate compared to stabilized ones. Since lactate is accumulated in anaerobic metabolism as well as aerobic conditions, the significance of increased lactate in stabilized fractures need to be further determined. In conclusion, results from current study are not sufficient to conclude on the relationship between angiogenesis and tissue oxygenation and stem cell fate decision.

It is worth mentioning that factors affect angiogenesis may have direct effects on osteoblasts and chondrocytes. Receptors for VEGF are found on osteoblast progenitors [22] and can stimulate gene expression in osteoblasts [23, 24]. *Tsp2*, as described above, is anti-angiogenic, but also has direct effect on skeletal progenitor cells and may be expressed in prechondrogenic and preosteogenic cells during fracture repair [20].

Mechanisms that underlie the effects of mechanical environment on angiogenesis

Post-injury angiogenesis is a complicated process, which involves activity of many growth factors and enzymes, such as VEGFs, Hif-1, and angiopoeitins. We used microarray and ELISA to determine the effects of mechanical stability on the expression of important angiogenesis-related factors. Hif-1 is a dimeric transcription factor comprised of Hif-1a and Hif1b sub-units and controls the expression of VEGF[25]. Hif-1a was up-regulated in fractured legs at both 2 and 7 days after bone injury. VEGF is a family of proteins that regulate angiogenesis and lymphoangiogenesis [26]. Among them VEGFa and VEGFb are regulators of vascular repair. VEGFc and VEGFd are also involved in lymphoangiogeneis. Our ELISA showed that total VEGF protein was high in non-stabilized fractures at 10 days after injury. Further micro-array analysis found that mRNA of VEGFd, but not of VEGFa, VEGFb, and VEGFc, was mildly up-regulated after bone injury. The increased expression of VEGFd in fractured limbs suggests that lymphoangiogenesis could play a role in bone repair or occurs concomitantly. Among VEGF receptors, the expression of neuropilin-2 was increased after fracture. VEGFR1 (flk-1) was decreased at day 7 after fracture and VEGFR2 (flt-1) remained unchanged during early fracture healing. Angiopoietins are another family of angiogenesis-related growth factors. However, we did not detect expression of angiopoietin 1 and 2, neither their receptors (Tie1 and Tie2) in this work. Levels of lactate, another regulator of angiogenesis, were also changed during the process of fracture healing, however, the changes were not correlated with changes in VEGF expression.

The mechanical environment did not significantly alter the major signaling pathways of angiogenesis. Our analyses did not reveal any significant differences in angiogenic gene expression profiles between stabilized and non-stabilized fractures. However, mechanical stimuli may have direct effects on endothelial cells and angiogenesis. There are reports showing that endothelial cells exposed to shear stress align with the direction of mechanical loading [27] and the endothelial cytoskeleton may undergo spatial reorganization [28]. Further, expression of manganese super-oxide dismutase and cyclooxygenase-2 in cultured endothelial cells is up-regulated by steady laminar shear stress [29], which supports a model whereby mechanical stimuli directly affect vascularity.

In summary, the mechanical environment affects tissue vascularization during fracture healing. Mechanical instability promotes angiogenesis at early stages of bone repair. The significance and role of the vasculature and oxygen supply in stem cell differentiation and later callus formation and the underlying mechanisms need to be further analyzed.

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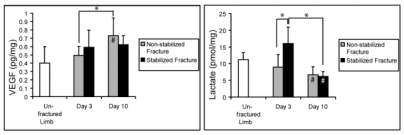

Fig. 1.

Non-stabilized tibia fractures were more vascularized at 3 days after injury. (A) PECAM immunohistochemistry showed blood vessels (black color) in the fracture site of non-stabilized fractures. (B) Less blood vessels were detected at the fracture site of stabilized fractures. (C) Length density and (D) surface density of blood vessels in the fractured tibiae. Scale bars in A and B = 500um. In C and D, * $p<0.05$, ** $p<0.01$.

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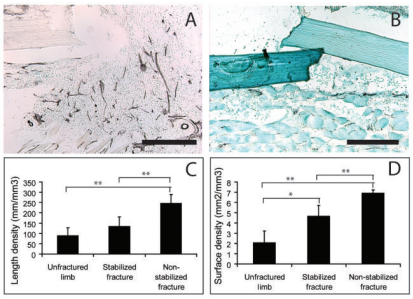

Fig. 2.

Tissue levels of VEGF and lactate in stabilized and non-stabilized fractures. * $p<0.05$. # $p<0.05$ compared to unfractured controls.

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Table 1

Oxygen levels in unstable and stabilized fractures.

Mechanical Environment	O_2 (mmHg) at the time of injury	O_2 (mmHg) at Day 1	O_2 (mmHg) at Day 3
Non-stabilized	5.7±1.7	48.2±25.6	45.3±20.9
Stabilized	10.4±5.7	27.0±11.1	41.8±19.2

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Table 2

A list of important angiogenesis-related genes and their differential expression. Un2d: non-stabilized fractures at day 2. S2d: stabilized fractures at day 2. Un7d: non-stabilized fractures at day 7. S7d: stabilized fractures at day 7.Ctrl: unfractured legs. DE: differential expression.

Gene	Full Name	un2d/ctrl	un7d/ctrl	s2d/ctrl	s7d/ctrl	un2d/s2d	un7d/s7d
Angpt1	Angiopoietin-1	No DE	No DE	No DE	-0.82	No DE	No DE
Angpt2	Angiopoietin-2	No DE	No DE	No DE	No DE	No DE	No DE
Col18a1	Collagen, type XVIII, alpha 1	1.4	**2.89**	1.12	**2.49**	No DE	No DE
Ctgf	Connective tissue growth factor	No DE	**2.36**	**2.69**	**2.09**	No DE	No DE
Ecgf1	Endothelial cell growth factor-1	No DE	0.57	No DE	0.64	No DE	No DE
Edg5	Endothelial differentiation gene-5	1.18	1.19	1	0.98	No DE	No DE
Egf	Epidermal growth factor	No DE	**-2.16**	No DE	-1.51	No DE	No DE
Fgf2	Fibroblast growth factor-2	No DE	No DE	No DE	No DE	No DE	No DE
Fgf21	Fibroblast growth factor-21	No DE	2.2	No DE	**3.64**	No DE	No DE
Fgfr1	Fibroblast growth factor receptor-1	No DE	1.38	No DE	No DE	No DE	No DE
Fgfr2	Fibroblast growth factor receptor-2	No DE	1.38	No DE	No DE	-0.01	0.6
Fgfr3	Fibroblast growth factor receptor-3	No DE	**2.3**	No DE	No DE	-0.04	1.43
Fgfr4	Fibroblast growth factor receptor-4	No DE	**2.25**	No DE	1.55	No DE	No DE
Flt1	VEGFR1	No DE	-1.08	No DE	-1	No DE	No DE
Flk-1	VEGFR2	-1.03	No DE	No DE	No DE	No DE	No DE
fzd1	Frizzled-1	0.86	**2.14**	No DE	1.46	No DE	No DE
Hgf	Hepatocyte growth factor	0.78	0.81	0.63	1.08	No DE	No DE
Hif1a	Hypoxia inducible factor-1, alpha unit	1.87	**2.08**	1.7	1.61	No DE	No DE
Hif1an	Hif-1a inhibitor	-0.78	-0.81	-1.14	No DE	No DE	No DE
Mmp2	Matrix metallopeptidase 2	No DE	1.85	No DE	No DE	0.18	1.05
Timp1	Tissue inhibitor of metalloproteinase 1	**4.19**	**4.24**	**4.27**	**4.22**	No DE	No DE
Nrp1	Neuropilin1	1.58	No DE	No DE	No DE	No DE	No DE
Nrp2	Neuropilin2	1.46	1.51	0.96	1.13	No DE	No DE
Pdgfa	Platelet derived growth factor a	No DE	No DE	No DE	No DE	No DE	No DE

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Gene	Full Name	un2d/ctrl	un7d/ctrl	s2d/ctrl	s7d/ctrl	un2d/s2d	un7d/s7d
Pdgfc	Platelet derived growth factor b	No DE	1.51	No DE	No DE	No DE	No DE
Tgfb1	Transforming growth factor, beta 1	No DE	No DE	No DE	No DE	No DE	No DE
Tgfb2	Transforming growth factor, beta 2	-1.14	No DE	No DE	No DE	No DE	No DE
Tgfb3	Transforming growth factor, beta 3	No DE	0.92	-1.38	0.79	No DE	No DE
Tie-1	TIE tyrosine kinase	No DE	No DE	No DE	No DE	No DE	No DE
Tie-2	The TEK receptor tyrosine kinase	No DE	No DE	No DE	No DE	No DE	No DE
Vegfa	Vascular endothelial growth factor, a	-1.02	-1.01	-1.05	-1.04	No DE	No DE
Vegfb	Vascular endothelial growth factor, b	-1.37	-1.02	-1.84	-1.11	No DE	No DE
Vegfc	Vascular endothelial growth factor, c	No DE	No DE	No DE	No DE	No DE	No DE
Vegfd	Vascular endothelial growth factor, d	1.76	1.07	1.39	1.27	No DE	No DE

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