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Temporary inhibition of the plasminogen activator inhibits periosteal chondrogenesis and promotes periosteal osteogenesis during appendicular bone fracture healing

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Abstract

Introduction—Aminocaproic acid is approved as an anti-fibrinolytic for use in joint replacement and spinal fusion surgeries to limit perioperative blood loss. Previous animal studies have demonstrated a pro-osteogenic effect of aminocaproic acid in spine fusion models. Here, we tested if aminocaproic acid enhances appendicular bone healing and we sought to uncover the effect of aminocaproic acid on osteoprogenitor cells (OPCs) during bone regeneration.

Methods—We employed a well-established murine femur fracture model in adult C57BL/6J mice after receiving two peri-operative injections of aminocaproic acid. Routine histological assays, biomechanical testing and micro-CT analyses were utilized to assess callus volume, and strength, progenitor cell proliferation, differentiation, and remodeling in vivo. Two disparate ectopic transplantation models were used to study the effect of the growth factor milieu within the early fracture hematoma on osteoprogenitor cell fate decisions.

Results—Aminocaproic acid treated femur fractures healed with a significantly smaller cartilaginous callus, and this effect was also observed in the ectopic transplantation assays. We hypothesized that aminocaproic acid treatment resulted in a stabilization of the early fracture hematoma, leading to a change in the growth factor milieu created by the early hematoma. Gene and protein expression analysis confirmed that aminocaproic acid treatment resulted in an increase in Wnt and BMP signaling and a decrease in TGF- β -signaling, resulting in a shift from chondrogenic to osteogenic differentiation in this model of endochondral bone formation.

Conclusion—These experiments demonstrate for the first time that inhibition of the plasminogen activator during fracture healing using aminocaproic acid leads to a change in cell fate decision of periosteal osteoprogenitor cells, with a predominance of osteogenic differentiation,

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resulting in a larger and stronger bony callus. These findings may offer a promising new use of aminocaproic acid, which is already FDA-approved and offers a very safe risk profile.

Graphical abstract

Keywords

amicar; osteoblast; osteoprogenitor cell; bone; periosteum; fracture hematoma

Introduction

With an ever-increasing number of fractures, orthopaedic surgeons are constantly faced with delayed unions or non-unions. Orthopaedic research has focused on the discovery of pro-osteogenic molecules for decades, but we are still left with only a few FDA-approved adjuvants with often questionable efficacy. Therefore, it is of utmost importance to identify mechanisms or molecules that offer a safe and cost-effective alternative to the current choices. *ε*-Aminocaproic (EACA) acid has been approved for use in joint replacement surgery and spinal fusion as an antifibrinolytic to limit perioperative blood loss [1–3]. The efficacy and safety of EACA has been studied extensively, and EACA has been shown to have a very safe and favorable risk profile [2, 3]. Recently, Cuellar et al. examined the effect of various antifibrinolytics on bone formation in a spinal fusion mouse model. Here, systemic treatment with EACA significantly enhanced the bony fusion in a dose-dependent manner, while another antifibrinolytic, tranexamic acid, did not affect fusion when compared to saline controls [4]. Previous studies have shown that mice, lacking the plasminogen activator or urokinase-type plasminogen activator, demonstrated enhanced osteoblast differentiation and thus increased bone mass [5, 6]. EACA inhibits the activation of plasminogen to plasmin, therefore mimicking the mechanism found in the plasminogen activator deficient mice, albeit only for a short time frame compared to the genetic mutations used to study the effects of plasminogen activator and urokinase-type plasminogen activator on bone healing. These previous established findings led us to hypothesize that EACA exerts a pro-osteogenic effect on osteoblast and thus may lead to enhanced fracture healing in EACA-treated animals.

While animal models mimic the clinical procedure of spinal fusion very well, these models are not ideal at determining the precise mechanism of action of a given drug on the

osteogenic response. Therefore, we utilized a well-described and reproducible model of bone regeneration in conjunction with in vivo transplantation experiments and in vitro assays to further understand the effect of EACA on bone healing of the appendicular skeleton. From these experiments, we learned that EACA exerts a very unique effect on appendicular fracture healing. EACA inhibits chondrogenesis during the soft callus phase and accelerates and enhances osteogenesis during the hard callus phase of endochondral bone formation during fracture healing.

Materials and Methods

Aminocaproic acid treatment

All procedures were approved by the New York University Committee on Animal Research and comply with the ARRIVE guidelines. Studies were conducted on 12-week-old, male C57BL/6 male mice (*Mus musculus*) purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-hour light/dark cycle with food and water provided ad libitum.

Aminocaproic acid, 100mg/kg was delivered via intraperitoneal injection 30 minutes prior to the surgical procedure and then again 60 minutes after the 1st dose in order to simulate the clinical dosage and timing of administration, and to match the administration schedule that was used in the spine fusion model resulting in enhanced osteogenesis [4, 7]. Mice were randomly selected to either receive EACA (treatment) or saline (control). Body weight of the mice was recorded before, during and after treatment, and was found to be unaffected by the treatment (data not shown).

Surgical procedures

In order to model bone repair through endochondral ossification, an established femur fracture model with intramedullary stabilization was employed [8, 9]. Briefly, after anesthesia was induced with Isoflurane inhalation (1–5%), an incision was made along the anterolateral femur. A 27-gauge syringe needle was used to open up the medullary canal in a retrograde fashion. Once opened, a 25-gauge needle was inserted up to the mid-diaphysis, the diaphysis was transected with surgical scissors and the needle was advanced into the proximal femur to stabilize the fracture. The free edge of the muscle flap was placed over the injury site with a single stitch, and skin was closed. Mice received buprenorphine as analgesic for up to 3 days post-operatively. Mice were euthanized by CO₂ asphyxiation, followed by cervical dislocation on post-operative day (POD) 2, 7, 14, 21 and 28.

The periosteal scratch injury was performed on the anterolateral surface of the proximal tibia. After skin incision, a muscle flap was raised exposing the periosteal surface of the tibia. An 18-gauge needle was used to create a longitudinal scratch along the anatomical axis. A 2 μm^3 clot, harvested from the injury site of EACA-treated or control tibial injury sites from syngeneic donor mice (24h post-injury), was laid over the periosteal scratch, and covered by the previously elevated muscle flap, which maintained the clot in its location. Mice were euthanized after 7 days and tibia were harvested and processed for histology.

The renal capsule assay was performed as previously described [10]. After elevation of the capsule, 2 μm^3 clot, harvested from the injury site of EACA-treated or control tibial injury sites (24h post-injury) from syngeneic donor mice was mixed with periosteal cells from syngeneic donor mice and then placed under the renal capsule. Mice were euthanized after 7 days and kidneys were processed for histology.

Periosteal cell harvest

After careful dissection, tibiae with intact periosteum were submitted to four serial collagenase digestions in 0.2% collagenase type 2 (Thermo Fisher Scientific) in DMEM at 37°C for 20 minutes with gentle rocking. After the last digestion, bones were centrifuged and the pelleted cells were considered periosteal progenitor cells [11], which were then used in the above mentioned renal capsule experiment.

Histology and histomorphometry

Femurs, tibiae and kidneys were dissected and fixed overnight in 4% paraformaldehyde at 4°C. Samples were decalcified in 19% ethylenediaminetetraacetic acid (EDTA) for approximately 3 weeks at 4°C. Decalcified samples were dehydrated in a graded ethanol series, embedded into paraffin, and cut into 10 μm thick sections. Pentachrome, aniline blue and alkaline phosphatase staining were used to detect osseous tissues as previously described [8, 12–14].

Micro-CT Analyses

Femur fractures from control and aminocaproic acid-treated animals were analyzed with micro-CT at 7, 14, 21 and 28 days post-fracture. Femurs were carefully dissected and fixed in 4% paraformaldehyde overnight. Specimens were scanned using a high-resolution SkyScan micro-CT system (SkyScan 1172, Kontich, Belgium). Images were acquired at 10 μm isotropic resolution using a 10MP digital detector, 10W energy (100kV and 100A), and a 0.5 mm aluminum filter with a 9.7 μm image voxel size. A fixed global threshold method was used based on the manufacturer's recommendations and preliminary studies, which showed that mineral variation between groups was not high enough to warrant adaptive thresholding. The following parameters were analyzed: total bone volume (BV), total tissue volume (TV), respective mineralized volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp) following the guidelines described by Buxsein et al. [15].

The volume of interested (VOI) included a region of the femur with the fracture centered, including 110 CT cuts above and below the fracture, resulting in a total region of interest of 220 CT cuts. We utilized the callus contouring methods rather than using a cylinder as a VOI to establish callus volume. Total volume (TV) therefore represents the callus volume, including calcified and non-calcified components.

Structural and compositional callus strength assessment

The maximum and minimum bending moment of inertia (I_{max} and I_{min} , respectively), as well as the polar moment of inertia (J), where $J = I_{\text{max}} + I_{\text{min}}$, were measured for the femur diaphysis, using CTan software (Bruker microCT). The volume of interest (VOI) consisted

of the entire callus in the longitudinal direction, and in the transverse direction. Thresholding was performed on the VOI to include all of the volume inside the perimeter of the fracture callus as described in [16]. The I_{max}, I_{min}, and J were averaged from all cross-section microCT images in the VOI using the CTan software. I_{max} and I_{min} represents the capacity of the bone to resist bending. J indicates the capacity of the bone to resist torsion.

Biomechanical whole-bone testing

Fractured and non-fractured femurs from EACA-treated and control animals were subjected to biomechanical testing. Harvested femurs were wrapped in PBS- soaked gauze and stored at -20°C before biomechanical testing. Femurs were slowly returned back to room temperature in a humidity controlled environment. Four-point bending analysis to failure were conducted using a materials-testing-machine (ElectroForce 3200, BOSE, MN) with a 245 N load cell and a linear variable displacement transducer (LVDT) with a range of +/- 6 mm. The upper span (loading) width was 6 mm and the lower span (support) width was 6.5 mm based on previous studies on 12-week-old mice [8, 17]. Both the upper and lower contact anvils had a radius of 1.0 mm. Femurs were positioned posterior side down on the support anvils to cause bending in the sagittal plane. Four-point-bending was performed with a displacement control and linear loading ramp waveform at a 0.01 mm/s loading rate and data acquisition of 125 Hz.

Load-displacement curves were analyzed and maximum load (N), maximum displacement (mm), structural stiffness (N/mm) and toughness (Nmm) were assessed and normalized to body weight.

SDS-PAGE and Western Blotting

Callus from femurs at POD 2 and 7 was collected from control and EACA-treated animals. Tissue collection for the injured samples included a 3-mm bony segment centered around the fracture. After dissection, the harvested tissues were immediately frozen in liquid nitrogen then homogenized in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Total protein was quantified with Bradford reagent using a BSA standard curve as reference. SDS-polyacrylamide gels, at 8–16% concentration, were loaded with 60 µg of lysate and proteins were blotted onto polyvinylidene fluoride (PVDF) membranes that were subsequently incubated for phosphorylated SMAD 2/3 receptor (Santa Cruz Biotechnology), phosphorylated SMAD 1/5/9 (Santa Cruz Biotechnology), SMAD 1 (Santa Cruz Biotechnology). GAPDH was probed as endogenous control (0.2 µg/ml concentration, mouse anti-GAPDH, 60004-1, Proteintech, Chicago, IL). After overnight primary antibody incubation at 4 °C, membranes were incubated with HRP-conjugated secondary antibody (BioRad, Hercules, CA) and bands were detected with Clarity Western ECL Substrate (BioRad) in an Odyssey Fc imaging system (LI-COR Biosciences, Lincoln, NE) with 30–90 seconds exposure time. Protein expression was analyzed by measuring the intensity of detected bands compared to intact femur samples and normalized to GAPDH with ImageJ software (U. S. National Institutes of Health, Bethesda, MD).

Bone marrow harvest for in vitro experiments

For the in vitro experiments, tibial and femoral bone marrow was extracted by centrifugation from untreated 12-week-old mice [18]. Cells were re-suspended in growth media (GM), which consisted of DMEM (Thermo Fisher Scientific) containing 10% FBS (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific), followed by plating in 75 ml tissue culture flasks. Media was replenished every 2 days. Approximately 1 week from harvest, the cells were trypsinized and passaged for the following assays.

Cellular proliferation assays

Cells were seeded in 96-well plates at a density of 2×10^4 cells per well in growth media alone or containing 150, 200 or 250 $\mu\text{g/ml}$ of aminocaproic acid (Sigma). On day 3, a BrdU assay (Abcam, Cambridge, UK) was performed according to the manufacturer's instruction. Wells were read on a Flex Station 3 microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Data were collected with Soft Max Pro (Molecular Devices, Sunnyvale, CA, USA) software. Means and standard deviations were calculated in GraphPad Prism 7 software.

Osteogenic differentiation

Cells were plated in 24-well plates with a density of 10,000 cells/well. After overnight attachment in growth media, cells were treated with osteogenic differentiation media (OM) containing DMEM, 10% FBS, 100 $\mu\text{g/ml}$ ascorbic acid 10 mM β -glycerophosphate and 1% penicillin/streptomycin. Cells were cultured in OM alone or containing 150, 200 or 250 $\mu\text{g/ml}$ aminocaproic acid. Media was replenished every 3 days. After 21 days, Alizarin Red staining (ARS) was performed. ARS was quantified as described in [19]. The samples were read on a Flex Station 3 microplate reader at 405 nm in 96-well opaque-walled transparent-bottomed plates. Data were collected with Soft Max Pro software. Means and standard deviations were calculated in GraphPad Prism 7 software.

RNA isolation and quantitative real-time PCR

Callus from femurs at POD 2 and 7 was collected from control and EACA-treated animals. Tissue collection for the injured samples included a 3-mm bony segment centered around the fracture. After dissection, RNA was isolated (RNeasy Kit, Qiagen, Germantown, MD), genomic DNA was removed (RNase-free DNase set, Qiagen) and the RNA reverse-transcribed (Omniscript RT Kit, Qiagen) with oligo-dT and random primers (Promega, Madison, WI). Quantitative real-time PCR was carried out using the Applied Biosystems Step One Plus detection system (Thermo Fisher Scientific) and RT² SYBR Green ROX PCR Master Mix (Qiagen). Specific primers were designed based on PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>) sequence and are listed in table 1. Results are presented as relative expression ratio, values are normalized to the expression of 18S and negative control samples [20]. All reactions were performed in triplicate; means and standard deviations were calculated in GraphPad Prism 7 software.

Measurement of fibrinogen degradation product

Plasma levels of fibrinogen degradation products were measured using an ELISA kit purchased from Cloud-Clone Corp. (Wuhan, China). Plasma samples were collected from mice following the surgical procedure previously described. Briefly, saline or aminocaproic acid was delivered via intraperitoneal injection 30 minutes prior to femur fracture procedure and again 60 minutes after the first dose. Thirty minutes after the second injection, peripheral blood was collected from mice via retro-orbital bleeding and combined with 3.8% sodium citrate in a ratio of 9:1. Blood samples were centrifuged at 1,500g for 15 minutes at 4°C. Resulting supernatant was collected and immediately stored at -80°C until use.

Statistical analysis

Prism 7 (GraphPad Software, Inc., La Jolla, CA) was used for statistical computations. A Student's t test was used for all comparisons in which there were two groups; ANOVA analyses followed by the Holms-Sidak correction for post-hoc testing was applied for analyses in which there were two or more comparisons being made. A statistician was consulted and verified all calculation reported in this manuscript. Error bars represent standard deviation. An asterisk symbol (*) denotes a p value of less than 0.05.

Results

Perioperative aminocaproic acid inhibits chondrogenesis and enhances osteogenesis during endochondral ossification

In order to better understand whether EACA use during fracture care influences bone regeneration, we employed a well-established murine model of endochondral ossification; a femoral shaft fracture stabilized with an intramedullary nail. Skeletally mature mice received aminocaproic acid 30 minutes prior to surgery and 60 minutes after surgery. We chose this EACA administration schedule as it closely mimics the clinical schedule of EACA administration, and matches the dose and schedule of the recent publication showing increased bone formation in a spinal fusion model [4]. Histologic analysis after 7 days revealed a large cartilaginous soft callus in the control animals (Fig. 1A), while the EACA-treated animals demonstrated a smaller callus with fewer chondrocytes (Fig. 1B). At two weeks, the cartilaginous callus in the control animals began to mineralize in the periphery with a large cartilaginous center (Fig. 1C). In contrast, in the EACA-treated animals, the cartilaginous center had almost disappeared, and most of the soft callus had been replaced by mineralized osteoid (Fig. 1D). After three weeks, the control animals showed a mostly ossified callus with a small center of remaining chondrocytes (Fig. 1E), and the EACA-treated animals exhibited a hard callus composed only of osteoid and woven bone (Fig. 1F). Histomorphometric measurements of the callus at 7, 14 and 21 days revealed a significantly smaller cartilage volume at all analyzed time points in the EACA-treated animals (Fig. 1G). Histomorphometric analysis of the osseous callus of fractures treated with EACA versus vehicle revealed a significant larger bony callus at 7, 14 and 21 days post-fracture (Fig. 2A–G). These data provide evidence that peri-operative inhibition of the plasminogen activator exerts a long-lasting effect on appendicular fracture healing by shifting cell fate towards osteogenesis.

Blood clots from aminocaproic acid treated animals establish a pro-osteogenic microenvironment

EACA inhibits fibrin degradation by blocking the binding of plasminogen to fibrin [7]. This results in stabilization of the initial fracture hematoma. We confirmed that our model in fact reliably reproduces this mechanism of action of EACA. Fibrinogen degradation products (FDP) can be detected in the circulation after coagulation and degradation of the resultant clot. During lysis of the clot FDPs are released into the blood stream. With EACA treatment FDP concentration should decrease, if in fact the hematoma is stabilized. We confirmed this by ELISA, which revealed a significant drop in FDP concentration after aminocaproic acid treatment (Suppl. Fig. 1). With this experiment, we confirmed that in fact our systemic concentration is effective at (1) inhibiting plasmin, and at (2) preventing fibrinolysis. Our first set of experiments revealed a shift from chondrogenesis to osteogenesis after aminocaproic acid treatment. Due to the short half-life and the short treatment interval (30 minutes prior to and 30 minutes post-fracture) we hypothesize that the treatment resulted in a biologically relevant change in the microenvironment of the early hematoma, that would then later trigger the observed cell fate shift (Fig. 1&2) [21]. To test this hypothesis, we employed two experimental strategies. Both involved the harvest of the early hematoma (24h post-injury) from bony injury sites from control- and EACA-treated animals. The blood clots were then either transplanted onto a periosteal scratch injury or together with periosteal cells under the renal capsule. From these experiments, we expected to learn whether the early hematoma from EACA-treated animals exerts a pro-osteogenic effect on untreated periosteal cells, similar to the effect observed in our femur fracture model, however excluding a potential direct effect of EACA on periosteal cells. Histologic evaluation of the periosteal scratch injury exposed to blood clots from control animals failed to show a regenerative response at day 7 after injury (Fig. 3A, C). In contrast, periosteal scratches exposed to EACA-treated blood clots showed an osteogenic response within the periosteum, suggesting a pro-osteogenic microenvironment created by the EACA-treated hematoma (Fig. 3B, D). We completed our analysis with an alkaline phosphatase assay. Mature osteoblasts express alkaline phosphatase once matrix deposition is initiated, and we observed a thin layer of alkaline phosphatase activity in the control group (Fig. 3E, between red dotted line), while the EACA- treated group exhibited robust alkaline phosphatase activity within the periosteum (Fig. 3F, between red dotted line). We then confirmed this observation utilizing an ectopic model, devoid of a pro-osteogenic injury environment, the subrenal capsule assay. Early hematoma from control or EACA-treated animals was transplanted together with periosteal cell from untreated animals under the renal capsule of syngeneic mice. After 7 days, histology of the control clot/periosteal cell mix showed a large soft tissue mass without significant bone formation (Fig. 3G). In contrast, the EACA-treated clot/periosteal cell mix showed a large regenerate with exuberant extracellular matrix production and osteoid, characteristic of early osteogenesis (Fig. 3H). Immunohistochemistry for collagen type 1 confirmed the presence of osseous tissue. While the control clot and periosteal cell mix barely exhibited any collagen type 1 staining (Fig. 3I), the EACA-treated clot and cell mixture showed abundant staining (Fig. 3J). These two transplantation experiments suggest that EACA- treated clots provide a pro-osteogenic environment that induces osteogenic differentiation in periosteal cells. The next sets of experiments were aimed at understanding how this pro-osteogenic response was regulated.

TGF- β -, BMP- and Wnt-signaling orchestrate the endochondral healing response after fracture healing in aminocaproic acid-treated animals

Our experiments so far have demonstrated that EACA-treatment resulted in a shift from chondrogenic differentiation to osteogenic differentiation in a femur fracture model and in an osteogenic response in two ectopic clot transplantation assays. Next, we sought to identify the molecular machinery that was responsible for the shift from chondrogenesis to osteogenesis as a result of EACA-treatment. During fracture healing, the first commitment of cells towards an osteogenic or chondrogenic lineage occurs around day 2 and 3 after injury. During this time, cells are actively dividing, and daughter cells begin to differentiate. At this early time point, we harvested fracture callus from control and EACA-treated animals and RNA and protein were isolated. qRT-PCR was performed and revealed significantly higher expression of the skeletal stem cell markers *osterix* and *sox9*, and elevated expression of the osteogenic differentiation markers *runx2*, *col1* and *opn* (Fig. 4A), further confirming the osteogenic shift in the EACA treated animals. Next, we aimed at identifying a mechanism responsible for this fate shift. qRT-PCR and Western blots for TGF- β - and BMP-signaling components were performed. At post-fracture day 2, transcriptional analysis by qRT-PCR did not show any significant differences between the two groups in regards to Wnt signaling (*axin2*), BMP signaling (*bmp2* and BMP-receptors) (Fig 4B). Western blot for p-SMAD2/3 (TGF- β -signaling) and p-SMAD1/5/9 (BMP-signaling) revealed no significant difference between the groups (Fig. 4 C, D). At post- fracture day 7, we observed a trend towards increased Wnt signaling, shown by an increase in *axin2* expression (Fig. 4E) and a significant increase in *bmp2* expression (Fig. 4E) in the EACA treated animals. Both the Wnt and BMP signaling pathways are implicated in osteogenesis in periosteal progenitor cells in response to injury [22]. Western blot demonstrated increased p-SMAD 2/3 activity at post-fracture day 7 in the control animals (Fig. 4F), suggesting active TGF- β -signaling, which is essential for chondrogenic differentiation during endochondral bone formation [23]. There was no significant difference in BMP signaling at day 7 between the two groups, shown by p-SMAD1/5/9 levels (Fig. 4G). These molecular data point towards a chondrogenic milieu in the early fracture hematoma in control animals, while the EACA treatment induced Wnt and BMP signaling, resulting in a pro-osteogenic environment.

Femoral shaft fractures in mice treated with aminocaproic acid exhibit biomechanically stronger callus

In the previous experiments we have established that EACA treatment results in a shift from chondrogenesis to osteogenesis in periosteal cells after fracture. We next set out to study the biomechanical properties of the fracture callus. Four- point bending analyses of early callus (day 7–21) often results in inconclusive data due to the heterogeneity of the woven bone. Therefore, we chose to calculate the moment of inertia from the micro-CT data sets for these early time points. At 28 days after fracture, the callus has matured enough to allow for four-point bending analysis. Micro-CT analysis of the fractured femurs at day 7 (Fig. 5A,B), day 14 (Fig. 5C,D), day 21 (Fig. 5E,F), and day 28 (Fig. 5G,H) revealed a continuous increase in callus volume up to post-operative day 28 in the EACA-treated animals, while the control animals demonstrated a bell-shaped distribution of callus volume with maximal callus volume at day 21, followed by a decline likely due to osteoclastic remodeling (Fig. 5I).

Callus volume was expected to be larger in the control animals, because of the exuberant cartilaginous soft-callus template, which was significantly smaller in the EACA- treated animals (Fig. 1). The bone volume within the entire callus, calculated as the BV/TV was comparable between the two experimental groups (Fig. 5J). In line with this finding, trabecular thickness and number was similar between the groups as well (Fig. 5K,L). The polar, min. and max. moment of inertia (MOI) were significantly greater in EACA treated femur fractures at day 7 (Fig. 5M–O). Biomechanical testing of the femur fractures 28 days after the injury using 4- point bending revealed a significantly higher yield force in EACA-treated femurs (Fig. 6A), while displacement (Fig. 6B), toughness (Fig. 6C) and stiffness (Fig. 6D) showed a trend towards stronger callus in EACA-treated animals, but these values did not reach significance ($p=0.059$ to $p=0.725$). In summary, these data provide convincing evidence that peri-operative inhibition of plasminogen results in a more osteogenic callus formation during the early stages of repair, and this effect is maintained throughout the healing process, culminating in an overall stronger bony regenerate.

Discussion

In light of the aging population and increased fracture incidence, the development of safe and efficient strategies for stimulating bone healing should remain the focus of translational orthopaedic research. While many pro-osteogenic agents have been discovered in basic research, their clinical translation is often hampered by safety concerns, most commonly due to fears of tumor induction [24, 25]. Therefore, recent interest has been focused on drugs or drug components that have been proven to be safe in the clinical setting, and investigators now test whether these components exert pro-osteogenic effects when used in fracture models. One such drug, aminocaproic acid, has been used for decades as an antifibrinolytic, and has proven to be clinically safe in multiple large clinical trials [26]. Interestingly, despite being more cost-effective, EACA has been completely replaced by tranexamic acid, which has a slightly different mechanism of action with a similar safety profile. At this point, most orthopaedic surgeons in subspecialties such as spine, joint reconstruction and trauma have adopted the transition to tranexamic acid, which may explain why we have not observed a similar clinical change in bone healing mode and activity as noted in this animal study with EACA.

Antifibrinolytics inhibit the function of plasmin, a key protease of the coagulation cascade. While fibrinolysis is one of the functions of plasmin, other roles include degradation of extracellular matrix proteins, activation of precursor forms of growth factors and bioactive molecules [27, 28]. Tranexamic acid, a lysine analog, inhibits plasmin activation by binding to the lysine-binding-site, and once bound inhibits plasmin activation by preventing it from binding to its substrate. In addition, tranexamic acid weakly binds to the proteolytic site of plasmin, inhibiting the secondary role of plasmin, which is responsible for the activation of growth fractures [29]. Therefore, tranexamic acid inhibits both the fibrinolytic and other proteolytic functions of plasmin, while EACA only inhibits the physical binding of plasmin to fibrin, while leaving the proteolytic site unaffected, thus maintaining its secondary role as a growth factor activator. This mechanism of action may explain why in vitro experiments using EACA, lacking an activated coagulation cascade and fibrin, failed to show a significant effect on osteoblast proliferation, differentiation and mineralization (Suppl. Fig.

2). However, in an in vivo environment, where fibrinolysis is inhibited by EACA treatment, the effect on bone healing may be exerted through a mechanism involving hematoma stabilization. Our data provide evidence that EACA treatment results in a decreased chondrogenic response during endochondral ossification while promoting an earlier onset of osteogenic differentiation, resulting in an overall stronger callus. This finding is in alignment with a study published by Rundle et al., who showed that plasminogen activator inhibitor-1 (PAI-1) knock out mice exhibit a large cartilaginous soft callus [30]. Aminocaproic acid functions as a plasminogen activator inhibitor, and thus one would expect the opposite effect of the PAI-1 KO mouse model in response to EACA treatment. Our data confirm this assumption: aminocaproic acid treatment resulted in a smaller cartilaginous callus. In a similar fashion to PAI-1, EACA mediates fibrinolysis and in doing so, not only stabilizes the early hematoma, but also the subsequent injury induced regenerate. The early injury hematoma is known to contain a variety of growth factors critical for wound healing and fracture healing [31]. If in fact EACA stabilizes the early fracture hematoma, then our data provide evidence that it may be beneficial for fracture healing to delay the release of the sequestered growth factors from the extracellular matrix of the hematoma to a later time point, at which the injury site may be populated by a higher density of effector cells, such as osteoprogenitor cells and osteoblasts [32].

A previous report by Cuellar et al. has shown a pro-osteogenic effect of EACA in a murine model of spine fusion [4]. We aimed to further understand and characterize this intriguing finding. First, we discovered that similar to regeneration in the axial skeleton, EACA treatment resulted in enhanced regeneration in the appendicular skeleton. Second, our results revealed a profound change in cell fate decision of osteochondroprogenitor cells within the periosteum. Here, EACA treatment resulted in a switch from chondrogenesis to osteogenesis. Our previous work has shown a tight regulation of chondrogenesis and osteogenesis in the periosteum through Wnt and BMP signaling [33]. Therefore, we analyzed the callus tissue for components of these pathways, and the TGF- β -pathway. Not surprisingly, the callus of the EACA treated animals exhibited increased Wnt and BMP-signaling and reduced TGF- β -signaling at post-fracture day 7, which may explain the switch from early chondrogenesis to osteogenesis in response to EACA treatment. The exact mechanism by which the pathway components are regulated in response to EACA treatment has to be further analyzed and will be the focus of future experiments. Speculations include degradation of the growth factors within the early hematoma during the hematoma remodeling phase, and this process may be decreased or abolished by the hematoma-stabilizing effect of EACA.

Accidental trauma continues to be on the rise, and thus the number of fractures seen by orthopaedic traumatologist will increase over the next decades. With an increase in fracture incidence, an increase of delayed unions and nonunions will follow. If we had a safe and efficacious adjunct that could be administered just minutes prior to fracture care and will result in a faster and more reliable union, then theoretically, we would be able shorten the overall recovery time, return patients to work earlier and thus reduce the overall impact of fractures on the US economy. EACA would be a prime candidate, if found to be pro-osteogenic, as it presents a very beneficial risk profile with only limited contra-indications and is relatively inexpensive and thus very cost-effective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Tzortzopoulou A, et al. Antifibrinolytic agents for reducing blood loss in scoliosis surgery in children. *Cochrane Database Syst Rev*. 2008; 3:CD006883.
2. Urban MK, et al. The efficacy of antifibrinolytics in the reduction of blood loss during complex adult reconstructive spine surgery. *Spine (Phila Pa 1976)*. 2001; 26(10):1152–6. [PubMed: 11413430]
3. Thompson GH, Florentino-Pineda I, Poe-Kochert C. The role of amicar in decreasing perioperative blood loss in idiopathic scoliosis. *Spine (Phila Pa 1976)*. 2005; 30(17 Suppl):S94–9. [PubMed: 16138073]
4. Cuellar JM, et al. The effects of Amicar and TXA on lumbar spine fusion in an animal model. *Spine (Phila Pa 1976)*. 2014; 39(19):E1132–7. [PubMed: 24979407]
5. Daci E, et al. Increased bone formation in mice lacking plasminogen activators. *J Bone Miner Res*. 2003; 18(7):1167–76. [PubMed: 12854826]
6. Furlan F, et al. Urokinase plasminogen activator receptor affects bone homeostasis by regulating osteoblast and osteoclast function. *J Bone Miner Res*. 2007; 22(9):1387–96. [PubMed: 17539736]
7. Schoenecker J, et al. 2010 Young Investigator Award winner: Therapeutic aprotinin stimulates osteoblast proliferation but inhibits differentiation and bone matrix mineralization. *Spine (Phila Pa 1976)*. 2010; 35(9):1008–16. [PubMed: 20407341]
8. Bradaschia-Correa V, et al. The Selective Serotonin Reuptake Inhibitor Fluoxetine Directly Inhibits Osteoblast Differentiation and Mineralization During Fracture Healing in Mice. *J Bone Miner Res*. 2017; 32(4):821–833. [PubMed: 27869327]
9. Bonnarens F, Einhorn TA. Production of a standard closed fracture in laboratory animal bone. *J Orthop Res*. 1984; 2(1):97–101. [PubMed: 6491805]
10. Abou-Khalil R, Colnot C. Renal capsule transplantations to assay skeletal angiogenesis. *Methods in molecular biology (Clifton, NJ)*. 2014; 1130:99–110.
11. Leucht P, et al. Embryonic origin and Hox status determine progenitor cell fate during adult bone regeneration. *Development*. 2008; 135(17):2845–54. [PubMed: 18653558]
12. Kim JB, et al. Bone regeneration is regulated by wnt signaling. *J Bone Miner Res*. 2007; 22(12):1913–1923. [PubMed: 17696762]
13. Leucht P, et al. Wnt3a reestablishes osteogenic capacity to bone grafts from aged animals. *The Journal of bone and joint surgery American volume*. 2013; 95(14):1278–1288. [PubMed: 23864176]
14. Minear S, et al. Wnt proteins promote bone regeneration. *Science translational medicine*. 2010; 2(29):29ra30.
15. Bouxsein ML, et al. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res*. 2010; 25(7):1468–86. [PubMed: 20533309]

16. O'Neill KR, et al. Micro-computed tomography assessment of the progression of fracture healing in mice. *Bone*. 2012; 50(6):1357–67. [PubMed: 22453081]
17. Jepsen KJ, et al. Establishing biomechanical mechanisms in mouse models: practical guidelines for systematically evaluating phenotypic changes in the diaphyses of long bones. *J Bone Miner Res*. 2015; 30(6):951–66. [PubMed: 25917136]
18. Kelly NH, et al. A method for isolating high quality RNA from mouse cortical and cancellous bone. *Bone*. 2014; 68:1–5. [PubMed: 25073031]
19. Hill CR, et al. Jagged1 is essential for osteoblast development during maxillary ossification. *Bone*. 2014; 62:10–21. [PubMed: 24491691]
20. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*. 2002; 30(9):e36. [PubMed: 11972351]
21. Kindell DG, Keck RW, Jankun J. Comparison between the clot- protecting activity of a mutant plasminogen activator inhibitor-1 with a very long half-life and 6-aminocaproic acid. *Exp Ther Med*. 2015; 9(6):2339–2343. [PubMed: 26136983]
22. Wang T, Zhang X, Bikle DD. Osteogenic Differentiation of Periosteal Cells During Fracture Healing. *J Cell Physiol*. 2017; 232(5):913–921. [PubMed: 27731505]
23. Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. *J Bone Miner Res*. 2002; 17(3):513–20. [PubMed: 11874242]
24. Bess S, et al. Does recombinant human bone morphogenetic protein-2 use in adult spinal deformity increase complications and are complications associated with location of rhBMP-2 use? A prospective, multicenter study of 279 consecutive patients. *Spine (Phila Pa 1976)*. 2014; 39(3): 233–42. [PubMed: 24477081]
25. Epstein NE. Complications due to the use of BMP/INFUSE in spine surgery: The evidence continues to mount. *Surg Neurol Int*. 2013; 4(Suppl 5):S343–52. [PubMed: 23878769]
26. European_Medicines_Agency. Antifibrinolytics Art. 2013; 31
27. Jenkins G. The role of proteases in transforming growth factor-beta activation. *Int J Biochem Cell Biol*. 2008; 40(6–7):1068–78. [PubMed: 18243766]
28. Toriseva M V, Kahari M. Proteinases in cutaneous wound healing. *Cell Mol Life Sci*. 2009; 66(2): 203–24. [PubMed: 18810321]
29. Law RHP, et al. X-ray crystal structure of plasmin with tranexamic acid- derived active site inhibitors. *Blood Advances*. 2017; 1(12):766–771. [PubMed: 29296720]
30. Rundle CH, et al. Fracture healing in mice deficient in plasminogen activator inhibitor-1. *Calcif Tissue Int*. 2008; 83(4):276–84. [PubMed: 18820962]
31. Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. *Nat Rev Rheumatol*. 2015; 11(1):45–54. [PubMed: 25266456]
32. Bohm AM, Dirckx N, Maes C. Recruitment of osteogenic cells to bone formation sites during development and fracture repair. *Z Rheumatol*. 2016
33. Minear S, et al. α BMP represses Wnt signaling and influences skeletal progenitor cell fate specification during bone repair. *Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research*. 2010; 25(6):1196–207.

Figure 1.

Aminocaproic acid induces osteogenic shift during endochondral bone formation. (A-F) Pentachrome staining of control and aminocaproic acid treated femur fractures at day 7, 14 and 21 days post fracture (n=5 for each time point). (G) Bone histomorphometric analysis revealing increased osteogenesis after aminocaproic acid treatment. (H) Aminocaproic acid treated fractures exhibit decreased chondrogenesis after treatment. Abb.: bm, bone marrow; c, cortical bone; ca, callus; imn, intramedullary nail; m, muscle. Scale bar A-F 500 μ m. Asterisk denotes statistical significance $p < 0.05$.

Figure 2.

Osteogenic differentiation is accelerated and enhanced in femur fractures of aminocaproic acid-treated mice. (A-F) Aniline blue staining of femur fractures treated with control or aminocaproic acid at the time of injury (n=5 for each time point). (G) Aminocaproic acid treated fractures exhibit larger bony calluses at all time points, confirmed by histomorphometry. Red dashed line outlines cartilaginous callus and yellow dashed line outlines bony callus. Abb.: bm, bone marrow; c, cortical bone; ca, cartilaginous callus; imn, intramedullary nail; m, muscle. Scale bar A-F 500 μ m. Asterisk denotes statistical significance $p < 0.05$.

Figure 3.

Hematoma from aminocaproic acid-treated injuries provide pro-osteogenic microenvironment in two disparate models of bone regeneration. (A-D) Pentachrome staining of periosteal scratch injuries covered with hematoma (outlined with red dashed line) from control and aminocaproic acid-treated animals 7 days post injury and transplant (n=5 for each time point). Aminocaproic acid-treated groups demonstrate increased bone formation (outlined with black dashed line). Black box denotes area viewed under high power magnification (arrowheads indicate osteoblasts lining woven bone) (C,D). (E,F) Alkaline phosphatase stain demonstrating increased osteogenic response in aminocaproic acid-treated hematoma group. Red dotted line outlines periosteal reaction. (G,H) Pentachrome staining of day 7 regenerate resulting from transplanted control/aminocaproic acid-treated hematoma and periosteal cells under the renal capsule (n=5 for each time point). Aminocaproic acid-treated groups demonstrate osteogenic response compared to fibrous tissue formation in the control group. (I,J) Immunohistochemistry for collagen type 1 of the regenerate formed from control (I) and EACA-treated clot/periosteal mix (J). Abb.: bm, bone marrow; c, cortical bone; h&p, hematoma and periosteal cells; k, kidney. Scale bar A,B 50 μ m, C-F 25 μ m, G-J 100 μ m.

Figure 4.

Increased in BMP and Wnt signaling and decreased TGF- β -signaling responsible for osteogenic shift in aminocaproic acid-treated femur fractures. qRT PCR and Western Blot of fracture callus at post-operative day 2 and 7. (A) At POD 2, EACA treated callus shows significantly increased expression of the skeletal stem cell markers *osx* and *sox9*, as well as *runx2*. *Col1* and *opn* expression show a trend towards significant elevation in the EACA treated animals. (B) qRT-PCR analysis did not show any significant differences between the two groups in Wnt signaling (*axin2*), BMP signaling (*bmp2* and BMP- receptors). (C-D) Western blot for p-SMAD2/3 and p-SMAD1/5/9 at POD 2 reveals no difference in TGF- β , and BMP activation at the early time point. (E) At POD 7 aminocaproic acid injected mice exhibit elevated levels of *axin2* and *bmp2* expression. (F,G) Western blot for p-SMADs revealed decreased p-SMAD 2/3 in the EACA group and no difference in p-SMAD 1/5/9 expression. Asterisk denotes statistical significance $p<0.05$.

Figure 5.

Micro-CT analysis confirms pro-osteogenic effect of aminocaproic acid in fracture healing. (A-H) Representative 3D renderings of femur fractures in mice treated with control and aminocaproic acid injection at the time of fracture (n=5 for each time point). (I) Callus volume at 1 and 4 weeks post-fracture is significantly increased in animals receiving aminocaproic acid. (J-L) Trabecular thickness, number and separation. Trabecular number and thickness were increased at POD 28 and 21, respectively. (M-O) Aminocaproic acid-treated femur fractures exhibit greater moment of inertia POD 7. Asterisk denotes statistical significance $p < 0.05$.

Figure 6.

Femoral shaft fractures in mice treated with aminocaproic acid exhibit biomechanically stronger callus. (A-D) Biomechanical testing of the femur fractures 28 days after the injury using 4-point bending (n=5). Aminocaproic acid treated group demonstrated a larger yield force (A) and a trend in increased toughness (C) and stiffness (D) in aminocaproic acid-treated animals. Asterisk denotes statistical significance $p<0.05$.

Table 1

Name	Sequence
18S FOR	ACGAGACTCTGGCATGCTAACTAGT
18S REV	CGCCACTTGTCCTCTAAGAA
col I FOR	CAGTCGATTACCTACAGCACG
col I REV	GGGATGGAGGGAGTTACACG
osx FOR	GGAGACCTTGCTCGTAGATTTT
osx REV	GGGATCTTAGTGACTGCCTAAC
runx2 FOR	CGGTCTCCTTCCAGGATGGT
runx2 REV	GCTTCCGTCAGCGTCAACA
sox9 FOR	TACGACTGGACGCTGGTGCT
sox9 REV	TTCATGGGTCGCTTGACGT
osteopontin FOR	TGCACCCAGATCCTATAGCC
osteopontin REV	CTCCATCGTCATCATCATCG