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Lack of ADAM10 in endothelial cells affects osteoclasts at the chondro-osseous junction

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Abstract

Purpose—Mice lacking ADAM10 in endothelial cells (*Adam10ΔEC* mice) have shorter femurs, tibiae and humeri than controls, raising questions about how endothelial cells could control long bone growth.

Methods—We performed a histopathological evaluation of the femur and tibia growth plates at different postnatal stages, and assessed the distribution of TRAP-positive osteoclasts and endothelial cells at the growth plate.

Results—The growth plates in *Adam10ΔEC* mice appeared normal at P7 and P14, but a thickened zone of hypertrophic chondrocytes and increased trabecular bone density were apparent by P21 and later. The number of TRAP+ cells at the COJ was normal at P7 and P14, but was strongly reduced at P21 and later. Moreover, the density of endomucin-stained endothelial cells at the COJ was increased starting at P7.

Conclusion—The defects in long bone growth in *Adam10ΔEC* mice could be caused by a lack of osteoclastogenesis at the COJ. Moreover, ADAM10 appears to regulate endothelial cell organization in the developing bone vasculature, perhaps in a similar manner as in the developing retinal vascular tree, where ADAM10 is thought to control Notch-dependent endothelial cell fate decisions. This study provides evidence for the regulation of osteoclast function by endothelial cells *in vivo*.

Keywords

ADAM10; endothelial cells; osteoclasts; growth plate; chondro-osseous junction

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Introduction

The chondro-osseous junction (COJ) is the site of dynamic interactions between several different cell types that drive bone growth during endochondral ossification (1; 2). The most prominent cell types at the growth plate are the hypertrophic chondrocytes on one side of the COJ, and invading osteoclasts and endothelial cells as well as osteoblasts on the other side (1; 2). A considerable amount of information is available on the regulation of chondrocytes, osteoclasts and osteoblasts at the COJ during endochondral ossification (1; 2), but only little is known about the role of endothelial cells in this process. We have previously identified a defect in long bone growth in mice lacking the cell surface metalloprotease ADAM10 in endothelial cells (3), raising questions about the underlying cause of this growth defect. ADAM10 is a principal regulator of Notch signaling (4–7), and mice lacking ADAM10 in endothelial cells (*Adam10 Δ EC* mice) display a characteristic increase in vascular branching in the developing retinal vasculature that is a hallmark for defects in Notch signaling (3). *Adam10 Δ EC* mice also exhibited additional defects in specialized vascular niches, including enlarged glomeruli, filled with endothelial cells, large vein-like structures on the liver surface and in the subepicardium, intestinal polyps filled with endothelial cells, and significantly shortened femurs, tibiae and humeri, whereas shorter long bones such as metatarsals did not seem affected, and the overall length of these animals was normal (3). Most *Adam10 Δ EC* mice survive for many months, providing a unique opportunity to study how the lack of ADAM10 in endothelial cells affects the growth of long bones. The main goal of this study was to examine the long bones of *Adam10 Δ EC* mice at different times of development compared to controls, with an emphasis on identifying possible abnormalities in the appearance and distribution of endothelial cells, chondrocytes and osteoclasts at the COJ.

Materials and Methods

Reagents and antibodies

All reagents were from Sigma-Aldrich, unless indicated otherwise. Rat anti-mouse endomucin antibodies were from eBioscience (San Diego, CA). Fluorescent mounting media was from Dako (Glostrup, Denmark), MERCOX II methacrylate casting resin was from Ladd Research (Williston, VT).

Adam10 Δ EC mice

Adam10 Δ EC mice were generated by mating *Adam10lox/lox* mice (8) with transgenic mice expressing the endothelial-specific *Tie2-Cre* transgene (9), and maintained as previously described, with *Adam10lox/lox* mice serving as littermate controls (3). The animals were of mixed genetic background (129P2/OlaHsd/C57BL6). All animal experiments were approved by the Internal Animal Care and Use Committee of the Hospital for Special Surgery.

Faxitron analysis

Digital faxitron images were generated from disarticulated limbs. The images were imported and individual bone length was measured using NIH ImageJ software. The femur length was determined by drawing a line from the most proximal edge of the femoral head to the distal end of the femur; for the other bones, the length was determined by measuring a straight line connecting the midpoint of the ends of each individual bone.

Histological analysis

Sample processing—Samples were fixed overnight at 4°C in 4% Paraformaldehyde (PFA). The next day, PFA was removed and replaced with 10% EDTA in 0.1M Tris buffer,

pH 7.4 for decalcification. Samples were decalcified for 6 to 14 days depending on the age of the pups. Samples were rinsed in running, distilled water for at least four hours before dehydration and embedding in paraffin through immersion in a graded series of alcohol, xylene and paraffin. 6 μ m sections were floated onto Superfrost Plus® microscope slides (Cardinal Health) in a 42°C water bath. Slides were dried at 37°C overnight before further treatment.

Staining—Hematoxylin and Eosin (H&E), Safranin O and fast green staining were performed according to standard protocols.

Endomucin staining—Sections were de-paraffinized and rehydrated, blocked with 2% BSA in PBS for 30 minutes to 1 hour, incubated with rat anti-mouse endomucin antibody at a 1:100 dilution at 4°C overnight, washed with PBS, then stained with Cy3 anti-rat antibody for 1 hour at room temperature. The samples were mounted in Fluorescent mounting media (Dako), or counterstained in mounting media with DAPI (Vectashield, Vector), imaged using a Nikon Eclipse E600 microscope, photographed using a Retiga Exi camera and processed with the accompanying software from QImaging.

Quantification of endothelial cell density at the COJ—Digital images of endomucin-stained sections were evaluated using Adobe Photoshop (Adobe Systems, Inc). The intensity of staining was determined by the magic wand tool in Adobe Photoshop with a tolerance setting of 35, and histogram function as previously described for retinal staining (10).

Tartrate Resistant Acid Phosphatase (TRAP) staining—TRAP staining was performed on de-paraffinized and rehydrated sections using Acid Phosphatase, Leukocyte TRAP kit (Sigma-Aldrich) according to the manufacturer's instructions. TRAP+ cells were counted along the COJ interface and the numbers were divided by the length of COJ to generate the relative density of TRAP+ cells at the COJ. To investigate the proximity of endothelial cells and osteoclasts, additional endomucin staining was done on some TRAP-stained samples and images were obtained and merged using Photoshop software.

Osteoclast differentiation assay

Murine bone marrow cells were harvested from tibiae and femora, and cultured overnight in Petri dishes (Midwest Scientific) in a-MEM medium (Invitrogen) with 10% FBS (Hyclone) and 20 ng/ml of M-CSF (Peprotech). Non-adherent cells were then re-plated and cultured in the same medium with 20 ng/ml of M-CSF for 3 days to obtain bone marrow derived macrophages (BMMs), which are capable of differentiating into osteoclasts and thus were used as osteoclast precursors. The attached BMMs were scraped, seeded at a density of $4.5 \times 10^4/\text{cm}^2$ and cultured in a-MEM medium with 10% FBS and 20 ng/ml of M-CSF for 1 day. The cells were then treated without or with optimized concentrations of RANKL (80 ng/ml) in the presence of M-CSF (20 ng/ml) for 3 days. TRAP staining was performed with an acid phosphatase leukocyte diagnostic kit (Sigma-Aldrich) in accordance with the manufacturer's instructions.

RNA isolation and Real time RT-PCR (RT-qPCR) analysis

For RT-qPCR analysis, total RNA was isolated from the hypertrophic and terminally differentiated (COJ) areas of the growth plates from the hind-limbs and forelimbs of 2 wild-type (cre-) and 2 *Adam10* Δ EC knockout P28 mice, and processed as described previously (11).

Vascular casts

Vascular casts were generated following a protocol described by Aharinejad et al. (12), using MERCOX II methacrylate resin as casting material according to the manufacturer's instruction. A 5% KOH solution and 10% EDTA solution were used alternately to accelerate the maceration and decalcification process.

Results

Characterization of the femur and tibia growth plate at different developmental stages by faxitron and H&E staining

To establish when the defect in the femur growth plate of *Adam10ΔEC* mice first develops, and how it progresses over time, we analyzed bones from *Adam10ΔEC* mice and littermate controls at different postnatal ages (P7, P14, P21, P28 and P42) by faxitron and by examination of H&E-stained sections. We found that *Adam10ΔEC* bones were slightly shorter than those of littermate controls at P7, and that this growth defect persisted at all time points shown here (Fig. 1A), but was most severe at 6 months of age (Fig. 1C). The histopathological analysis of the growth plate at P7 and P14 did not uncover any evident abnormalities in *Adam10ΔEC* femurs at these early time points compared to controls (Fig. 1B). At P21, the posterior part of the *Adam10ΔEC* distal femur growth plate had developed a discontinuity in three out of four samples (representative sample shown in Figure 1, discontinuity pointed by black arrowhead). By P28, the discontinuity of the posterior aspect of the distal femur growth plate had progressed to a point where the entire growth plate was bisected in *Adam10ΔEC* mice (black arrow), whereas the anterior aspects of the growth plate had irregular enlargements of the zone of hypertrophic chondrocytes compared to controls (indicated by a black line, the red line marks the length of zone of proliferating and hypertrophic chondrocytes; quantification of the ratio of the hypertrophic zone to proliferating zone at different time points is shown in Fig. 1D). In addition, *Adam10ΔEC* femurs showed an increase in the density of trabecular bone under the growth plate at P28, which was more pronounced towards the cortical aspects of the bone (yellow arrows), and resulted in narrowing of the central bone marrow cavity compared to wild type controls. By P42, several large discontinuities spanning the entire zone of chondrocytes had developed (black arrows), and there was an overall increase in the density of trabecular bone (yellow arrows). The increased density of the trabecular bone in *Adam10ΔEC* femurs was also visible on faxitron images at P28 and P42 (Fig. 1A, black arrows, quantification of images from P42 animals shown in Fig. 1E; *Adam10ΔEC* n=10, control n=10).

When a similar analysis of tibiae was performed by faxitron and H&E staining at different stages of development (Suppl. Fig. 2), the growth plate in *Adam10ΔEC* mice appeared normal at P7, but had an increased size of the central part of the zone of hypertrophic chondrocytes starting at P14 (black arrow, the zone of hypertrophic chondrocytes is marked by a black line, and the entire zone of chondrocytes by a red line). The tibia growth plates also displayed an increase in the density of trabecular bone at P21 and later stages of development (black arrows in the top panels, yellow arrows in the bottom panels, quantification of the ratio of the hypertrophic zone to proliferating zone is shown in Suppl. 1C). Quantification of 10 faxitron images per genotype of the tibial growth plate at P42 confirmed a significant increase in bone density in the posterior portion of *Adam10ΔEC* tibiae compared to controls (Suppl. Fig. 1D). However, unlike at the distal femur growth plate, there were no discontinuities of the proximal tibial growth plate. Measurements of the length of the tibiae by faxitron analysis showed that there was a significant growth defect at all stages from P7 to P42 in *Adam10ΔEC* mice compared to littermate controls (Suppl. Fig. 1E). The length of the humerus, ulna and radius were comparable at P7, although these long bones were slightly, but significantly shorter in *Adam10ΔEC* mice compared to control

littermates starting at P14 and at all later stages until P42 (Suppl. Fig. 2A–C). At P183, the tibiae, humeri and radii were slightly, but significantly shorter in *Adam10ΔEC* mice than in wild type controls, whereas there was no difference in the length of the ulna at that stage. The length of the metacarpals of *Adam10ΔEC* mice was comparable to that of controls at all stages of development (Suppl. Fig. 2D).

Analysis of the number of TRAP+ cells to the COJ

The increased density of trabecular bone in *Adam10ΔEC* femurs and tibiae suggested the possibility of a defect in osteoclasts. To address this possibility, we stained sections of the growth plate of *Adam10ΔEC* femurs and tibiae to identify Tartrate-resistant Acid Phosphatase positive (TRAP+) osteoclasts. We found similar numbers and distribution of TRAP stained cells in the growth plates of *Adam10ΔEC* femurs compared to controls at P7 and P14 (Fig. 2A,B). However, at P21, the distal femoral growth plate in *Adam10ΔEC* mice had a significant reduction in the number of TRAP-stained cells at the COJ compared to controls (Fig. 2A,B), and this difference persisted at P28 (Fig. 2B). Similar observations were made in an analysis of the proximal growth plate of the tibia, where the number of TRAP+ cells was comparable at P7 and P14 in *Adam10ΔEC* mice and controls, but was significantly reduced in *Adam10ΔEC* mice from P21 onward (Suppl. Fig. 3). When we cultured osteoclast precursors from *Adam10ΔEC* mice, we found that the osteoclastogenesis was slightly delayed compared to the control mice, resulting in a small but significant reduction in the number of TRAP+ multinucleated osteoclasts (Suppl. Fig. 4A, B). However, there was no significant difference in the RANKL/OPG ratio as determined by qPCR in samples extracted from the zone of hypertrophic and terminally differentiation/COJ zones in the hindlimbs and forelimbs of *Adam10ΔEC* or control mice (Suppl. Fig. 5).

Analysis of endothelial cells at the COJ and their distribution compared to TRAP+ cells

We next analyzed the distribution of endothelial cells at the COJ of *Adam10ΔEC* and control mice using an antibody against the endothelial cell marker endomucin, which allows detection of endothelial cells in bone sections (13). We found that the endomucin staining at the COJ at P14 was stronger in samples from *Adam10ΔEC* mice compared to controls, and that there was an increased density of endomucin-positive cells at the COJ (Fig. 3A, yellow arrows). Co-staining of these samples for TRAP (Fig. 3A, middle panels) followed by merging of the images (Fig. 3A, right panels) revealed a tight association of TRAP+ cells (red arrows) with endothelial cells (yellow arrows) at the COJ at P14 in *Adam10ΔEC* mice and controls. A quantification of the vessel density at the COJ at P14 confirmed the increase in *Adam10ΔEC* femurs at P14 compared to controls. At P28, there was a similar increase in the endomucin-positive cells at the COJ in femurs from *Adam10ΔEC* mice, but very few TRAP+ cells could be detected at the COJ (Fig. 3B, yellow arrows, endothelial cells, red arrows, TRAP+ cells). Taken together, these findings suggest that there is an increase in vascular density at the COJ at the distal femoral growth plate in *Adam10ΔEC* mice, and that the lack of ADAM10 in endothelial cells affects the distribution of osteoclasts at the COJ at P28.

Methacrylate casting of the bone vasculature in *Adam10ΔEC* mice

To better visualize the three-dimensional organization of the bone vasculature in *Adam10ΔEC* mice, we generated vascular casts using a methacrylate casting resin (see materials and methods for details). Examination of the vascular casts of the femur under a stereo microscope showed a large central artery with increased branching towards the growth plates that ended in regularly distributed fine vascular loop structures in control mice (Figure 4, top row). By comparison, the vascular casts from *Adam10ΔEC* mice showed that the large central artery branched out in vascular loops that were less parallel and less well

organized and had numerous small bulb-like expansions, further corroborating the presence of defects in the bone vasculature and microvasculature in these animals. In vascular casts from *Adam10 Δ EC* mice, the epiphyseal and metaphyseal vasculature in the distal femur was often connected by vessels spanning the usually avascular growth plate (not shown). These observations confirmed the results of the histopathological analysis, which demonstrated that the discontinuity in the zone of hypertrophic chondrocytes in the femur of *Adam10 Δ EC* mice usually contains endothelial cells with red blood cells (data not shown).

Discussion

The findings presented here suggest that ADAM10 in endothelial cells is required for the proper development and function of the specialized vasculature in the bone during the process of endochondral ossification. The altered vasculature at the COJ in *Adam10 Δ EC* mice is reminiscent of the increased density in the developing retinal vasculature in these animals (3). These results raise the question of whether ADAM10 could potentially regulate endothelial cell branching and density in the bone vasculature in a similar manner as in the retinal vasculature, where ADAM10 is thought to be required for the processing and activation of Notch (3). Consistent with this, mice lacking the Notch-response gene *RBPJ* in endothelial cells have a similar defect in bone development as *Adam10 Δ EC* mice, with discontinuous growth plate and increased density of trabecular bone (Glomski et al., manuscript in preparation). These findings suggest that the reduced number of osteoclasts at the COJ in joints of long bones of *Adam10 Δ EC* mice are likely the consequence of alterations in Notch-dependent differentiation of endothelial cells in the absence of ADAM10, instead of defects in release of a soluble factor from endothelial cells by ADAM10. The observation that the growth of metatarsal bones appears normal in *Adam10 Δ EC* mice, and that the number of osteoclasts at the femur COJ is not detectably affected at P7 and P14, suggests that the role of bone vessels at the growth plate in regulating the function of osteoclasts only manifests itself in the large long bones such as the femur, humerus, tibia, ulna and radius at the later stages of their growth.

These studies raise interesting questions about the role of the ADAM10/Notch/RBPJ signaling pathway in the development of the bone vasculature and perhaps also in determining the fate of developing bone endothelial cells. Previous studies on the contribution of the VEGF-signaling axis to endochondral ossification have identified an important role of endothelial cells in regulating the apoptosis and resorption of hypertrophic chondrocytes (14–16). These findings bring up the possibility that the increased size of the zone of hypertrophic cells towards the center of the femur and tibia growth plate in *Adam10 Δ EC* mice could be caused by an inability of the endothelial cells at the COJ to cause resorption and apoptosis of these chondrocytes. However, blockade of VEGF signaling usually also decreases the density of trabecular bone, whereas *Adam10 Δ EC* mice have increased trabecular bone density adjacent to the distal femoral and proximal tibial growth plates. Additionally, the reason for the existence of discontinuities in the zone of chondrocytes in the distal femur growth plate remains unclear. One hypothesis is that the discontinuities are caused by the mechanical stresses of ambulation in this particular growth plate, although it is also possible that endothelial cells at the COJ of *Adam10 Δ EC* mice are able to invade the normally avascular zone of hypertrophic chondrocytes.

Previous studies have reported an effect of endothelial progenitor cells on the survival, migration and proliferation of RAW monocytic cells (17), so the increased bone density could be due to a lack of osteoclast function that is caused by the ADAM10-deficient endothelial cells, or to increased osteoblast function. Since much remains to be learned about the crosstalk between endothelial cells and osteoclasts or osteoblasts, further studies on the bone vasculature of *Adam10 Δ EC* mice (and *Rbpj Δ EC* mice), and how it interacts with the

closely associated osteoclasts and osteoblasts, could help provide a better understanding of the role of endothelial cells, one of the major cell types at the COJ, in endochondral ossification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of the femur length and growth plate of *Adam10ΔEC* mice and controls at different stages of postnatal development

A) Representative faxitron images of *Adam10ΔEC* and littermate control femurs at the time points indicated (P7, P14, P21, P28, P42). Arrows indicate increased trabecular bone density in *Adam10ΔEC* femurs at P28 and P42. **B)** H&E stained sections of the distal femur growth plate from P7 to P42. A discontinuity of the distal femur growth plate of *Adam10ΔEC* mice first became evident at P21 (black arrowhead). At P28, the discontinuity completely bisected the growth plate of *Adam10ΔEC* mice (black arrow), and several discontinuities were present at P42 (black arrows). The zone of hypertrophic chondrocytes was enlarged towards the inner portion of the growth plate at P21 and P28 (marked by a black line, red lines indicate the overall length of the zone of proliferating and hypertrophic chondrocytes). An increase in trabecular bone density was evident in *Adam10ΔEC* femurs at P28 and P42 (yellow arrows) with a correspondingly narrowed bone marrow cavity. Red lines indicate the height of a given growth plate and black lines mark the zone of hypertrophic chondrocytes. **C)** *Adam10ΔEC* femurs were shorter compared with those of littermate controls at all stages of development examined here by Faxitron. P7, *Adam10ΔEC*, n=14, controls, n=5; P14, *Adam10ΔEC*, n=11, controls, n=10; P21, *Adam10ΔEC*, n=5, controls, n=5; P28, *Adam10ΔEC*, n=8, controls, n=8; P42, *Adam10ΔEC*, n=6, controls, n=4 **D)** Quantification of the ratio of the hypertrophic zone to the proliferating zone of chondrocytes shows a significant increase in hypertrophic chondrocytes in *Adam10ΔEC* femurs at P21, P28 and P42 compared to controls. **E)** Measurements of bone density of trabecular bone under the distal femur growth plate by faxitron showed a significant increase in *Adam10ΔEC* mice compared to controls. All images are representative of at least 3 samples

from different individuals of a given genotype, in E), the n was 10 each for *Adam10* Δ EC and control mice. * indicates $p < 0.05$ in a two-tailed student's t-test.

Figure 2. Osteoclast number and distribution at the COJ in the distal femur joint of *Adam10ΔEC* mice and controls

A) TRAP staining of osteoclasts (purple dots) along the distal femur COJ showed no obvious difference in the number or distribution of osteoclasts at P14 in *Adam10ΔEC* mice versus controls, whereas significantly less TRAP+ cells were visible at P21 at the COJ of *Adam10ΔEC* mice than in controls. **B)** Quantification of the number of osteoclasts at the COJ showed no significant difference of TRAP+ cell number in *Adam10ΔEC* mice compared to controls at P7 and P14, whereas the TRAP+ cells at the COJ were significantly reduced in *Adam10ΔEC* mice at P21 and P28 versus controls. The number of samples analyzed per time point was as follows: P7, *Adam10ΔEC* n=8, control n=3; P14, *Adam10ΔEC* n=5, control n=4; P21, *Adam10ΔEC* n=4, control n=4; P28, *Adam10ΔEC* n=4, control n=2. NS indicates no significant difference, * indicates $p < 0.05$ in a two-tailed student's t-test.

Figure 3. Increased vascular density precedes the defect in osteoclastogenesis at the distal femoral COJ in *Adam10ΔEC* mice

Endothelial cells were labeled with antibodies against endomucin (red staining, marked by yellow arrows) followed by TRAP staining of osteoclasts (purple staining, marked by red arrows) along the COJ. Merged images show proximity of endothelial cells and osteoclasts in all samples (right panels). An increased density of the endomucin+ endothelial cells at the COJ was evident in *Adam10ΔEC* mice at P14 and P28 compared to controls (yellow arrows, quantification at P14 shown on the right, * indicates $p < 0.05$ in a two-tailed student's t-test). At P14, the distribution and number of TRAP+ cells at the COJ were comparable in *Adam10ΔEC* samples and controls, whereas significantly less TRAP+ osteoclasts were present at the COJ in *Adam10ΔEC* samples compared to controls at P28. Note that endomucin staining at the COJ was also stronger in *Adam10ΔEC* samples than in controls. All images are representative of the data obtained from at least 3 separate individuals for each genotype and time point.

Figure 4. Vascular casting shows disorganized vasculature at the distal femoral growth plate of *Adam10ΔEC* mice

Methacrylate casts of the vasculature at the distal femur growth plate of *Adam10ΔEC* mice had a less regular appearance compared to controls, with less parallel capillaries that had numerous small bulb-like enlargements (pointed by red arrows in the 4× and 8× images) that were rarely seen in controls, as shown in photographs of vascular casts at low magnification (1×, black arrow) and at higher magnifications (4× and 8×). All images are representative of those taken from 4 different individuals per genotype.