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Characterization of Osteoclasts from Patients Harboring a G215R Mutation in ClC-7 Causing Autosomal Dominant Osteopetrosis Type II

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Autosomal dominant osteopetrosis II (ADOII) is a relatively benign disorder caused by a missense mutation in the *ClCN7* gene. In this study, we characterize the osteoclasts from patients with ADOII, caused by a G215R mutation, and investigate the effect on osteoclast function *in vitro*. Osteoclasts from ADOII patients and healthy age- and sex-matched controls, were used to evaluate osteoclastogenesis, cell fusion, acidification, and resorptive activity. ADOII osteoclasts *in vivo* have increased number and size. However, *in vitro* we observed no significant changes in the osteoclast formation rate, the morphology, and the expression of markers, such as cathepsin K and tartrate-resistant acid phosphatase. When mature ADOII osteoclasts were investigated on mineralized bone, they degraded the bone material, however only to 10 to 20% of the level in controls. We show by acridine orange, that the reduced chloride transport leads to reduced acidification. We show that the residual activity is sensitive to inhibitors of cathepsins and chloride channels, confirming that resorption is reduced but present. In conclusion, this is the first functional *in vitro* study of human ADOII osteoclasts. We show normal osteoclastogenesis in ADOII osteoclasts. However, the residual activity of the ClC-7 channel in ADOII osteoclasts does not allow sufficient acidification and thereby resorption. *(Am J Pathol 2004, 164:1537–1545)*

The osteoclasts ability to dissolve the inorganic phase of bone is essential for the degradation of the organic bone matrix, and thereby for the maintenance of the skeleton. The inorganic phase of bone consists of hydroxyapatite crystals, and the dissolution of this matrix requires a decrease in pH, which is facilitated by active transport of protons into the resorption lacunae.[1] The proton transport is mediated by a specialized osteoclastic V-ATPase.[2] To preserve the electroneutrality in the resorption compartment a passive transport of chloride through chloride channels takes place.[3] Chloride channels in osteoclasts have been studied extensively, and recently it was demonstrated that the chloride channel ClC-7 is involved in bone resorption. ClC-7 is a member of the chloride channel (CLC) family of voltage-gated chloride ion channels, which have been shown to have 16 transmembrane domains, and to form structural dimers.[4] These channels are involved in the regulation of membrane potentials, in various processes such as secretion and absorption of salts and organellar acidification.[5] Recent studies have shown that ClC-7 is mainly expressed in the osteoclasts in the bones and in neurons in the brain.[6,7] The major phenotype of the ClC-7-deficient mice is the complete absence of bone resorption by the osteoclasts.[6] The absence of bone resorption was shown to lead to a severe osteopetrosis, very similar to that observed in patients with autosomal recessive osteopetrosis (ARO).[6] In a few cases of ARO, the phenotype was shown to be caused by mutations in ClC-7.[6,8] The osteopetrosis was speculated to be caused by an intrinsic defect in the osteoclasts, which correlates with the expression pattern. ClC-7 was shown to be expressed at high levels at the ruffled border of the osteoclasts, where it participates in the secretion of acid into the resorption lacunae.[6]

Two other forms of osteopetrosis related to defects in ClC-7 have been found. One of these is the intermediate form of autosomal recessive osteopetrosis (IARO), which is a less severe form of osteopetrosis than ARO. However, still caused by a mutation in ClC-7 and with the same hereditary pattern.[9] The other is the autosomal dominant osteopetrosis type II (ADOII), which is a clinically less severe form often discovered by coincidental radiography.[8,10,11] The ADOII phenotype is thought to be caused by a dominant-negative effect, related to the dimeric property of the ClC-7 channel.[4,8] The ADOII phenotype has been characterized in detail with respect to histology, response to hormonal treatments, and expression of both osteoclast and osteoblast markers. Among the characteristics are a marked increase in bone mass,

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increased numbers of abnormally large osteoclasts that express unusually high levels of tartrate-resistant acid phosphatase (TRAP) and creatine kinase BB (CKBB), and that have accumulations of lysosomal vesicles containing an unidentified material.[12–16] In contrast, osteoblast markers and activity appeared to be normal.[11,17–19] Some ADOII patients also develop premature osteoarthritis of unknown reason.[20] Moreover, cranial nerve compression is observed in some ADOII patients, however this is a secondary effect caused by the increased thickness of the skull.[21]

In this study we dissect the effect of the G215R mutation in ClC-7 on osteoclastic processes. We show that osteoclastogenesis and expression of markers are normal in the ADOII patients, and the expression and localization of ClC-7 are unchanged in ADOII osteoclasts as well. We demonstrate that ADOII osteoclasts have decreased acidification leading to reduced resorption of mineralized bone.

Materials and Methods

Patients and Ethics

Patients were ascertained from a Danish family with ADOII previously shown to be caused by a G215R mutation in the *ClCN-7* chloride channel gene.[8] Nine mutation-positive members (four women and five men) aged 28 to 60 years were included as well as nine age- and sex-matched controls. The study was approved by the Danish Regional Ethical Committee (registration number 2473-03).

Drugs and Chemicals

The chloride channel inhibitor NS5818 (*N*-(3,5-dichlorophenyl)-*N′*-[2-(1H-tetrazol-5-yl)-biphenyl-4-yl-4′-carboxylic acid dimethylamide] urea; patent reference, WO 0024707) is a close, but more potent, analog of the previously described NS3736 (1-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-3-(4-chloro-3-trifluoromethyl-phenyl)-urea),[7] which was synthesized at Neurosearch A/S, Ballerup, Denmark. Bafilomycin A1 and E64 were purchased from Calbiochem (San Diego, CA, USA). The broad-spectrum matrix metalloproteinase (MMP) inhibitor GM6001 was from AM Scientific (Pleasant Hill, CA, USA). The remaining chemicals were from Sigma-Aldrich (Copenhagen, Denmark) and the culture media were from Life Technologies (Taastrup, Denmark) unless specified.

Cell Culture

Isolation of CD14+ Human Monocytes

The CD14+ isolation was performed as previously described.[22] Briefly, the monocytes were isolated from peripheral blood by centrifugation on a Ficoll-Paque gradient (Amersham Pharmacia, Hillerød, Denmark), and magnetically sorted using a CD14+ magnetic bead isolation kit (Dynabeads M-450; Dynal Biotech, Oslo, Norway). The cells were then seeded in 75-cm^2 flasks, and cultured in α-minimal essential medium containing 10% serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 ng/ml of macrophage-colony stimulating factor (M-CSF) (R&D Systems) for 3 days, then they were lifted, reseeded, and cultured until day 10 in the presence of 25 ng/ml of M-CSF and 25 ng/ml of receptor activator of nuclear factor-κB ligand (RANKL) (R&D Systems, Minneapolis, MN, USA) unless stated.

Cell Fusion

Cell fusion was determined by seeding equal numbers of CD14+ monocytes isolated from either the ADOII patients or healthy controls, and culturing them for 5, 7, 9, or 11 days in the presence of RANKL and M-CSF. The conditioned media were removed and stored for measurement of TRAP activity. The cells were fixed using 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes, and subsequently in pure methanol for 5 minutes. The nuclei of the cells were visualized using Ehrlich's hematoxylin (BDH Laboratory Supplies, Poole, England) for 2 minutes, followed by washing in tap water. Fusion scorings were performed using an Olympus IX-70 light microscope, and a video camera linked to a computer using CAST-Grid software (Olympus, Glostrup, Denmark). Osteoclast fusion was detected by the formation of cells with three or more nuclei.

TRAP Activity Measurements

The TRAP activities were measured using a colorimetric assay. The conditioned media were tested for TRAP activity by addition of 6 mmol/L 4-nitrophenylphosphate and 25 mmol/L sodium tartrate at pH 5.5. The reaction products were quantified by measuring absorbance at 405 nm using an optical plate reader.

Immunoblotting

Total cell lysates were prepared by lysing the cells in RIPA+++ buffer[22] for 5 minutes. The lysates were centrifuged at 15,000 $\times$ *g* for 30 minutes to remove any leftover cell debris. Protein concentrations were measured using the Bio-Rad DC protein measurement assay (Bio-Rad Laboratories, Hercules, CA, USA).

Thirty μg of total protein in sample buffer containing 40 mmol/L dithiothreitol was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and then electroblotted onto nitrocellulose membranes. The quality of the protein loading was always checked by Ponceau Red (Sigma-Aldrich) staining. The membranes were then blocked in 50 mmol/L Tris-base, pH 7.5, 100 mmol/L NaCl, 0,1% Tween-20 containing 5% skim milk powder for 1 hour at ambient temperature. This was followed by overnight incubation at 4°C with the correct dilution of the primary antibodies against either CKBB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), TRAP (Zymed, South San Francisco, CA, USA), cathepsin K (Chemicon International, Temecula, CA, USA), ClC-7 (Nordic Bio-

science A/S, Merlev, Denmark), and p38 MAPK (Cell Signaling Technology, Beverly, MA, USA), which was used as a constant control.[22] This was followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 hour at ambient temperature. Finally, the results were visualized using the ECL kit (Amersham Pharmacia Biotech).

Immunocytochemistry

Mature CD14+ isolated osteoclasts were seeded on Osteologic coverslips (BD Biosciences, Brøndby, Denmark), and cultured for 2 days, fixated in 3.7% formaldehyde in PBS for 20 minutes, and then washed thoroughly in PBS. The coverslips were blocked in Tris-buffered saline containing 2.5% casein and 0.1% Triton X-100 for 30 minutes at ambient temperature. This was followed by incubation with the primary antibody (ClC-7) or the corresponding preimmune serum overnight at 4°C in a moist atmosphere, after washing in Tris-buffered saline the coverslips were incubated in Rabbit EnVision (DakoCytomation, Glostrup, Denmark) for 30 minutes at ambient temperature. Finally, the peroxidase activity was visualized using DAB+, and the nuclei were counterstained using Ehrlich's hematoxylin. The coverslips were then dehydrated through a gradient of alcohol (70 to 99%) and toluene, and mounted in DPX. The pictures were taken using an Olympus BX-60 light microscope equipped with an Olympus C-2000 Zoom digital camera.

Acridine Orange Accumulation

Mature CD14+ sorted human osteoclasts were seeded on Osteologic coverslips, allowed to attach, and were cultured for 2 days. The cells were then incubated with acridine orange [3,6-bis(dimethylamine)acridine, 2 μg/ml] and the additives described in the figure legends for 45 minutes, the dye was then removed by intensive washing, and the accumulation was visualized using an Olympus IX-70 microscope equipped with Olympus U-MWB filter, a $\times$20 objective and an Olympus C-2000 Zoom digital camera.

Area and Frequency of Resorption Pits on Osteologic Coverslips

Mature CD14+ sorted human osteoclasts were seeded on the coverslips, and they were cultured for 48 hours in the presence of RANKL and M-CSF, and then fixed in 3.7% formaldehyde in PBS for 20 minutes at room temperature followed by extensive washing. Finally, the coverslips were stained with Ehrlich's hematoxylin for 2 minutes, and washed in running tap water for 5 minutes. The coverslips were dehydrated through a gradient of alcohol (70 to 99%) and toluene, and mounted in DPX. The frequency of resorption was scored using an Olympus IX-70 equipped with a video camera. The frequency was determined as the number of osteoclasts that had formed a visible pit in percentage of the total number of osteoclasts ($\sim$1000 osteoclasts per condition). The area of the resorption pits was determined using an Olympus BX-60 microscope, a $\times$20 objective, and an Olympus C-2000 Zoom digital camera. Osteoclasts with more than three nuclei, that had formed a visible resorption area, were selected. The resorbed area and the osteoclast area were measured using Imagepro (Media Cypernetics, Silver Spring, MD, USA). The results are shown as resorption area relative to the osteoclast area, to avoid any influence from the size of the osteoclasts (no significant differences in size between the ADOII and the control osteoclasts were observed at these time points).

Resorption Assays

Bone resorption was measured as formation of resorption pits on cortical bovine bone slices. Mature osteoclasts (day 10) were cultured for the indicated time periods and at the end of the culture period, adherent cells were removed using a cotton swab. The bone slices were washed in distilled water, and the pits visualized by staining with Mayer's hematoxylin (Bie & Berntsen A/S, Rødovre, Denmark), followed by washing in water. The resorbed area was measured using CAST-Grid (Olympus). The results are shown as resorbed area relative to the total area of bone. The release of the c-terminal type I collagen fragments (CTX) from mineralized bone slices was determined using the CrossLaps for Culture kit (Nordic Bioscience Diagnostics), which was used according to the manufacturer's instructions. In all of the resorption assays, the dye AlamarBlue (Trek Diagnostics Systems Inc., West Sussex, England) was used as a control for equal cell numbers, and we observed no significant differences in cell numbers during the 10-day resorption experiments.

Statistics

Statistical analyses were performed using an unpaired Student's *t*-test, assuming normal distribution with equal variance. Statistical significance is shown by the number of asterisks, $P < 0.05 = *$, $P < 0{,}01 = **$, and $P < 0001 = ***$.

Results

Osteoclastic Fusion Is Normal in ADOII Osteoclasts

Because ADOII patients have increased TRAP activity in serum and a higher number of osteoclast profiles,[12] we tested whether the fusion of osteoclasts cultured from CD14+-isolated peripheral blood monocytes was changed. We measured the formation of multinucleated osteoclasts as seen in Figure 1a. We did not detect differences in the formation of osteoclasts between the ADOII cells and controls. However, the TRAP activity levels measured in the conditioned media were slightly higher in the controls than the ADOII cells (Figure 1b). We observed no morphological differences between ADOII

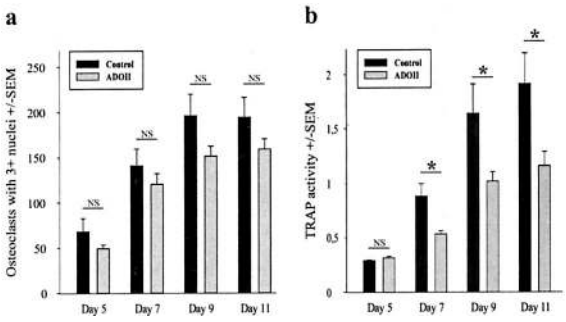

Figure 1. Fusion is unchanged in ADOII osteoclasts. Peripheral blood was obtained from ADOII patients and age-matched control individuals, and the monocytes were isolated by Ficoll-Paque gradient centrifugation and CD14 magnetic bead isolation. The monocytes were cultured for 3 days in the presence of 25 ng/ml of M-CSF and then lifted and reseeded at a cell density of 100,000 cells/cm^2 and cultured for 5, 7, 9, or 11 days with M-CSF and 25 ng/ml of RANKL. The cells were then fixed in 3.7% formaldehyde and the number of fused cells was scored. **a:** The formation of osteoclasts with three or more nuclei. **b:** The TRAP activity was measured in the conditioned media from the fusion experiments. The results are pooled from six ADOII individuals and six controls, and they represent four replications for each individual.

and control osteoclasts, neither with respect to formation of actin rings nor with respect to the localization of the nuclei (data not shown).

Expression of TRAP and CKBB Is Normal in Differentiating ADOII Osteoclasts

TRAP and CKBB activity levels have previously been found to be elevated in ADOII patients.[12,14,15] The expression of CKBB was examined in CD14+ cells stimulated either with a combination of RANKL and M-CSF, or

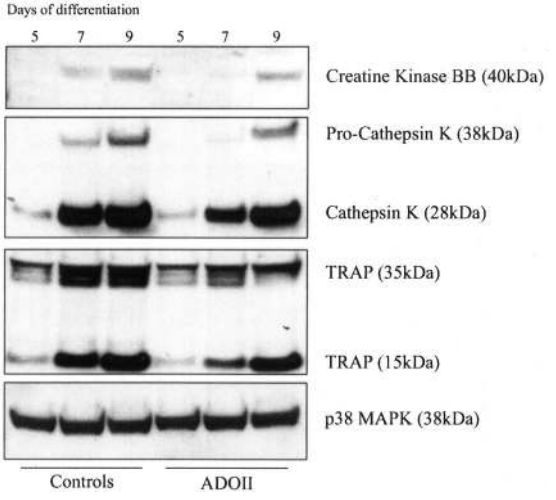

Figure 2. Expression of TRAP and CKBB appears normal in ADOII osteoclasts. Peripheral blood was obtained from ADOII patients and age-matched control individuals, and the monocytes were isolated by Ficoll-Paque gradient centrifugation and CD14 magnetic bead isolation. The monocytes were cultured for 3 days in the presence of 25 ng/ml of M-CSF and then lifted and reseeded at a cell density of 100,000 cells/cm^2 and cultured for 5, 7, or 9 days with M-CSF and 25 ng/ml of RANKL. This was followed by lysis of the cells in RIPA+++ buffer at the indicated time points. Thirty μg of total cell lysate was subjected to sodium dodecyl sulfate gel-electrophoresis and immunoblotting with antibodies against creatine kinase BB, TRAP, cathepsin K, and the p38 MAPK as described in the Materials and Methods section. The immunoblots are representative of five different individuals in each group.

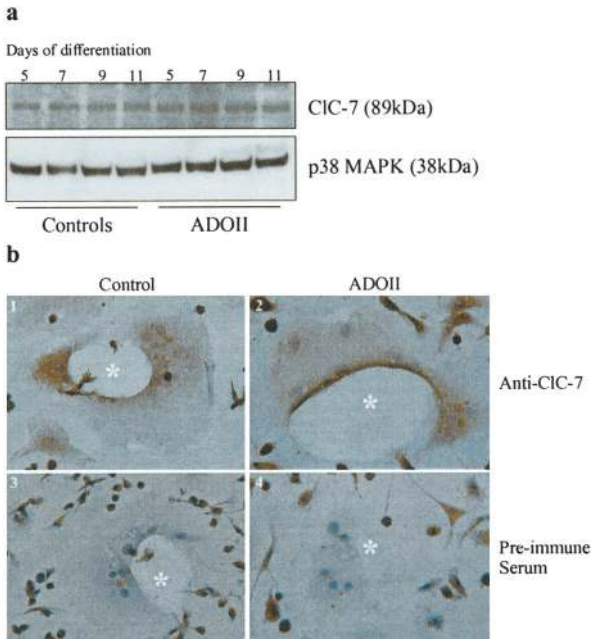

Figure 3. The expression and localization of ClC-7 is unchanged in ADOII cells. Peripheral blood was obtained from ADOII patients and age-matched control individuals, and the monocytes were isolated by Ficoll-Paque gradient centrifugation and CD14 magnetic bead isolation. The cells were cultured in the presence of 25 ng/ml of M-CSF and 25 ng/ml of RANKL. **a:** The lysates for the immunoblots were prepared as described in Figure 2, and then 50 μg of total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted for ClC-7 and p38 MAPK. **b:** Mature osteoclasts were cultured as described in Materials and Methods, lifted by trypsin digestion, and reseeded on Osteologic coverslips. The cells were cultured for 48 hours, fixed, and immunostained using the ClC-7 antibody or the corresponding preimmune serum as described in the Materials and Methods section. The **asterisks** indicate the localization of the resorbed area. The immunoblots are representative of five different individuals in each group.

with M-CSF alone. The expression of CKBB was induced by osteoclastic differentiation as indicated before.[23,24] Subsequently, we tested the expression of TRAP and CKBB in differentiating osteoclasts from both ADOII patients and controls. As seen in Figure 2, the expression of both TRAP and CKBB increases with osteoclast differentiation, as does the expression of the osteoclastic marker cathepsin K. We observed some person-to-person variance (data not shown). However, we did not observe significant differences in the expression levels of these markers between the ADOII and control cells, indicating that the differentiation of the ADOII osteoclasts is normal *in vitro*. Furthermore, these data show that CKBB can be used as an osteoclast marker.

Expression and Localization of ClC-7 Are Unchanged in ADOII Osteoclasts

ADOII patients have defective resorption because of mutations in the *ClC-7N* gene. However, whether the mutation affects the expression, the localization, or the transport activity of ClC-7, has not previously been investigated. Hence, we studied the effect of the G215R mutation with respect to the expression and localization of ClC-7 in ADOII osteoclasts. We found that the level of ClC-7 expression is unchanged in ADOII osteoclasts

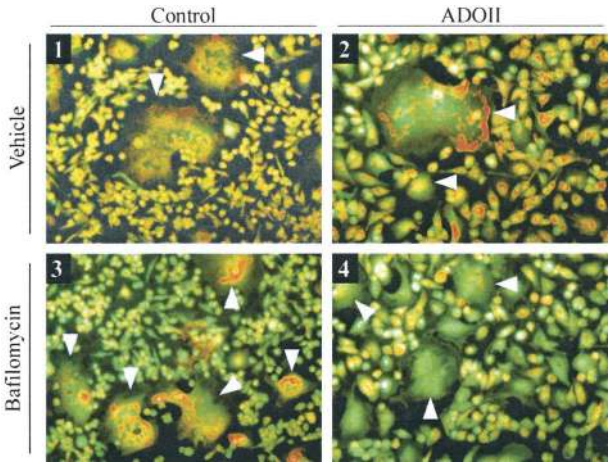

Figure 4. Acidification in ADOII osteoclasts is more sensitive to bafilomycin A1 treatment. Mature osteoclasts from ADOII patients and healthy controls were cultured as described in Materials and Methods, and then lifted by trypsin digestion and reseeded onto the Osteologic coverslips, where they were cultured for 48 hours. Controls (**1** and **3**) or ADOII cells (**2** and **4**) were incubated with acridine orange either in the absence (**1** and **2**) or presence (**3** and **4**) of 50 nmol/L of bafilomycin A1 for 45 minutes, and the accumulation of acridine orange was followed using a fluorescence microscope and a digital camera. The **white arrowheads** indicate multinuclear osteoclasts.

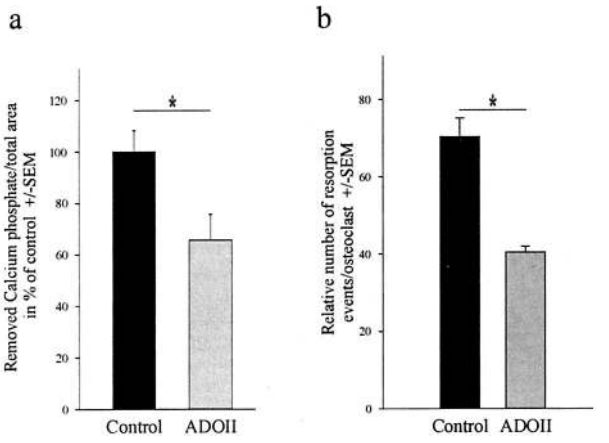

Figure 5. The number and area of resorption events on Osteologic coverslips are reduced in ADOII osteoclasts. Mature osteoclasts from ADOII patients and healthy controls were cultured as described in Materials and Methods, and then lifted by trypsin digestion and reseeded onto the Osteologic coverslips, where they were cultured for 48 hours. The osteoclasts were fixed and the cells and the hydroxyapatite layer were visualized by staining with hematoxylin. **a:** The area of removed hydroxyapatite (40 osteoclasts with area were measured for each group). **b:** The number of osteoclasts that formed visible resorption areas relative to the total number of cells (~1000 cells were scored for each group).

(Figure 3a). Thus, indicating that the mutation does not affect protein expression. The localization of ClC-7 was studied in osteoclasts seeded on calcium phosphate-covered coverslips, where osteoclasts have been shown to actively resorb the calcium phosphate material.[25] We found that ClC-7 localizes in a gradient toward the resorption zone in both ADOII and normal osteoclasts (Figure 3b, 1 and 2) as expected for normal osteoclasts.[6,7] No staining was observed using the preimmune serum (Figure 3b, 3 and 4). Thereby, showing that the G215R mutation does not affect the localization of the channel.

Secretion of Acid Is Impaired in ADOII Osteoclasts

Because ClC-7 was shown to be essential for acid secretion in murine osteoclasts,[6] we analyzed the acidification in osteoclasts isolated from ADOII patients and healthy controls. The osteoclasts were seeded on the Osteologic coverslips, and the acridine orange accumulation was followed by microscopy. As presented in Figure 4 the acidification of ADOII osteoclasts (Figure 4, panel 2) appears to be reduced. To further characterize the defect we used the proton pump inhibitor bafilomycin A1, which has previously been shown to inhibit osteoclastic acidification.[26] Using 50 nmol/L of bafilomycin acidification in ADOII osteoclasts was completely abolished (Figure 4, panel 4), whereas it was only partially reduced in the controls (Figure 4, panel 3), thus confirming that the acidification levels in the ADOII osteoclasts are reduced. Interestingly, the acidification level in the ADOII osteoclasts was different from patient to patient (data not shown), thereby possibly explaining the differences in penetrance of the ADOII phenotype.[27,28]

ADOII Osteoclasts Have Defective Dissolution of Calcium Phosphate

To further evaluate the defective acidification in the ADOII osteoclasts we measured the dissolution of the calcium phosphate layer on the Osteologic coverslips. Firstly, we measured the area of calcium phosphate that the osteoclasts removed, and we found that the area removed by the ADOII osteoclasts was ~60% of the area removed by the control osteoclasts (Figure 5a). Next, we evaluated the percentage of osteoclasts that formed a visible resorption area on the Osteologic coverslips, and we found that only 40% of the ADOII osteoclasts formed a visible zone, whereas 70% of the controls formed a visible resorption zone (Figure 5b). This further supports our evidence that the acidification, and thereby the demineralization, is impaired in osteoclasts from ADOII patients. Because less ADOII osteoclasts form resorption areas, this suggests that a threshold of acidification is needed for resorption to occur.

ADOII Osteoclasts Have Severely Impaired Bone Resorption

To further investigate the reduction in acidification in the ADOII osteoclasts with respect to bone matrix degradation, we cultured mature osteoclasts on cortical bone slices, and monitored their ability to degrade the bone by measuring the release of the CTX fragment of type I collagen, and by scoring the total area of resorption pits formed on the bone. As shown in Figure 6a we found that the volume of the resorption pits, measured by CTX levels is severely reduced in the ADOII cells. In correlation with the CTX data, we found that the ADOII osteoclasts form pits, however the total area of the pits is reduced to 10 to 20% of the control levels (Figure 6b), although the same

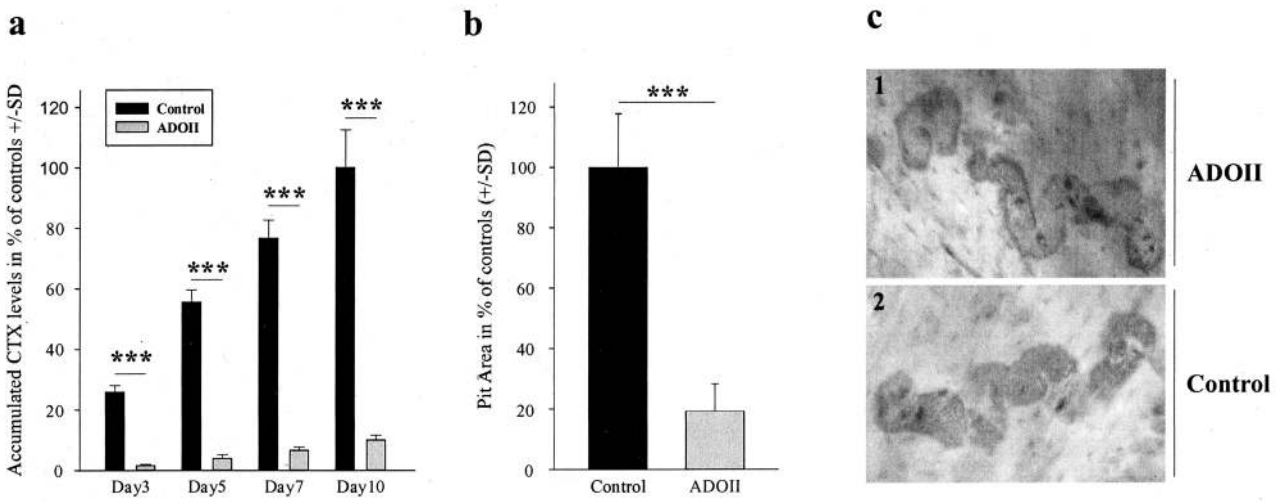

Figure 6. Resorption is severely impaired in ADOII osteoclasts. Mature osteoclasts from ADOII patients and healthy controls were cultured as described in Materials and Methods, lifted by trypsin digestion, and then reseeded on cortical bone slices. **a:** Resorption of bone measured as CTX fragments released into the culture supernatant by the CrossLaps ELISA. The data are shown as accumulated resorption in percentage of endpoint values in the controls. **b:** Pit scorings from the endpoint (day 10) of the experiment. Results are shown as pit area in percentage of controls. **c:** Pictures of hematoxylin-stained pits at the endpoint (day 10) of the culture from ADOII (**1**) and control (**2**) osteoclasts. The results are representative of two ADOII and two control individuals, with each condition performed in pentaplicates.

number of osteoclasts was present (data not shown). As shown in Figure 6c, the individual pits formed by the ADOII osteoclasts are similar to those formed by the controls, however it is the total number of pits that is reduced. This further suggests that a certain level of acidification is a prerequisite for resorption to be initiated. Taken together, these data clearly show that the *in vivo* phenotype is, as expected,[6,8] caused by a reduction in the osteoclastic resorptive activity because of the mutated ClC-7.

ADOII Osteoclasts Respond to Inhibitors of Resorption

To further characterize the deficient resorption in the ADOII osteoclasts we investigated the effects of a chloride channel inhibitor (NS5818), a cathepsin K inhibitor (E64) and a broad spectrum MMP inhibitor (GM6001) on the ability of the ADOII osteoclasts to degrade mineralized bone.

As shown in Figure 7a1, the chloride channel inhibitor NS5818 completely blocked the resorption of bone, by both ADOII and control osteoclasts, thereby clearly demonstrating that although the acidification is dramatically reduced, the remaining resorption in the ADOII osteoclasts is sensitive chloride channel inhibition, and therefore is likely to be mediated by ClC-7. When we tested the cathepsin inhibitor, we obtained similar results, namely that E64 abolishes the CTX-release, as expected[29,30] in both the ADOII and the control osteoclasts (Figure 7a2). This further suggests that despite a reduction in resorption level, the remaining resorptive activity is normal with respect to the proteins involved in it. The broad-spectrum MMP inhibitor (GM6001) did not inhibit resorption (Figure 7a3) as expected.[31] When the pit area was investigated, the values correlated with the CTX values for the NS5818 and the GM6001 treatments. In contrast, when osteoclasts were exposed to inhibition of resorption by E64, the area of the pits was unchanged, whereas the volume of the pits was reduced, as estimated by the CTX measurements. This finding was expected, because inhibition or absence of cathepsin K only leads to impaired degradation of the organic phase of bone,[31] and therefore the acidification is still present, and thus pits are formed.

This finding highlights the need for a careful study of both volume and area, to understand the true effects of both genotype and effects of inhibitors. In summary, these data show that although severely reduced, the resorption performed by the ADOII osteoclasts is performed by the normal resorption machinery.

Discussion

To our knowledge, this is the first *in vitro* study of osteoclasts from ADOII patients. We used cells from patients with a G215R mutation. In these patients the ADOII phenotype is caused by a mutation in the *ClC-N7* gene, and it is manifested by the presence of ~44% more bone, although high numbers of large and TRAP-positive osteoclasts are present.[12] The serum levels of both TRAP and CKBB were also shown to be highly elevated.[14,15] This led us to speculate, whether the differentiation of the osteoclasts was affected by the reduction in acidification.

To investigate osteoclastogenesis we used a fusion assay previously described.[22] However, we found no changes in the cell fusion of the ADOII osteoclasts, although the TRAP levels were slightly reduced. We speculate that this reduction is because of decreased acidification of lysosomes, and because TRAP is a lysosomal enzyme, this could possibly impair the release of TRAP in the ADOII osteoclasts. Furthermore, we show that the formation of resorption pits on bone is reduced by 80%. Finally, we found that the ADOII osteoclasts were indis-

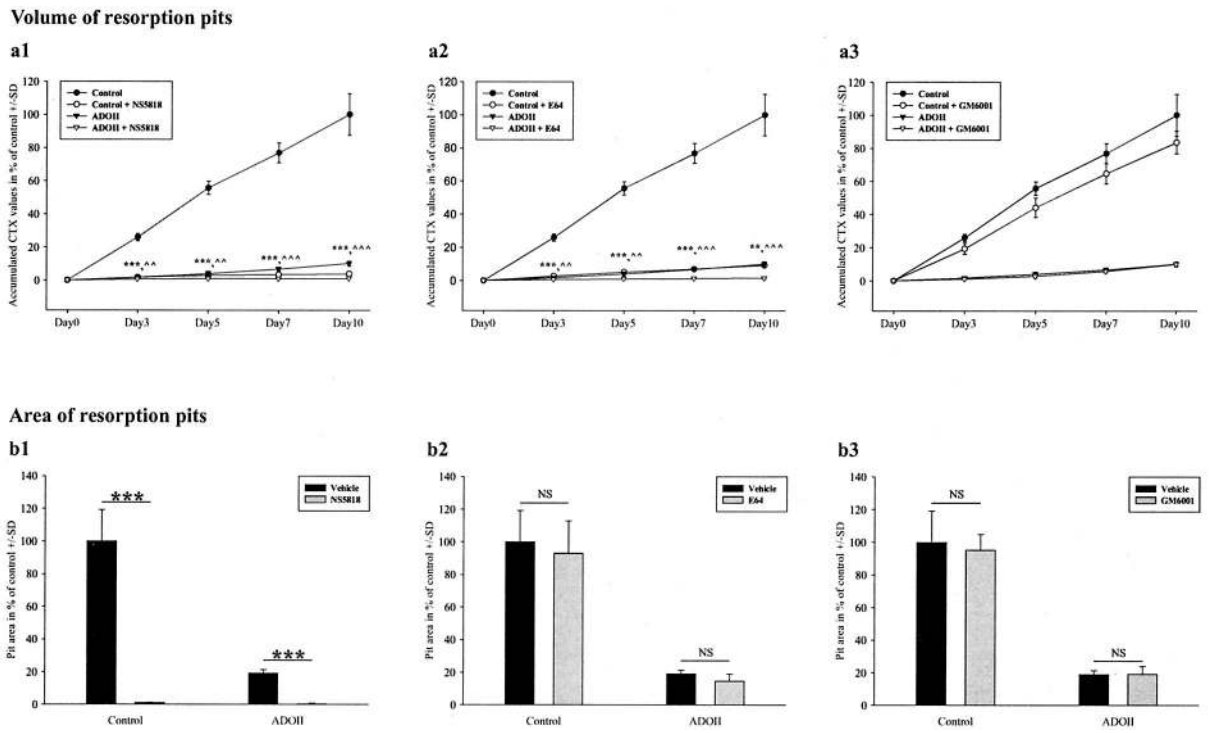

Figure 7. Resorption of bone by ADOII osteoclasts is sensitive to inhibitors of chloride channels and cathepsins. Mature osteoclasts from ADOII patients and healthy controls were cultured as described in Materials and Methods, lifted by trypsin digestion, and then reseeded on mineralized bone. The volume of the resorption pits, measured by the CrossLaps ELISA, is shown in **a**, and the area of the pits, measured by stereology is shown in **b**. Controls and ADOII osteoclasts were cultured in the presence of either 30 μmol/L of NS5818 (**a1**, **b1**), 40 μmol/L E64 (**a2**, **b2**), or 10 μmol/L GM6001 (**a3**, **b3**). The data are presented as accumulated CTX release in percentage of endpoint values of the controls, and represent data from two ADOII patients and two matched controls, with each condition performed in pentaplicates. NS5818 significantly inhibited the CTX release in both controls (*) and ADOII cells (^). E64 significantly inhibited the CTX release in both controls (*) and ADOII cells (^). GM6001 did not change the CTX release from either controls or ADOII cells significantly. Endpoint measurements of the pit formation are shown as the percentage of nontreated control osteoclasts. Only NS5818 significantly reduced the area of the pits, when compared to the nontreated conditions.

tinguishable from the controls because they formed actin rings and their nuclei located similarly in both cell types. These data indicate that the abnormally sized osteoclasts *in vivo* are likely caused by factors present in the bone, but not in the *in vitro* cell fusion assay.

To further investigate the phenotype of the ADOII osteoclasts and compare this to the *in vivo* ADOII phenotype, we investigated expression of TRAP and CKBB, and we found that the expressions of both increase during osteoclastogenesis. However, we observed no differences in the expression levels between ADOII and control osteoclasts. Thus, showing that the high levels of both enzymes *in vivo* in ADOII patients are likely caused by the increased size and number of osteoclasts. Our finding that expression of CKBB is up-regulated during osteoclastogenesis correlates well with previous studies showing that CKBB is highly expressed in osteoclasts.[23] Thus, corroborating that CKBB can be used as an osteoclast marker.[24,32]

In this study we used cells from patients that harbor a G215R mutation. The effect of this mutation on ClC-7 is unknown with regards to the function of ClC-7. Thus, we analyzed the expression level and the protein localization of ClC-7 in the ADOII osteoclasts. We found that the ClC-7 expression level and pattern were similar in ADOII and control osteoclasts, indicating that the mutation does not affect the expression and localization, but the chloride transport directly. This correlates well with the localization and nature of the mutation (G215R), which was estimated to be located near the chloride pore.[8] Furthermore, the substitution leads to insertion of a positive charge in a negatively charged area close to the chloride pore,[4] and therefore it is not surprising that it affects the chloride transport directly.

To characterize the defect in the ADOII osteoclasts with respect to acid transport, we used the dye acridine orange that accumulates in acidic vesicles, both inside the osteoclasts and in the resorption lacunae.[33] We found that the acidification level in ADOII cells was lower than in controls. This was illustrated by an increased sensitivity to the proton pump inhibitor bafilomycin A1. We also found that the reduction in the acidification level in osteoclasts from different patients was of differing severity (data not shown), which could potentially explain the nonhomogenous penetrance of the ADOII phenotype.[27,28,34] These data correlate very well with the data from the ClC-7 knockout mice, demonstrating an almost absent acidification in the osteoclasts.[6] Furthermore, we measured the area of removed calcium phosphate on the Osteologic coverslips, and we found that the area, as well as the number of resorption events was reduced in the ADOII cells, thereby supporting our evidence that the acidification is defective in these osteoclasts. Interestingly, we observed the largest difference between the

number of cells that had formed pits relative to the total number of cells (70% in controls *versus* 40% in ADOII cells), indicating that a certain level of acidification has to be present to initiate and continue the resorption event, and that this level is displaced in the ADOII osteoclasts. These data are supported by two previous publications,[35,36] showing that not all osteoclasts resorb. Furthermore, these publications also demonstrate that the size of the osteoclasts and activity of the proton pump is related to the ability of the individual osteoclast to resorb bone, supporting the presence of a threshold. To further study the implications of the reduced acidification on bone resorption, we measured the total volume and total area of the resorption pits, and we found that both are reduced to 10 to 20% of the control values. However, the size of the individual resorption pits appeared to be the same. Thus, demonstrating that the ADOII phenotype is caused by defective osteoclastic resorption, and again indicating that a threshold level of acidification in the individual osteoclast is necessary for the initiation and formation of a resorption pit, and that the percentage of ADOII cells capable of reaching the threshold is displaced.[37,38]

To examine the residual resorptive activity of the ADOII osteoclasts, we investigated resorption in the presence of protease and chloride channel inhibitors. We found that resorption was dependent on both chloride channels and cathepsins, but not MMPs, thus confirming, that although reduced, the resorption process takes place, and it utilizes the normal components of the resorption machinery.[6,31,39,40]

In conclusion, we have shown that the osteoclasts from ADOII patients have defective acidification, and thereby defective resorption of mineralized bones. We demonstrated that the fusion of these osteoclasts is normal, which is in contrast to *in vivo* data, in which the osteoclasts are larger and more TRAP-positive than in the controls.[12] We speculate that the increase in size of the ADOII osteoclasts *in vivo* could be caused by an increased survival of the osteoclasts, because of a change in release of factors during resorption of bones, which could possibly be involved in an autoregulatory control of the osteoclastic activities. Interestingly, recent publications showed that the number of osteoclasts is increased both *in vivo* and *in vitro* in patients and mice with a defective a3 subunit of the proton pump.[41] Thereby making it likely that acidification is directly linked to the life span of the osteoclasts.[41] Another very interesting feature of reduced acidification present in both patients and animals treated with inhibitors of acidification, is the presence of highly active osteoblasts, thus indicating that although the resorption is reduced, the formation is unaltered.[7,17,41,42] Hence, it is possible that the coupling of bone resorption to bone formation is directly linked to the acidification of the resorption lacunae.

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