**Osteoclasts from blood of symptomatic and asymptomatic carriers of cherubism-causing SH3BP2 mutations are extremely large and aggressive**

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Abstract

Cherubism is a rare autosomal dominant bone disease of the maxilla and mandible whose severity ranges widely, from asymptomatic to life-threatening. While almost all cherubism patients carry a mutation in a specific hexapeptide in the gene encoding SH3 binding protein 2 (SH3BP2), the factors contributing to genetic penetrance and severity of clinical symptoms are still unknown. In mice the cherubism mutation causes systemic inflammation mediated by increased TNFα levels, however the etiological role of TNFα in cherubism in humans is still under debate. Recent studies have suggested that enrichment of multinucleated osteoclasts, but not macrophages, in cherubism bone lesions correlates with the severity of the symptoms. However, it is unknown whether this higher level of giant cells in cherubism lesions derives from increased differentiation of osteoclast progenitors or specific tissue environmental factors that control osteoclast formation. To address these questions, we compared the differentiation and resorption of osteoclasts derived from blood of two symptomatic and one asymptomatic carrier of the same cherubism mutation. Peripheral blood mononuclear cells (PBMCs) from all carriers formed osteoclasts that were an order of magnitude larger than osteoclasts from healthy donors when cultured with either the receptor activator of the NF-kB ligand (RANKL) or tumor necrosis factor alpha (TNFα). When cultured on bone slices, osteoclasts from cherubism mutation carriers resorbed significantly more bone only when cultured with RANKL. Our data suggest that factors driving genetic penetrance and severity of symptoms in carriers of the SH3BP2 cherubism mutations are not intrinsic to osteoclasts and probably derive from other cells or tissue microenvironmental factors. Our data also suggest that giant cell formation due to TNFα is not correlated with enhanced osteoclast aggression in humans, which is in accordance with a study showing reduced multinucleated giant cells but no clinical improvement in cherubism patients treated with anti-TNFα drugs.

Introduction

Cherubism is a rare autosomal dominant bone disorder characterized by symmetrical fibrotic bone lesions in the maxilla and mandible (JONES et al., 1950).

Clinical symptoms of cherubism appear in patients between 2 and 5 years of age, progress until puberty, and then usually regress in adulthood (Katz et al., 1992). The clinical symptoms include swelling and expansion of the affected bones, destruction of cortical bones, tooth displacement, and tooth agenesis. The severity of the clinical symptoms varies and can range from asymptomatic bilateral swelling in the jaws to life threatening bone lesions (Pontes et al., 2007; Khirani et al., 2013; Machado et al., 2017). Although much knowledge has been gained about the underlying pathological mechanisms driving cherubism, the factors that determine the appearance of symptoms and their severity are still unknown.

Histologically, cherubism granulomas are filled with a dense fibrotic tissue that is rich in multinucleated giant cells (MGCs), some of which are in contact with the bone while others are scattered throughout the fibrotic tissue (Yamaguchi et al., 1999).

Cherubism is caused by mutations in the gene encoding the adaptor protein SH3 domain-binding protein 2 (SH3BP2) (Ueki et al., 2001). These mutations can be inherited or occur sporadically (Chrcanovic et al., 2021). In most cases of cherubism, the mutations are missense mutations clustered within a hexapeptide with the amino acid sequence RSPPDG located at position 415-420 in the protein (Ueki et al., 2001). The most common mutation is c.1244G>A resulting in p.R415Q in both familial and sporadic cases. (Chrcanovic et al., 2021). SH3BP2 is an adaptor protein which coordinates signals from integrins, colony-stimulating factor-1 receptors (c-fms), immunoreceptor tyrosine-based activation motifs (ITAMs), and the receptor activator of the NF-kB (RANK) with downstream activation of Src, Syk and Vav-family protein kinases followed by activation of the osteoclast (OC) master transcription factor nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (Deckert et al., 1998; Maeno et al., 2003; Foucault et al., 2005; Deckert and Rottapel, 2006; Levaot et al., 2011a). The causative role of SH3BP2 in cherubism has been confirmed in a mouse model harboring a P416R knock-in mutation (KI) in murine SH3BP2 (equivalent to P418R in humans). Mice homozygous for the mutation show extensive bone resorption and recapitulate the bone lesions in the maxilla and mandible seen in cherubism patients (Ueki et al., 2007). We have shown that SH3BP2 serves as a substrate for the poly-ADP-ribosyltransferases Tankyrase 1 and 2. Tankyrases bind and poly-ADP-ribosylate (Parsylate) SH3BP2. Parsylation of SH3BP2 is followed by ubiquitination by the E3 ligase RNF146 and subsequent proteasomal degradation. Each of the mutations in the SH3BP2 hexapeptide prevent Tankyrase binding, which is followed by the stabilization of SH3BP2 and an enhanced signaling cascade involving Src and Syk kinases. These changes result in hyperactive and aggressive OCs with enhanced bone resorption (Levaot et al., 2011b). Unlike human SH3BP2 heterozygotes, heterozygous mice do not exhibit the cherubism phenotype. The homozygous KI mouse has high circulating tumor necrosis factor alpha (TNFα) concentrations and suffer from systemic inflammation with macrophage infiltration into tissues. This systemic inflammation is rescued when SH3BP2 KI mice are crossed with either TNFα or MYD88 (a mediator of toll like receptor signaling) indicating that cherubism is an auto-inflammatory bone disease (Ueki et al., 2007; Yoshitaka et al., 2014). Recently it was shown that there is a correlation between the number of multinucleated giant cells (MGCs) expressing OC markers and the aggressiveness of the bone lesions (Kadlub et al., 2018). However, it is not known if increased OC numbers in more aggressive cherubism bone lesions is a result of an increased differentiation potential of OC precursors or increased local osteoclastogenic signals.

To address this question, we compared the differentiation and resorption potential of OC progenitors derived from peripheral blood of symptomatic and asymptomatic carriers of the SH3BP2 P418R mutation.

**Materials and methods**

*Ethics statement*

This study was carried out according to protocols approved by the Soroka Medical Center (No. 0166-17 SOR).

*Study population and Samples*

The cells described in the study were derived from blood donated by two sisters, aged 15 and 19 years, who were diagnosed with cherubism. The diagnosis was confirmed by a genetic examination that showed a P418R mutation in SH3BP2. Cells were also taken from the blood of their father who was found to be an asymptomatic carrier of this mutation. Blood from three donors, one man and two women ages 26, 24 and 25 respectively was used as controls.

*In vitro generation of human osteoclasts*

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples and differentiated to mature OCs as follows: whole blood samples were collected to CPT™ tubes and monocytes were separated according to the manufacturer’s protocol (*BD Vacutainer, 362782*). To enrich for monocytes, 25 x 106 PBMCs were seeded in T75 culture flasks supplemented with 25 ng/ml M-CSF (R&D Systems, 216-MC) for two days, then cells were detached from the flask using Accutase (Sigma-Aldrich, [A6964](https://www.sigmaaldrich.com/catalog/product/sigma/a6964?lang=en&region=IL)), counted and re-seeded in 96-well plates for either differentiation or bone resorption assays (see below).

*Osteoclast differentiation assay*

To assess OC differentiation 75 x 103 cells from PBMC cultures enriched for monocytes were cultured in 96-well plates in differentiation media (αMEM, Sigma-Aldrich, M8042; 10% FBS, 5% Pen/Strep and l-glutamine, Biological Industries) that was supplemented with 25 ng/ml M-CSF and either 25 ng/ml RANKL (R&D Systems, 390-TN) or 100 ng/ml TNFα (R&D Systems, 210-TA) Media was changed every two to three days and differentiation was observed. Differentiated cells (days 11-13) were fixed with 4% paraformaldehyde (PFA, manufacturer?) and stained using a tartrate resistant acid phosphatase (TRAP) staining kit (Sigma-Aldrich, 387A-1KT) according to the manufacturer’s protocol with additional staining of the nuclei with DAPI. OC parameters were obtained by analysis of 20 images from random areas in each well; the OCs were observed with an Olympus ×83 microscope. A total of 480 frames were analyzed for 6638 OCs and 82,430 nuclei. OCs were defined as TRAP-positive cells harboring three or more nuclei and were counted in a double-blind manner, and the number of nuclei in the OCs and the total OC surface area were determined using ImageJ software.

*Bone resorption assays*

To assess bone resorption, 200 x 103 cells from PBMC cultures enriched for monocytes were cultured on 0.4 mm thick cortical bovine bone slices (BoneSlices.com, Jelling, Denmark) in differentiation media with either RANKL or TNFα (same conditions as in the differentiation assay above). Media was changed every two to three days and resorbtion was measured after 14 days. On day 14, bone slices were washed with PBS, cells were scraped, and the bone slices were stained with toluidine blue solution (Sigma-Aldrich, T3260)*.* At the end of the resorption experiment, the bone slice were imaged using an Olympus ×83 microscope. The eroded surface/bone surface (ES/BS) percentage was manually quantified. For each treatment, three bone slices were used.

*Statistics*

The data from the differentiation and resorption assays were analyzed for column statistics with GraphPad Prism version 8.4.3 for Windows (La Jolla, CA, USA). Data is shown as means ± SEM. Statistical significance was determined by column statistics (t-test) and ANOVA test analysis. A *p* value < 0.05 was considered statistically significant.

**Results**

The cherubism patients who donated their blood were two sisters aged 15 (P#1) and 19 (P#2). Both were diagnosed with cherubism and both exhibited classical symptoms including lesions in both jaws, mandibular dentition loss and hyperplastic appearance of the molars and mandibular rami and body. P#1 has lesions in both jaws, causing a hyperplastic appearance of the molars and mandibular rami and body. When P#2 began treatment at the clinic, she had already lost most of her mandibular dentition, presenting with only 12 remaining teeth intraorally. The detailed clinical, radiological and histological information of these specific patients has been described in a previous publication (Bar Droma et al., 2020). The father, who is asymptomatic, is 50 years old and while genetically diagnosed as a cherubism patient, he has no clinical symptoms now nor had in the past.

In humans, the physiological conditions characterized by enhanced resorption *in vivo* are reflected by enhanced fusion and resorption of OCs derived from blood monocytes (Møller et al., 2020a, 2020b). The differentiation potential and characteristics of PBMCs from human cherubism patients have not been studied before. For this purpose, we examined OC differentiation of PBMCs from the two cherubism patients, the asymptomatic carrier and three healthy controls. PBMCs from the donors’ blood samples were cultured for two days in the presence of M-CSF to enrich for monocytes and then transferred to culture plates and cultured for additional 12 days in the presence of M-CSF and RANKL to induce OC differentiation. Osteoclasts were defined as TRAP-positive cells containing more than two nuclei. The number of OCs, the area of OCs and the total number of nuclei were manually quantified. A total of 240 frames were analyzed for 3245 osteoclasts and 48,347 nuclei. The OC parameters of number, area and nuclei for the two cherubism patients and the asymptomatic carrier were similar (Figure 1A-C). However, these parameters were significantly different when the patients and the asymptomatic carrier were compared to the controls, with three-fold more OCs (Figure 1A), which contained two-fold more total nuclei (Figure 1B) and whose area was four-fold greater (Figure 1C). The cherubism patients and the asymptomatic carrier had giant OCs that contained hundreds of nuclei that were in an order of magnitude larger than the largest OCs observed in the healthy controls (Figure 1D-G).

We investigated next whether osteoclasts derived from PBMCs of cherubism patients and the asymptomatic carrier are more aggressive. For this purpose, we compared OC resorption *in vitro*. Blood samples were cultured for two days in the presence of M-CSF to enrich for monocytes and then an equal number of PBMCs were seeded on bone slices, supplemented with M-CSF and RANKL. After 12 days, the eroded surface (ES) that was formed by OCs was visualized by toluidine staining and manually measured. As in the differentiation experiment, the resorption area of the symptomatic and asymptomatic carriers was similar (Figure 2A and B). The resorption area of all carriers was on average 5.5-fold higher than that of healthy controls (Figure 2A and B).

The role of TNF-α in the etiology of cherubism is under debate and could be different in murine and human cherubism. Mukai et al. showed that monocytes derived from heterozygous KI mice are sensitive to TNF-α and can differentiate into osteoclasts independently of RANK-L (Mukai et al., 2014). However the ability of TNF-α to induce osteoclast differentiation of cells from cherubism patients has not been tested before. In order to evaluate TNF-α’s effect on PBMCs from whole blood samples from cherubism patients, we used exactly the same conditions as in the differentiation experiment described above with the exception that RANKL was substituted with TNF-α. After 14 days of differentiation, the number of OCs with more than two nuclei, the area of OCs and the total number of nuclei were manually quantified. Each treatment was performed in triplicate. A total of 240 frames were analyzed for 3,393 OCs and 34,083 nuclei.

TNFα induced a profound and significant increase in all OC differentiation parameters in the mutation carreirs compared to control. OC number were 15-fold higher in P#2 and the asymptomatic carrier (Figure 3A). P#1 had the highest amount of OCs that were 25-fold greater than in the matched healthy control and even significantly higher than in patient #2 and the asymptomatic carrier (Figure 3A). The analysis of total nuclei numbers within OCs showed the greatest difference between the patients and asymptomatic carrier and their matched healthy controls. The patients and asymptomatic carrier had on average 200-fold more nuclei in their osteoclasts than the healthy controls (Figure 3B). OCs from P#2 had significantly more nuclei in OCs than P #1 and the asymptomatic carrier (Figure 3B). As for the other parameters, the area covered by OCs from the patients and the asymptomatic carrier was much greater (four-fold) than the area covered by OCs from the healthy controls. No differences in OC area between the two patients and the asymptomatic control were observed. (Figure 3C). As for RANKL, TNF α induced the formation of extremely large osteoclasts containing hundreds of nuclei in cultures from both patients and the asymptomatic carrier (Figure 3D-G).

To determine whether formation of numerous giant OCs in response to TNFα correlates with enhanced resorption by these cells, we compared the resorption area in cultures of OCs derived from PBMCs from the two patients and the asymptomatic carrier. For this purpose, we used exactly the same conditions as for the resorption assay above except that again RANKL was substituted with TNFα. After 14 days of culture on bone slices the ES that was formed by OCs was visualized by toluidine staining and manually measured. No significant differences between the two patients and the asymptomatic carrier were observed (Figure 4A and B). In addition, in contrast to the differentiation experiment, no differences between the SH3BP2 mutation carriers and the healthy controls were seen.

Finally, we compared the effects of TNFα and RANKL on differentiation and resorption of OCs from the two patients, the asymptomatic carrier and the healthy controls. In all experiments the same conditions were used and the only difference was whether RANKL or TNFα was added. As expected culturing of PBMCs from healthy controls with RANKL produced significantly more osteoclasts that had more nuclei, were larger and resorbed more bone than PBMCs cultured with TNFα (Figure 5A-D). The effects of these cytokines (TNFα and RANKL) on OC numbers in cultures of PBMCs from the two cherubism patients and the asymptomatic carrier varied. P#1 formed significantly more OCs when cultured with TNFα than with RANKL. P#2 had small but significantly higher amount of OCs when cultured with RANKL. PBMCs from the asymptomatic carrier formed a similar amount of OCs with either of the cytokines in the media (Figure 5A). There were significantly greater amount of OC nuclei in PBMCs cultured with RANKL than with TNFα, except in the case of the asymptomatic carrier where they were similar (Figure 5B). Differences in area were seen only in the controls and P#1, with RANKL treatment resulted in larger OCs than TNFα treatment (Figure 5C). Bone resorption analysis showed the greatest differences between the effects of RANKL and TNFα in cultures of PBMCs from cherubism patients and resorption was much greater in cultures with RANKL. The asymptomatic carrier had higher values for bone resorption in cultures with RANKL, but this did not reach statistical significance (p = X), probably because of higher variation in the bone slices (Figure 5D).

**Discussion**

In this case study we evaluated the differentiation potential and aggressiveness of OCs derived from the peripheral blood from two patients with clinical symptoms of cherubism and from an asymptomatic carrier of the same SH3BP2 P418R mutation. To the best of our knowledge, in this study we are first to explore the differentiation of osteoclasts derived from the peripheral blood of patients with cherubism.

It has been shown that age and menopausal status correlate with the aggressiveness of OCs derived from peripheral blood (Møller et al., 2020b). These OCs seem to be reprogrammed as a result of the physiological conditions that drive towards more aggressive osteoclast behavior. Therefore, we sought to determine if the appearance of clinical symptoms of cherubism is correlated to aggressive behavior of osteoclasts *in vitro*. We show that upon stimulation with RANKL or TNFα, monocytes from cherubism patients form more OCs which are larger and contain a higher number of nuclei. These results are in accordance with studies showing increased differentiation potential of OCs from cherubism KI mice (Ueki et al., 2007). The greatest differences seen between the SH3BP2 P418R carriers and the healthy controls were the size of OCs and nuclei number; in some cases OCs containing more than 100 nuclei were observed. This observation suggests that the SH3BP2 P418R mutation promotes OC fusion. We and others have shown that OC fusion is a cell heterotypic process involving "fusion founder" and "fusion follower" cells (Hobolt-Pedersen et al., 2014; Levaot et al., 2015). We have also shown that the size of an OC could be determined by the fusion potency of both the founder and the follower cells (Guterman-Ram et al., 2018). The cellular and molecular mechanisms underlying SH3BP2 regulation of osteoclast fusion are still elusive, therefore increased fusion potential of monocytes from the peripheral blood of cherubism patients could be attributed to enhanced potential of the fusion founders or the fusion followers and this remains to be determined.

Monocytes from the asymptomatic carrier also formed higher numbers of larger OCs, which were similar to the osteoclasts formed by the symptomatic carriers. These data suggest that in humans the cherubism mutations in SH3BP2 increase the differentiation potential of osteoclast precursors, but this differentiation potential does not correlate with genetic penetrance in cherubism patients.

TNFα plays a pivotal role in driving systemic inflammation in cherubism KI mice (Ueki et al., 2007). It was shown that monocytes derived from the bone marrow of the KI mice can differentiate and resorb bone *in vitro* when cultured with TNFα in a RANKL-independent manner (Mukai et al., 2014). It was also found that bone resorbing osteoclasts are formed independently of RANKL *in vivo* when cherubism KI mice are crossed with RANKL-deficient mice (Kittaka et al., 2020). In humans high TNFα expression has been reported in some studies while another study did not show increased TNFα expression (Hero et al., 2013; Mukai et al., 2014; Kadlub et al., 2018). Our data show in contrast to monocytes from healthy donors, monocytes from carriers of the SH3BP2 P418R mutation form high amounts of TRAP-positive multinucleated cells when RANKL is substituted with TNFα. The amount, multinucleation and size of these cells is similar to those of the cells formed when these monocytes are cultured with RANKL. However, monocytes from carriers of the SH3BP2 P418R mutation did not resorb more bone than those from the healthy donors when cultured with TNFα as they did in the presence of RANKL. Our findings indicate that as seen in mice, TNFα can potentiate monocyte precursors and drive differentiation of OCs independent of RANKL, but in contrast to mice, TNFα is not sufficient to induce aggressive resorption. Treatment of patients with anti-TNFα drugs decreased its expression in cherubism bone lesions without any apparent clinical improvement (Hero et al., 2013). On the other hand, treatment of cherubism patients with anti-RANKL antibody induced regression of bone lesions and improved the clinical outcome (Bar Droma et al., 2020). Thus, these studies and our observation underline the differences between mouse and humans in regard to the role of TNFα in the etiology of cherubism and suggest that while TNFα can induce OC differentiation, formation and progression of bone lesions, in cherubism these processes are TNFα-independent but RANKL-dependent.

**Study limitations**

Cherubism is a very rare disease with only a few hundred cases reported worldwide. Therefore, the sample size in this study and in other cherubism studies is low. The asymptomatic carrier in the study was a male in his fifties?, while the symptomatic carriers were younger females. It has been reported that aging is associated with higher aggressiveness of OCs and therefore the high aggressiveness seen in the OCs from the asymptomatic carrier could be due to his age. Nevertheless, the amount of nuclei and the size of OCs in both the symptomatic patients and the asymptomatic carrier was an order of magnitude higher than that previously reported for OCs from human donors of the age of the asymptomatic carrier and older. Thus, the close similarity between symptomatic and asymptomatic carrier.

Overall our study found that OCs derived from cherubism patients are extremely large and resorb greater amounts of bone *in vitro*. We showed that the aggressive activity of OCs derived from peripheral blood is similar in the patients and the asymptomatic carrier. These observations suggest that the genetic penetrance in cherubism is not intrinsic to the OC but is driven by the microenvironment or systemic factors. The observation that the asymptomatic carrier of the SH3BP2 P418R mutation had PBMCs with enhanced differentiation potential that produced extremely large and aggressive OCs *in vitro* raises the possibility that overall osteoclastogenesis is restrained by an unknown protective mechanism. Thus, systemic and/or micro-environmental factors likely determine the appearance and severity of symptoms in cherubism patients and should be the focus of future research.

# Conflict of Interest

*The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest*.

# Author Contributions

The Author Contributions section is mandatory for all articles, including articles by sole authors. If an appropriate statement is not provided on submission, a standard one will be inserted during the production process. The Author Contributions statement must describe the contributions of individual authors referred to by their initials and, in doing so, all authors agree to be accountable for the content of the work. Please see [here](http://home.frontiersin.org/about/author-guidelines#AuthorandContributors) for full authorship criteria.

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**Figure legends**

Figure 1: Osteoclasts from symptomatic and asymptomatic carriers of cherubism mutations are more frequent and larger compared to osteoclasts from healthy donors.

Human PBMCs were cultured in differentiation medium containing RANKL for 14 days. Cells were fixed and stained for TRAP and DAPI. (A-C) Cells were examined for (A) number of OCs/well, (B) number of nuclei within OCs/well, and (C) surface area of OCs/well. Data are means ± SEM of triplicates. A total of 60 frames were analyzed for each group and total of 180 frames were analyzed for the control. (D-G) Frequency distribution of number of nuclei per OC for each patient or healthy control (D, Control; E, P#1; F, P#2; G, asymptomatic). Data was analyzed by one way ANOVA; the relevant p value is present on each column and it is compared to the control sample.

Figure 2: Osteoclasts derived from carriers of cherubism mutations resorb more bone compared to osteoclasts from healthy donors.

Human PBMCs were cultured in differentiation medium containing RANKL for 14 days. (A) Representative bone slice (low and high magnification); scale bar length is 50 µm. (B) The percentage eroded surface (ES) per total bone surface (BS). Data are means ± SEM of triplicates. Data was analyzed by t-test; the relevant p value is present on each column and it is compared to the control.

Figure 3: TNFα induces OC formation in cultures of PBMCs from cherubism mutation carriers in a RANKL-independent manner.

Human monocytes were cultured in a differentiation medium containing TNFα instead of RANKL. After 14 days cells were fixed and stained for TRAP and DAPI. (A-C) Cells were examined for (A) Number of OCs per well, (B) Average number of nuclei within OCs per well, and (C) Average surface area of OCs per well. Data are means ± SEM of triplicates. A total of 60 frames were analyzed for each group (20 frames/well). (D-G) Frequency distribution of number of nuclei per OC for each patient or healthy control (D, Control; E, P#1; F, P#2; G, asymptomatic). Data was analyzed by one way ANOVA; the relevant p value is present on each column and it is compared to the control.

Figure 4: Osteoclasts derived from cherubism mutation carriers do not resorb more bone than osteoclasts from healthy donors when cultured with TNFα

Human monocytes were cultured in a differentiation medium containing TNFα for 14 days. (A) Representative bone slice (low and high magnification); scale bar length is 50 µm. (B) The percentage eroded surface (ES) per total bone surface (BS). Data are means ± SEM of triplicates. No significance differences were found between the different groups.

Figure 5: A comparison of TNF α and RANKL effects on osteoclast derived from PBMCs of symptomatic and asymptomatic cherubism patients and healthy controls.

Differentiation and resorbing data from Figures 1-4 are present as a comparison between TNF α and RANKL treatment on OC formation. (A) Average number of OCs, (B) Average number of nuclei within OCs, and (C) Average surface area of OCs (D) The percentage eroded surface (ES) per total bone surface (BS). Data was analyzed by t-test comparing treatment with TNF α and RANKL for each patient or healthy control. The relevant p value is present between columns.