**Osteoclasts derived from blood of either symptomatic or asymptomatic carriers of SH3BP2 mutations causing cherubism 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 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 and not macrophages in cherubism bone lesions is correlated to the severity of the symptoms. However, it is unknown whether the level of giant cell in cherubism lesions derive 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. PBMCs from all carriers formed osteoclasts that were an order of magnitude bigger than osteoclasts from healthy donors when cultured with either RANKL or 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 by TNFα is not correlated with enhanced osteoclast aggression in humans and in accordance with a study showing reduced multinucleated giant cells with 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 patients appear 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, tooth agenesis. The severity of the clinical symptoms is variable and could 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 on 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 and is rich in multinucleated giant cells (MGCs), some of which are in contact with the bone and 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, c-fms, Itams, and the RANK receptor with downstream activation of Src, Syk and Vav-family protein kinases followed by activation of the osteoclast master transcription factor NFATc1 (Deckert et al., 1998; Maeno et al., 2003; Foucault et al., 2005; Deckert and Rottapel, 2006; Levaot et al., 2011a). The causative role for 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 showed that SH3BP2 serve as a substrate for the poly-ADP-ribosyltransferases Tankyrase 1 and 2. Tankyrases bind SH3BP2 and poly-ADP-ribosylate it (Parsylate). 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 Tankyrases binding which is followed by the stabilization of SH3BP2 and enhanced signaling cascade involving Src and Syk kinases. These changes result in hyperactive and aggressive osteoclasts with enhanced bone resorption (Levaot et al., 2011b). Unlike human SH3BP2 heterozygotes, heterozygous mice do not exhibit any cherubism phenotype. The homozygous KI mouse has high circulating Tumer 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 osteoclast markers to the aggressiveness of the bone lesions (Kadlub et al., 2018). However, it is not known if increased osteoclast numbers in more aggressive cherubism bone lesions is a result of an increased differentiation potential of osteoclast precursors or increased local osteoclastogenic signals.

To address this question, we compared the differentiation and resorption potential of osteoclast 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 hospital (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, that were diagnosed with cherubism. The diagnostic was confirmed by a genetic examination which showed a P418R mutation in SH3BP2. Cells were also taken from blood of the father that was found to be an asymptomatic carrier of this mutation. For control blood from three donors, one man and two women ages 26, 24 and 25 respectively were used.

***In vitro generation of human osteoclasts***

Mononuclear cells (PBMCs) were isolated from peripheral blood samples and differentiated to mature osteoclasts (OCs) as follow: whole blood samples were collected to CPT tubes and monocytes were separated according to the manufacture protocol (*BD Vacutainer, 362782*). To enrich for monocytes 25x106 PBMCs were seeded in T75 culture flasks supplemented with 25ng/ml M-CSF (R&D systems, 216-MC) for 2 days, then cells were detached from the flask using Accutase (Sigma, [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).

**Osteoclasts differentiation assay**

To assess osteoclast differentiation 75x103 cells from PBMCs cultures enriched for monocytes were cultured in 96 well plates in differentiation media (αMEM; M8042 sigma, 10% FBS, 5% Penstrep and l-glu , BI) that was supplemented with 25ng/ml M-CSF (R&D systems, 216-MC) and either 25ng/ml RANKL (R&D systems, 390-TN) or TNFα 100ng/ml (R&D systems, 210-TA) Media was changed every 2-3 days and differentiation was observed. Differentiated cells (days 11-13) were fixed with 4% PFA 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. Osteoclast parameters were obtained by analysis of 20 images from random areas in each well; the osteoclasts were observed with an Olympus ×83 microscope. A total of 480 frames were analyzed for 6638 osteoclasts and 82,430 nuclei. Osteoclasts 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 osteoclasts and the total osteoclast surface area were determined using ImageJ software

**Bone resorption assays**

To assess bone resorption 200x103 from PBMCs 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 above). Media was changed every 2-3 days and resorbtion was measured after 14 days. At day 14 bone slices were washed with PBS, cells were scraped, and the bone slice were stained with Toluidine blue solution (Sigma, T3260)*.* At the end point of resorption experiment the bone slice were imaged using Olympus ×83 microscope. The percentage of eroded surface/bone surface (ES/BS) 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 blood donating cherubism patients were two sisters 15 and 19 years old ( i.e. P#1 and P#2 respectively). Both were diagnosed for cherubism and both exhibited classical symptoms like, lesions in both jaws, mandibular dentition loss and hyperplastic appearance of the molars and mandibular rami and body. P#1 have exhibited lesions in both jaws, causing a hyperplastic appearance of the molars and mandibular rami and body. While P#2 when starting the treatment in the clinic had already lost most of her mandibular dentition, presenting with only 12 remaining teeth intraorally. A detailed clinical, radiological and histological information of these specific patients have been described in a previous publication (Bar Droma et al., 2020). The father (i.e. asymptomatic) is 50 years old genetically diagnosed as a cherubism patient but did not have any clinical symptoms now or in the past.

In humans, physiological conditions characterized by enhanced resorption in vivo are reflected by enhanced fusion and resorption by osteoclasts derived from blood monocytes (Møller et al., 2020a, 2020b). The differentiation potential and characteristics of PBMCs from human cherubism patients has not been studied before. For this purpose, we examined osteoclast 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 osteoclast 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. Osteoclast parameters of the two cherubism patients and the asymptomatic carriers had similar OCs number, area and nuclei (Figure 1A-C). When compared to controls the patients and asymptomatic carriers had significantly 3 fold osteoclasts (Figure 1A) that had two fold total nuclei (Figure 1B) and were 4 fold area (Figure 1C).

The cherubism patients and the asymptomatic carrier had giant osteoclasts that contained hundreds of nuclei, that were in an order of magnitude bigger than the biggest osteoclasts observed in the healthy controls (Figure 1D-G).

We next asked if osteoclasts derived from PBMCs of cherubism patients and the asymptomatic carrier are more aggressive. For this purpose we compared osteoclast 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 slice, supplemented with M-CSF and RANKL.After 12 days eroded surface (ES) that was formed by OCs was visualized by toluidine staining and manually measured. As for 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 in average 5.5 folds higher then healthy control (Figure 2A and B).

The role of Tumor Necrosis Factor α (TNF-α) in the etiology of cherubism is under debate and could be different between 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 was not tested before. In order to evaluate TNF-α effect on PBMCs from whole blood samples of cherubism patients, we used exactly the same conditions as in the differentiation experiment described above with the exception that RANKL was substituted by TNF-α. After 14 days of differentiation the number of OCs with more than 2 nuclei, the area of osteoclasts and the total number of nuclei were manually quantified. Each treatment was performed in triplicates. A total of 240 frames were analyzed for 3,393 osteoclasts and 34,083 nuclei.

As compared to control TNFα induced a profound and significant increase in all osteoclast differentiation parameters. Osteoclasts number were 15 fold higher in patient #2 and the asymptomatic carrier (Figure 3A). Patient #1 had the highest amount of osteoclast numbers that were 25 fold higher than the healthy control and even significantly higher than their numbers in patient #2 and the asymptomatic carrier (Figure 3A). The analysis of total nuclei numbers within osteoclasts showed the biggest difference between the patients and asymptomatic carrier to the healthy control. The patients and asymptomatic had in average 200 folds more nuclei in their osteoclasts than the healthy control (Figure 3B). Osteoclasts from patient #2 had significantly more nuclei in osteoclasts than patient #1 and the asymptomatic control (Figure 3B). As for the other parameters, the area covered by osteoclasts from the patients and the asymptomatic control was much bigger (4 fold) than the area of the healthy controls. No differences in osteoclasts 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 control (Figure 3D-G).

To determine if formation of numerous giant osteoclasts by TNFα is in correlation with enhanced resorption by these cells we compared the resorption area in cultures of osteoclasts derived from PBMCs of 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 by TNFα. After 14 days of culture on bone slices eroded surface (ES) that was formed by OCs was visualized by toluidine staining and manually measured. No significant differences between the two patients to the asymptomatic carrier were observed (Figure 4A and B). In addition, as opposed to the differentiation experiment no differences between the SH3BP2 mutation carriers and the healthy controls were detected.

Finally, we compared the effects of TNFα and RANKL on differentiation and resorption of osteoclasts from the two patients, the asymptomatic carrier and healthy controls. In all experiments the exact conditions were used and the only difference was the addition of RANKL or TNFα. As expected culturing of PBMCs from healthy controls with RANKL produced significantly more osteoclasts that had more nuclei, were bigger and resorbed more bone than PBMCs cultured with TNFα (Figure 5A-D). The effects of the cytokines (TNFα and RANKL) on osteoclasts numbers in cultures of PBMCs from the tow cherubism patients and the asymptomatic carrier varied. Patient #1 formed significantly more osteoclasts when cultured with TNFα compared to RANKL. Patient two had slightly but significant higher amount osteoclasts in the cultures with RANKL. While PBMCs from the asymptomatic formed similar amount of osteoclasts regardless of the cytokine in the media (Figure 5A). Osteoclast nuclei number were significantly higher for PBMCs cultured with RANKL compared to cultures with TNFα, except for the asymptomatic carrier were they were similar (Figure 5B). Differences in area were seen only for the controls and P#1 were RANKL produced bigger osteoclasts than TNFα (Figure 5C). Bone resorption analysis showed the biggest differences between the effects of RANKL and TNFα in cultures of PBMCs from cherubism patients and was much higher in cultures with RANKL. The asymptomatic carrier had higher values of bone resorption in cultures of RANKL but it did not reach a statistical significant p value (X) probably because of increased variation in the bone slices Figure 5D).

**Discussion**

In this case study we evaluated the differentiation potential and aggressiveness of osteoclasts derived from peripheral blood of two patients with clinical symptoms of cherubism to an asymptomatic carriers 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 was shown that age and menopausal status correlate with the aggressiveness of osteoclasts derived from peripheral blood(Møller et al., 2020b). These osteoclasts are likely reprogrammed as a result of the physiological condition that push toward a 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 higher number of osteoclasts which are bigger and contain higher number of nuclei. These results are in accordance with studies showing increased differentiation potential of osteoclasts from the cherubism KI mice (Ueki et al., 2007). The biggest differences between the SH3BP2 P418R carriers to the healthy controls was the size of OCs and nuclei number , in some cases osteoclasts containing more than 100 nuclei were observed. This observation suggests that the SH3BP2 P418R mutation promote osteoclast fusion. We and others have shown that osteoclast fusion is a cell heterotypic process involving "fusion founder" and "fusion follower" cells (Hobolt-Pedersen et al., 2014; Levaot et al., 2015). We also showed that the size of osteoclast could be determined by both the fusion potency of founder and follower cells (Guterman-Ram et al., 2018). The cellular and molecular mechanisms underlying SH3BP2 regulation of osteoclast fusion are still elusiverefore, increased fusion potential of monocytes from the peripheral blood of cherubism patients could be attributed to either enhanced potential of fusion founder and fusion follower cells and remained to be.

Monocytes from the asymptomatic carrier also formed higher numbers of bigger osteoclasts 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 is not in correlation with genetic penetrance in cherubism patients.

 In Cherubism KI mice TNFα plays a pivotal role in driving systemic inflammation ((Ueki et al., 2007).It was shown that monocyte 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 shown 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 were 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 that as opposed to monocytes from healthy donors, monocytes from carriers of the SH3BP2 P418R mutation form high amount of TRAP positive multinucleated cells when RANKL is substituted by TNFα. The numbers, multinucleation and size of these cells is similar to 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 the healthy donor when cultured with TNFα as like they did when in the presence of RANKL. Our data show that as in mice TNFα can potentiate monocyte precursors and drive differentiation of osteoclasts independent of RANKL but opposed 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 the 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 human in regard to the role of TNFα in the etiology of cherubism and suggest that while TNFα can induce osteoclast differentiation, formation and progression of bone lesions in cherubism is TNFα independent and RANKL dependent.

Study limitations

Cherubism is a very rare disease with only a few hundreds of cases reported worldwide. Therefore, the sample size reported in this study and other cherubism studies is low. The asymptomatic carrier in the study was a male in his fiftieths? while the symptomatic carriers were younger females. It has been reported that aging is associated with higher aggressiveness of osteoclast and therefore the high aggressiveness of the males could be affected by his older age. Nevertheless, the nuclei number and size of osteoclasts in both the symptomatic and the asymptomatic carriers was in an order of magnitude higher than what has been reported for osteoclasts from human donors at the age of the asymptomatic and older. Thus, the close similarity between symptomatic and asymptomatic carrier.

Overall our study show that osteoclast derived from cherubism patients are extremely large and resorb higher amount of bone *in vitro*. We show that the aggressive activity of osteoclasts 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 osteoclast and driven by the microenvironment or systemic factors. The observation that the asymptomatic carrier of the SH3BP2 P418R mutation have PBMCs with enhanced differentiation potential that produce extremely large and aggressive osteoclasts 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 specific p value is present on each column and it is vs. 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 50µm. (B) the percentage eroded surface (ES) per total bone surface. Data are means ± SEM of triplicates. Data was analyzed by t-test, specific p value is present on each column and it is vs. the control.

Figure 3: TNFα induces osteoclast 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 specific p value is present on each column and it is vs. 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 50µm. (B) The percentage eroded surface (ES) per total bone surface. Data are means ± SEM of triplicates. No significance change was found between the different groups.

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

Differentiation and resorbing data from Figures 1-4 are present as a comparison between TNF α and RANKL treatment on OCs 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. Data was analyzed by t-test compering between the TNF α and RANKL treatment on each patient or healthy control. Specific p value is present between columns.