The effect of clodronate on a Mevalonate Kinase Deficiency cellular model.

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ABSTRACT

The clodronate, an aminobisphosphonate, showed a potential anti-inflammatory role, able to antagonize the pro-inflammatory effects of mevalonate pathway's block, the main feature of Mevalonate Kinase Deficiency (MKD). In order to assess the potential anti-inflammatory effect of clodronate used at several concentrations (1, 10 and 100 μM) we evaluated the nitric oxide production using Griess Reagents and programmed cell death by flow cytometry, commonly assumed as inflammatory parameters in Mevalonate Kinase Deficiency.

Preliminary data obtained on Raw 264.7 cell line and monocytes isolated from two mevalonate kinase deficiency patients, suggest that clodronate is not able to reduce the main mevalonate kinase deficiency markers, but it increases the epiphenomena of the disease. In particular MKD monocytes showed increased cell apoptosis and nitric oxide production in comparison with MKD cell patients treated with clodronate (cell apoptosis: 21.12 ± 2.08 % vs 46.39 ± 0.61 %; $p < 0.05$; nitric oxide production: 27.93 ± 0.59 μM vs 40.94 ± 4.39 μM; $p < 0.05$).

KEY WORDS:

Mevalonate kinase deficiency, clodronate, programmed cell death and nitric oxide.

INTRODUCTION

Mevalonate Kinase Deficiency (MKD) (OMIM #610377), a rare auto-inflammatory disease, is caused by mutations in the second enzyme of mevalonate pathway (mevalonate kinase, *MK*/*MVK*) resulting in reduced enzymatic activity and consequent shortage of downstream compounds. The main MKD pathogenic mechanism is lack of mevalonate intermediate compound geranylgeranyl-pyrophosphate, resulting in the increased caspase-1 activation and IL-1β release [1, 2].

We recently described *in vivo* and *in vitro* models of MKD [3, 4] chemically obtained treating mice and cells with several compounds such as aminobisphosphonate (i.e. alendronate) and statins (i.e. lovastatin), able to block the mevalonate pathway reproducing biochemical features similar to those found in MKD (Fig. 1). In these models, bacterial compounds such as muramyldipeptide or lipopolysaccharide (LPS) are capable to trigger strong inflammatory attacks, similarly to what described in the acute phase of MKD patients [5]. Moreover, we recently demonstrated that in the cellular model of MKD, LPS amplifies the effect of mevalonate pathway inhibition on both programmed cell death (PCD) and nitric oxide production (NO), and not only on IL-1ß production, as previously reported [6, 7].

Although in the last decade the knowledge of MKD pathogenesis has increased, an etiologic treatment for MKD is still unavailable and anti-inflammatory drugs [5, 8], as well as novel biologic therapies [8, 9], are currently being used with heterogeneous and often contradictory results.

Recently Shikama et al. [9] described the potential anti-inflammatory role of clodronate in an experimental condition comparable to our cellular MKD model obtained treating Raw 264.7 cell line with alendronate and LPS.

The observation that clodronate could antagonize the pro-inflammatory effects of the mevalonate pathway's block prompted us to evaluate the activity of this drug in our MKD *in vitro* model, and in monocytes isolated from two MKD patients, in order to determine if it could provide new insights for the treatment of MKD, a still orphan drug disease [3, 4, 7].

MATERIALS AND METHODS

Reagents. Lypopolysaccharide (LPS, E.Coli-serotype 055:B5, 10μg/ml in H_2O), alendronate endotoxin free (ALD) and clodronate endotoxin free (CLO) from Sigma-Aldrich (Milano, Italy) were dissolved in sterile saline solution (pH 7.0) (Diaco SpA, Trieste, Italy).

Raw 264.7 Cell Culture. $2x10^5$ Raw 264.7 cells (murine monocyte/macrophage cell line) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), clodronate (1, 10 and 100 μM) and alendronate (100 μM) for 20 hours. 10 μg/ml LPS were then added and the incubation continued for additional 24 hours.

Healthy Donors and MKD Human peripheral blood monocytes. Monocytes isolated from healthy subjects (n=5: 3 males, mean age 18.67 ± 0.33, and 2 females, mean age 18.5 ± 0.5) and monocytes isolated from 2 MKD subjects (Patient 1, P1: 6 years old, male, *MVK*: S135L/V377I; Patient 2, P2: 8 years old, male, *MVK*: G336S/G336S) were cultured at $2x10^5$ cells/well in RPMI 1640-10% FBS (Euroclone, Milano, Italy) with or without 100 μM ALD and/or (1, 10, 100 μM) CLO for 20 hours and then with 1 μg/ml LPS for additional 24 hours. Written informed consent was obtained from either donors or patients (or their parents) according to the ethical board protocol of the Institute for Maternal and Children Health – IRCCS "Burlo Garofolo" (Trieste, Italy) (n.185/08, 19/08/2008).

Evaluation of Programmed Cell Death. To evaluate apoptosis after culture, cells were stained with FITC-labelled Annexin V and analyzed by flow cytometry. The percentage of annexin positive cells (A+) was then determined using FlowJo software (Treestar Inc, Ashland, OR).

Determination of NO production. After cell incubation period, the supernatants were collected for the evaluation of Nitric Oxide production. NO was assayed on supernatants using Griess Reagents (Sigma Aldrich, USA) according to the instructions of the manufacturer.

Data analysis. All results are expressed as means ± SD. One-way analysis of variance (ANOVA) and Bonferroni's multiple comparisons test were used for statistical significance calculation. Statistical analyses were performed using the Graphpad Prism software (version 5.0).

RESULTS

We studied the programmed cell death and nitric oxide production as biological parameters to evaluate the capacity of clodronate to contrast the inflammatory alendronate/LPS-induced effect, by treating cells with incremental doses of clodronate (1, 10 and 100 μM). Clodronate concentrations higher than 100 μM, were cytotoxic both in Raw 264.7 cells, our *in vitro* model of MKD, and in monocytes from MKD patients. Consequently, a maximum concentration of 100 μM was used in all subsequent experiments.

When Raw 264.7 cells were treated with LPS, a significant increase in PCD was detected in comparison with untreated cells (10.81 ± 0.81 % vs. 4.71 ± 0.42 %; $p < 0.01$) and the addition of clodronate 100 μM to LPS treated cells further significantly increased PCD (25.21 ± 3.09 %; $p < 0.001$) (Fig. 2A). After the Raw 264.7 stimulation with ALD+LPS, a significant PCD increase was detected in comparison with untreated cells (39.35 ± 4.95 % vs. 4.71 ± 0.42 %; $p < 0.001$) (Fig. 2B). The addition of CLO induced an increased PCD, if compared to ALD+LPS-treated cells, that became significant only at the concentration of 10 and 100 μM (ALD+LPS+CLO 10: 51.40 ± 1.6 $p < 0.05$; ALD+LPS+CLO 100, $p < 0.001$).

Using the same experimental conditions, we found (Fig. 2C) a significantly augmented NO production in LPS-treated cells in comparison with untreated cells, even more pronounced after addition of increasing doses of clodronate (CLO 1 μM: 27.21 ± 0.62 μM, no significative, ns; CLO 10 μM: 30.88 ± 0.12 μM, $p < 0.05$ and CLO 100 μM: 33.31 ± 0.73 μM, $p < 0.01$). ALD+LPS-treated cells showed an increased NO production when compared with untreated cells (26 ± 1 μM vs. 10.22 ± 1.877 μM; $p < 0.001$). Once more CLO was able to increase NO production and the increase was statistically significant for all three concentrations of CLO when compared to ALD+LPS-treated cells (Fig. 2D) (CLO 1 μM: 32.29 ± 0.28 μM, $p < 0.05$; CLO 10 μM: 33.07 ± 0.071 μM, $p < 0.05$ and CLO 100 μM: 36.40 ± 0.4 μM, $p < 0.01$).

When treating monocytes from healthy donors with LPS and CLO 100 μM, a significant increase of PCD was found in comparison to monocytes treated only with LPS (45.81 ± 0.61 % vs. 23.03 ± 0.69 %; $p < 0.01$) (Fig. 3A). ALD+LPS induced a significant PCD increase, as expected [4], when comparing the results with those obtained on untreated cells (Fig. 3B). A significant NO production increase was detected in LPS (Fig. 3C) and LPS+ALD treated cells (Fig. 3D) in comparison with untreated cells. A further NO increase was detected when cells were treated with ALD+LPS+CLO 100 μM (39.80 ± 1.75, $p < 0.01$).

Since ALD was able to mimic the effect of genetic mutations in monocytes MKD patients, we considered ALD+LPS-treated healthy cells as mimicking LPS-treated MKD cells, due to the impairment of the mevalonate pathway deriving from ALD treatment.

LPS-treated MKD monocytes, showed increased cell apoptosis in comparison with untreated cells (21.12 ± 2.08 % vs. 7.59 ± 1.38 %; $p < 0.05$); a further PCD increase was detected in presence of LPS and CLO 100 μM (46.39 ± 0.61 %; $p < 0.05$) (Fig. 3E). Moreover, CLO (10 μM and 100 μM) induced an increased NO production in comparison with LPS-treated cells (CLO 10 μM: 34.57 ± 2.66 μM; CLO 100 μM 40.94 ± 4.39 μM vs. 27.93 ± 0.59 μM ; $p < 0.05$) (Fig. 3F).

DISCUSSION

Defective mevalonate kinase activity, leading to shortage of downstream compounds in the mevalonate pathway, such as isoprenoids (Fig. 1), characterizes Mevalonate Kinase Deficiency. *In vivo* and *in vitro* studies demonstrated that clodronate is able to reduce inflammation [10]. Based on these findings, we hypothesized that clodronate, rescuing inflammatory phenotype related to MKD, could represent a possible therapeutic compound for this disease. However, in our MKD cellular model using Raw 264.7 cell line and monocytes isolated from healthy donors, the *in vitro* treatment with clodronate was associated with increased levels of programmed cell death, suggesting the lack of anti-inflammatory action for this compound. This finding is further confirmed by experiments performed in the same cellular model aimed to detect NO production, which significantly increased when clodronate was used. Clodronate activity was subsequently tested in monocytes isolated from two patients with MKD. Despite the low number of patients, due to the difficulty in obtaining samples from individuals with this rare pathological condition, also in the cells of this disease-model from MKD patients, clodronate did not induce a decrease of PCD and NO production, but, in contrast, increased the percentage of apoptotic cells and NO production.

Based on our previous study, PCD and NO production [6, 7] are parameters suitable for assessing the inflammatory phenotype associated with MKD disease. Our findings give an overview of the inflammatory effects of clodronate and indicate that this compound, in our *in vitro* MKD cellular model, is not able to rescue the main marker of MKD but it increases the epiphenomena of the disease.

Finally, considering our findings, we suggest that clodronate is not able to antagonize the alendronate-dependent effect, and this lack of antagonist activity is also confirmed in monocytes of MKD patients, where clodronate increases the inflammatory effects due to the blockade of the endogenous pathway of mevalonate.

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LEGENDS

Fig.1 Schematic representation of mevalonate pathway

Schematic representation of the mevalonate pathway: mevalonate kinase is the second enzyme of the mevalonate pathway for the biosynthesis of cholesterol and non-sterol isoprenoids

Fig.2 Apoptosis (A+) and nitric oxide (NO) production after clodronate (CLO) treatment in Raw 264.7-treated cells

Raw 264.7 cells were stimulated with 1/10/100 µM CLO (2A, 2C) and/or with 100 µM ALD (2B, 2D) for 20 h; 10 µg/ml LPS was added for supplementary 24 h.

A+ and production of NO (µM) were measured and bars represent the means ± SD of 3 independent experiments.

(A, C) Analyses were performed with one way ANOVA and Bonferroni correction comparing LPS-treated cells with other experimental conditions; ** $p < 0.01$ and *** $p < 0.001$.

(B, D) Analyses were performed with one way ANOVA and Bonferroni correction comparing ALD-LPS treated cells with other experimental conditions; * $p<0.05$, ** $p < 0.01$ and *** $p < 0.001$

Fig.3 Apoptosis (A+) and nitric oxide (NO) production after clodronate (CLO) treatment in monocyte-treated cells

Monocytes from healthy donors were stimulated with 1/10/100 µM CLO (3A, 3C, 3E) and/or 100 µM ALD (3B, 3D, 3F) for 20 h. 1 µg/ml LPS was added for supplementary 24 h.

Monocytes from MKD patients (P1 and P2) were stimulated with 1/10/100 µM CLO (3E, 3F) for 20 h and/or with 10 µg/ml LPS for supplementary 24 h. Apoptosis (A+) and production of NO (µM) were measured and bars represent the means ± SD of 3 indipendent experiments.

(A, C, E, F) Analyses were performed with one way ANOVA and Bonferroni correction comparing LPS-treated cells with other experimental conditions; * $p<0.05$, ** $p < 0.01$.

(B, D) Analyses were performed with one way ANOVA and Bonferroni correction comparing ALD-LPS treated cells with other experimental conditions; * $p<0.05$ and ** $p < 0.01$

REFERENCES

1. Kuijk LM, Beekman JM, Koster J, et al. HMG-CoA reductase inhibition induces IL-1beta release through Rac1/PI3K/PKB-dependent caspase-1 activation. Blood 2008;112:3563-73.

2. Mandey SH, Kuijk LM, Frenkel J, et al. A role for geranylgeranylation in interleukin-1beta secretion. Arthritis Rheum. 2006;54:3690-5.

3. Marcuzzi A, Pontillo A, De Leo L, et al. Natural isoprenoids are able to reduce inflammation in a mouse model of mevalonate kinase deficiency. Pediatr Res. 2008;64:177-82.

4. Bodar EJ, van der Hilst JC, Drenth JP, et al. Effect of etanercept and anakinra on inflammatory attacks in the hyper-IgD syndrome: introducing a vaccination provocation model. Neth J Med. 2005;63:260-4.

5. Marcuzzi A, Piscianz E, Girardelli M, et al. Defect in mevalonate pathway induces pyroptosis in Raw 264.7 murine monocytes. Apoptosis 2011;16:882-8.

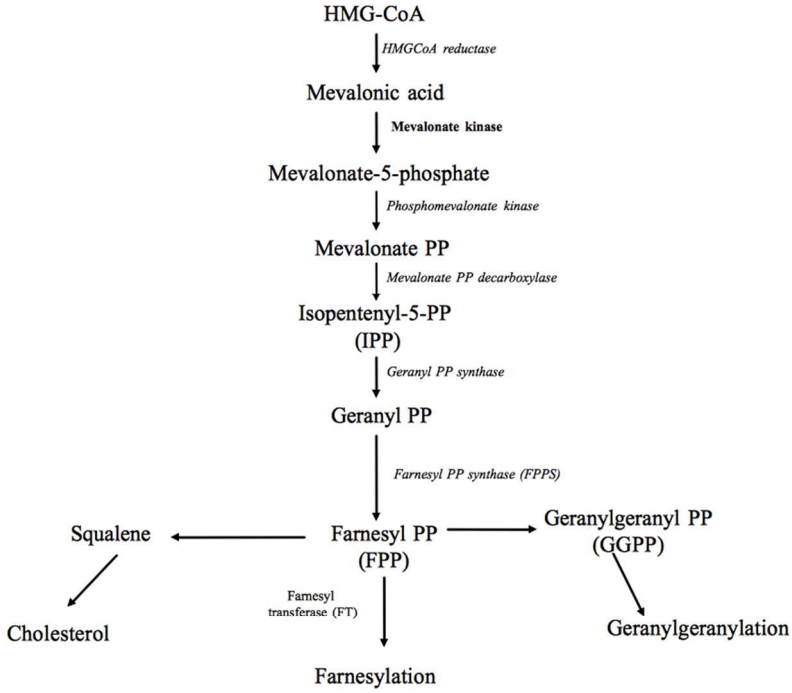
6. Marcuzzi A, Tommasini A, Crovella S et al. Natural isoprenoids inhibit LPS induced-production of cytokines and nitric oxide in aminobisphosphonate-treated monocytes. Int Immunopharmacol. 2010;10:639-42.

7. Cailliez M, Garaix F, Rousset-Rouviere C, et al. Anakinra is safe and effective in controlling hyperimmunoglobulinaemia D syndrome-associated febrile crisis. J Inherit Metab Dis. 2006;29:763.

8. van der Hilst JC, Bodar EJ, Barron KS, et al. Long-term follow-up, clinical features and quality of life in a series of 103 patients with hyperimmunoglobulinemia D syndrome. Medicine 2008;87:301-10.

9. Shikama Y, Nagai Y, Okada S, et al. Pro-IL-1beta accumulation in macrophages by alendronate and its prevention by clodronate. Toxicol Lett. 2010;199:123-8.

10. De Leo L, Marcuzzi A, Decorti G, et al. Targeting farnesyl-transferase as a novel therapeutic strategy for mevalonate kinase deficiency: in vitro and in vivo approaches. Pharmacol Res. 2010;61:506-10.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(IPP)
Geranyl PP synthase
Geranyl PP
Farnesyl PP synthase (FPPS)
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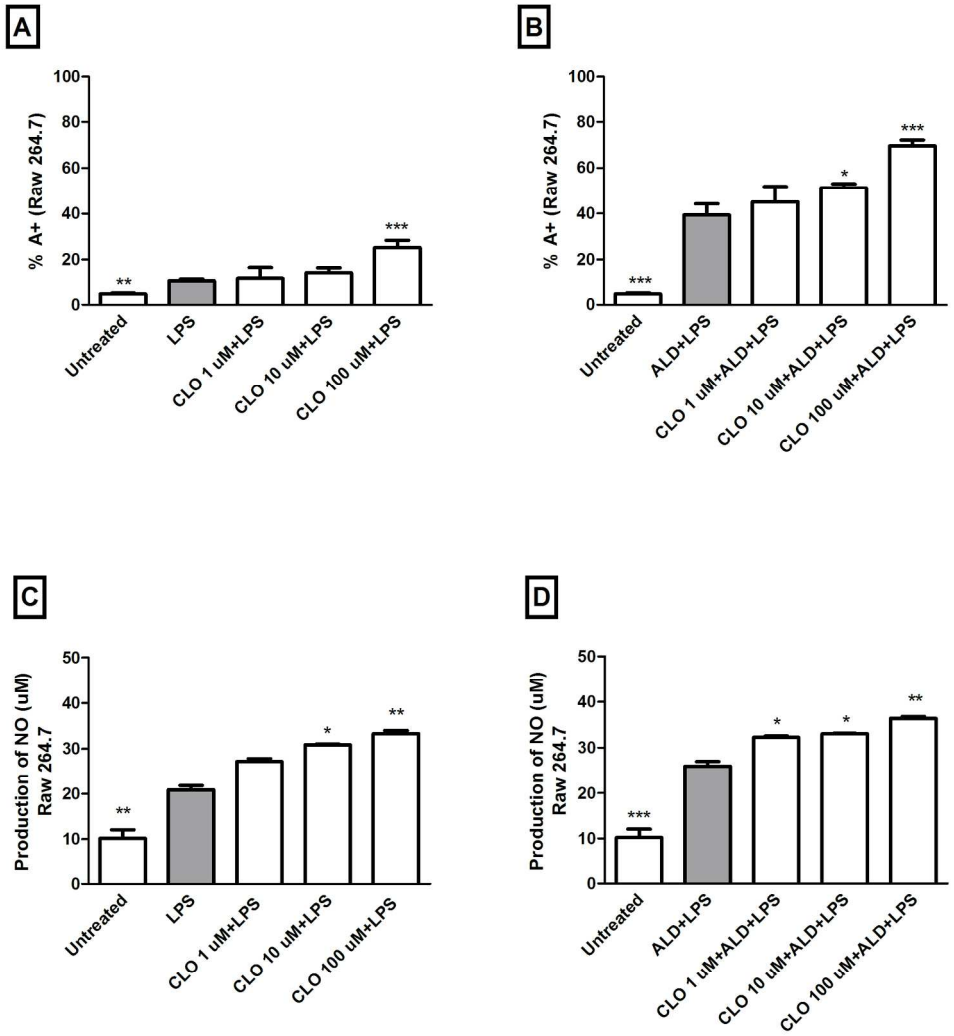

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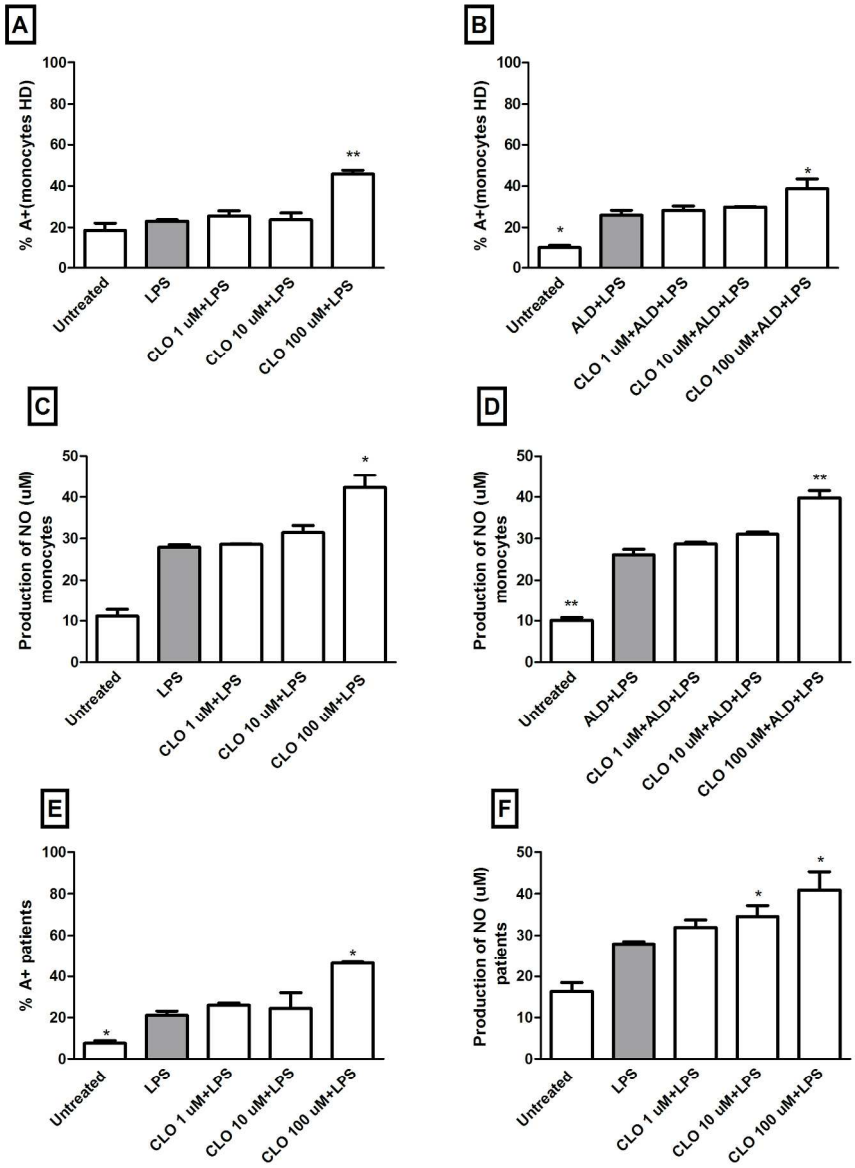

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