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Cholesterol-induced impact on murine macrophage responsiveness involves down-regulation of mevalonate pathway

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Abstract: Free cholesterol (Ch) and its oxidative derivatives, oxysterols, are often accumulated in macrophages during chronic inflammation and atherogenesis. The effects of Ch and oxysterols on the balance of pro- and anti-inflammatory cytokines in inflammatory response and the role of mevalonate pathway in the effects of these sterols are studied poorly. We studied the effects of cholesterol, oxysterols, atorvastatin, and mevalonic acid on the LPS-induced TNF-α, IL-10, and TGF-β1 production in macrophage cell culture. The study was carried out in murine peritoneal macrophages preincubated for 4 h with Ch (5 μg/mL), 25-hydroxycholesterol (25-OH-Ch) (5 μg/mL), 7-keto-Ch (5 μg/mL), farnesol (10 μM), or atorvastatin (5 μmol/mL) in the presence or absence of 1 mM of mevalonate. The cells were further incubated in the presence or absence of *E. coli* 0111:B4 lipopolysaccharide (LPS) for 24 h, and cytokine concentrations in incubation media were determined. Macrophages preincubation with Ch, 25-OH-Ch, or atorvastatin decreased LPS-induced TNF-α production in cell cultures, while supplementation of preincubation medium with mevalonic acid abrogated the effects of atorvastatin and Ch. The Ch, 25-OH-Ch, 7-keto-Ch and atorvastatin significantly reduced IL-10 production by LPS–stimulated macrophages, while farnesol had no effect. Supplementation of Ch or atorvastatin-containing preincubation medium with mevalonate restored IL-10 production. The TGF-β1 production was significantly enhanced by the presence of Ch or atorvastatin in preincubation medium as compared to the control level in non-treated macrophages, while 25-OH-Ch or farnesol decreased profoundly TGF-β1 production. Mevalonate abrogated the effect of Ch or atorvastatin but not of 25-OH-Ch or farnesol. These results allow to conclude, that Ch is able to promote anti-inflammatory and fibrogenic macrophage response, which is connected, at least in part, with the deficiency of mevalonate pathway intermediates, particularly to the deficiency of farnesol, whereas hydroxysterols produce tolerogenic, but not fibrogenic effect, independently of mevalonate pathway.

Keywords: Macrophage, Cytokines, Cholesterol, Oxysterols, Mevalonate Pathway

1. Introduction

Macrophages (Mf) are the major cell type involved in chronic inflammatory processes, such as atherogenesis, prolonged endotoxemia or immune response. They are the key cellular players able to acquire different functional phenotypes. These Mf phenotypes vary dynamically over a wide range: from predominantly biocidal-destructive to the opposite, mostly reparative-fibrotic conditions. The Mf of the 1st type (often termed M1 Mf) produce pro-inflammatory cytokines, such as TNF-α and IL-1β, whereas Mf of the opposite phenotypes (termed M2) produce anti-inflammatory cytokines, such as IL-10 and TGF-β. The course and the outcome of inflammatory processes are critically dependent on the Mf phenotypes acquired. For instance, the production of pro-inflammatory cytokines induces the increase of metalloproteinase activity and destabilization of atheromatous plaques, thereby eventually leading to the plaque rupture and to acute vascular catastrophes [1, 2]. On the other hand, anti-inflammatory cytokines are regarded as factors of plaque stabilization and reduction of the risks of atherosclerosis complications.

Chronic inflammation is typically associated with hypercholesterolemia. Cholesterol (Ch) and its oxidized derivatives, hydroxysterols (OS), were demonstrated to

accumulate in chronically inflamed tissues [3, 4] and in atherosclerotic plaques [5]. As we have shown previously [6], Ch and OS down-regulate pro-inflammatory cytokine production in Mf. At the same time, the effects of these sterols on anti-inflammatory cytokines IL-10 and TGF-β are poorly investigated. So, the effects of Ch and OS on Mf polarization are largely unknown.

The Ch impact on Mf phenotype formation can be exerted directly and/or through OS generated during the course of inflammation. The latter are the natural agonists of nuclear receptors liver X receptor (LXR) and produce their effects via LXR-dependent and LXR-independent mechanisms. Both Ch and OS are able to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) [7, 8], a key enzyme of mevalonate pathway. One cannot rule out the possibility that these sterols, similar to statins, modify Mf phenotype through mevalonate pathway down-regulation, although the contribution of Ch or OS-mediated mechanisms to HMG-CoA reductase inhibition can differ substantially. The role of mevalonate pathway in the effects of Ch and LXR agonists on Mf polarization is not clear so far.

This work aimed to determine the effects of Ch and LXR ligands on the lipopolysaccharide (LPS)-stimulated production of TNF-α, IL-10 and TGF-β1 in Mf cultures, and to assess the ability of mevalonate pathway activity to modulate these effects.

2. Materials and Methods

2.1. Chemicals

All reagents, if not otherwise stated, were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). 25-hydroxycholesterol (25-OH-Ch) and 7-keto-cholesterol (7-keto-Ch) were purchased from Steraloids Inc. and used as typical oxysterols derived from the enzymatic and non-enzymatic Ch oxidation, respectively, and able/unable to bind LXR. Farnesol, the cell-permeable analogue of farnesyl pyrophosphate, served as a mevalonate pathway intermediate, which can be involved in farnesylation process. We did not consider farnesol as FXR ligand because FXRs are not expressed in mouse peritoneal Mf [9] used in our experiments. Mevalonic acid lactone was used as the direct product of HMG-CoA reductase activity able to abolish the effects of HMG-CoA reductase inhibitors and to support descending reactions of mevalonate pathway.

2.2. Experimental Animals

The animals used in this work were cared for in accordance with the guidelines established by the Institutional Animal Care and Use Committee. All procedures were reviewed and approved by the Animal Care Committee at the FSBI of Internal and Preventive Medicine SB RAMS. Male C57BL/6 mice of 6-8 months of age obtained from breeding farms of the Institute for Cytology & Genetics SB RAS (Novosibirsk, Russia) were kept under vivarium conditions and were fed the standard laboratory chow and tap water ad libitum.

2.3. Cell Culture

The study was carried out on primary cultures of elicited peritoneal Mf harvested by lavage from C57BL/6 mice and isolated using the standard method of adhesion to the culture plastic surface. Mf were cultured at 37 °C, 5 % CO_2 and 95 % air in 24-well plates (250,000 cells/well) in RPMI-1640 with 2 mM HEPES, 10 % FCS, and a mixture of antibiotics for cell cultivation (MP Biomedicals) [10]. Cell monolayers were preincubated for 4 h with Ch (5 μg/mL), 25-OH-Ch (5 μg/mL), 7-keto-Ch (5 μg/mL), farnesol (10 μM), or 5 μmol/mL atorvastatin (Pfizer) in the presence or absence of 1 mM mevalonate lactone. No cell toxicity was observed for any treatments at the concentrations employed. The monolayers were further washed and 200 ng/mL of *E. coli* 0111:B4 LPS in a fresh incubation medium was added to the selected wells. The monolayers were incubated for 24 h, the incubation media were collected, frozen at - 20 °C, and stored for 1-1.5 weeks before the determination of cytokine concentrations.

2.4. Cytokine Determination

The concentrations of TNF-α, IL-10 and TGF-β1 in cell culture incubation media were measured by solid-phase ELISA with R&D Systems kits according to the manufacturer`s instructions. All experiments were repeated 3 times, and measurements were made in triplicates. The significance of differences between the groups was analyzed using paired Student's t-test.

3. Results

3.1. TNF-α Production

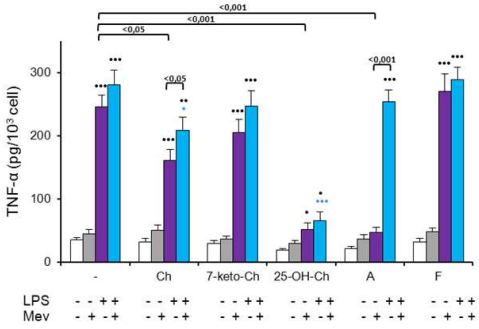

Figure 1. *Effects of Ch, 7-keto-Ch, 25-OH-Ch, atorvastatin (A) or farnesol (F) on LPS-induced production of TNF-α in Mf cultures preincubated with or without mevalonate lactone (Mev). White bars - cultures of elicited Mf preincubated with or without Ch, 7-keto-Ch, 25-OH-Ch, A or F; Grey bars – the same as white bars beside Mf are also preincubated with Mev; Violet bars - the same as white bars beside macrophages preincubation is followed by LPS stimulation; Blue bars - the same as grey bars beside Mf preincubation is followed by LPS stimulation. The differences between groups are significant at ● - P<0.05, ●● - P<0.01, ●●● - P<0.001 when the groups of comparison are elicited Mev-free and LPS-free Mf (white bars) within the same preincubation condition; ● - P<0.05, ●●● - P<0.001 when the group of comparison is LPS-stimulated Mf preincubated with Mev (blue) with no other additions.*

Stimulation of the control Mf monolayers with bacterial LPS caused an increase of TNF-α concentration in incubation medium by 7-fold (Fig. 1). Preincubation of Mf monolayers with Ch, 7-keto-Ch, 25-OH-Ch, farnesol or atorvastatin, per se, had no significant effects on the basal level of TNF-α production. The presence of mevalonate together with sterols, farnesol or atorvastatin caused an insignificant increase of TNF-α production. Mf preincubation with Ch, 25-OH-Ch, or atorvastatin induced a pronounced inhibition of LPS-stimulated TNF-α secretion, whereas the effects of farnesol or 7-keto-Ch were insignificant. Mevalonate added together with Ch or with atorvastatin abrogated their inhibitory effect and restored Mf responsiveness to LPS stimulation. By contrast, mevalonate practically did not affect 25-OH-Ch-induced inhibition of LPS-stimulated TNF-α production.

3.2. IL-10 Production

Addition of Ch, 7-keto-Ch, 25-OH-Ch, farnesol or atorvastatin to Mf cultures in the absence of LPS stimulation induced insignificant changes in the baseline IL-10 production level (Fig. 2). Mevalonate added together with these compounds actually did not change the levels of IL-10 production. LPS caused the 1.7 fold increase of IL-10 production in control Mf monolayers. LPS-stimulation of Mf preincubated with Ch, 7-keto-Ch, 25-OH-Ch, or atorvastatin decreased production of IL-10 significantly, whereas farnesol did not decrease IL-10 response. The presence of mevalonate in LPS-stimulated Mf preincubated with Ch or atorvastatin restored the level of IL-10 response down-regulated by preincubation with Ch and atorvastatin. Herewith, in atorvastatin-preincubated Mf mevalonate restored the IL-10 response completely, that is, to the normal level. In Mf preincubated with Ch mevalonate restored the level of LPS-stimulated IL-10 production partially, whereas in 7-keto-Ch-, 25-OH-Ch-, or farnesol-preincubated Mf effect of mevalonate was minimal.

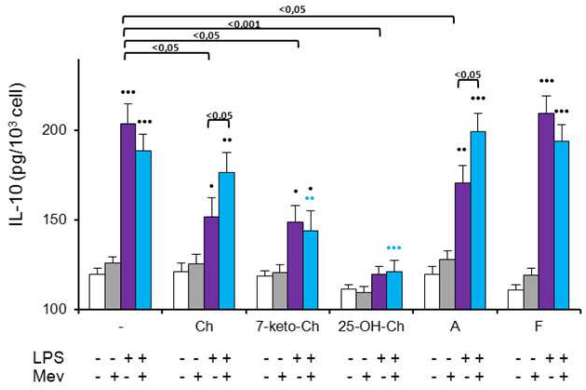

Figure 2. *Effects of Ch, 7-keto-Ch, 25-OH-Ch, A, or F on LPS-induced production of IL-10 in Mf cultures preincubated with or without Mev. Bar colors denote the same as in Fig. 1. The differences between groups are significant at ● - P<0.05, ●● - P<0.01, ●●● - P<0.001 when the groups of comparison are elicited Mev-free and LPS-free Mf (white bars) within the same preincubation condition; ●● - P<0.01, ●●● - P<0.001 when the group of comparison is LPS-stimulated Mf preincubated with Mev (blue) with no other additions.*

3.3. TGF-β1 Production

The TGF-β1 production was significantly enhanced by the presence of Ch or atorvastatin in preincubation medium as compared to the control level in non-treated Mf (Fig. 3). By contrast, 25-OH-Ch or farnesol induced a pronounced down-regulation of TGF-β1 secretion, while 7-keto-Ch had no effect. LPS stimulation of control Mf or 25-OH-Ch-pretreated Mf slightly increased the TGF-β1 level, while farnesol pretreatment induced the increase of TGF-β1 response. Vice versa, Mf preincubation with Ch, 7-keto-Ch or atorvastatin led to a decreased response to LPS. LPS stimulation of Mf preincubated with mevalonate and Ch, 7-keto-Ch or atorvastatin resulted in a decreased TGF-β1 production, as compared to the preincubated unstimulated Mf. In the control Mf or those preincubated with 25-OH-Ch or farnesol, the combination of mevalonate with LPS stimulation induced a slight increase of TGF-β1 response.

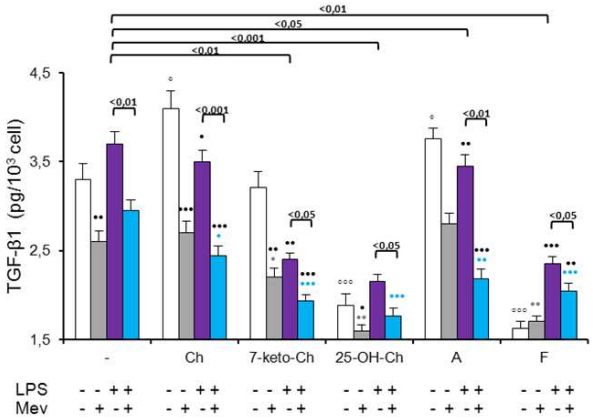

Figure 3. *Effects of Ch, 7-keto-Ch, 25-OH-Ch, A, or F on LPS-induced production of TGF-β1 in Mf cultures preincubated with or without Mev. Bar colors denote the same as in Fig. 1. The differences between groups are significant at ● - P<0.05, ●● - P<0.01, ●●● - P<0.001 when the groups of comparison are elicited Mev-free and LPS-free Mf (white bars) within the same preincubation condition; ○- P<0.05, ○○○ - P<0.001 when the group of comparison is Mf (white) preincubated with no other additions; ● - P<0.05, ●● - P<0.01 when the group of comparison is Mf (grey) preincubated with Mev with no additions, ● - P<0.05, ●● - P<0.01, ●●● - P<0.001 when the group of comparison is LPS-stimulated Mf preincubated with Mev (blue) with no other additions.*

4. Discussion

The balance between pro- and anti-inflammatory cytokines produced by Mf during inflammation determines the course and the outcome of the latter. Chronic inflammation is frequently associated with hypercholesterolemia and accumulation of Ch and OS in Mf [3]. Accumulation of these sterols in Mf can apparently impact on the formation of the functional phenotype of the cells, including formation of the balance between pro- and anti-inflammatory cytokines produced under inflammatory stimulation. Our experiments demonstrated the decrease of LPS-stimulated TNF-α production in Mf preincubated with Ch, 25-OH-Ch, or atorvastatin. These findings are in good

agreement with the literature data [11, 12] and can be potentially accounted for the inhibition of mevalonate pathway and for the effects of OS on LXR nuclear receptors. The absence of the effect of farnesol or 7-keto-Ch can be explained by the inability of these compounds to down-regulate HMG-CoA reductase and by inability of 7-keto-Ch to affect/bind LXR. We demonstrated the mevalonate-mediated restoration of the level of Mf response to LPS, inhibited by Ch or atorvastatin. Herewith mevalonate completely abolished the inhibitory effect of atorvastatin, thus, restoring the TNF-α production response to the control level. At the same time, mevalonate failed to completely restore the Mf response after preincubation with Ch. This apparently implies that Ch-induced inhibition of Mf response is partially dependent on the inhibition of mevalonate pathway. A minor effect of mevalonate on Mf hyporesponsiveness induced by 25-OH-Ch testifies to the minimal dependence of LXR-mediated down-regulation of TNF-α production on mevalonate pathway intermediates. Similarly, the lack of mevalonate effect on the LPS-stimulated response in Mf preincubated with farnesol testifies that farnesol change Mf responsiveness in a way similar to mevalonate.

Despite the fact that IL-10 is an anti-inflammatory cytokine typical for Mf phenotype of M2 character, IL-10 gene expression is stimulated by IFN-β and depends on TLR4-TRIF-TRAM signaling [13]. Thus, Mf stimulation with LPS can efficiently induce IL-10 production. Our data confirm this link, demonstrating the rather efficient LPS induction of IL-10 production in control Mf and in cultures preincubated with sterols, atorvastatin or farnesol. The only exception was Mf preincubation with 25-OH-Ch, which completely suppressed the LPS stimulation of IL-10 production (similar to its effect on TNF-α response). One can conclude that OS-induced LXR mediate Mf hyporesponsiveness most effectively. Mf pretreatment with sterols or atorvastatin also suppressed, though not completely, LPS-induced IL-10 production while farnesol exerted no suppressive effect at all. One apparent cause of suppressive effects of Ch or atorvastatin can obviously be the inhibition of HMG-CoA reductase, since those effects can be abolished by mevalonate (fully for atorvastatin and partially for Ch). The inability of mevalonate to modify the LPS-stimulated IL-10 production in farnesol-pretreated cells points to the involvement of mevalonate pathway in IL-10 response to LPS.

Our experiments demonstrated a relatively high baseline TGF-β1 production level in the control elicited Mf. This finding is in accord with the literature data showing the increase of TGF-β1 production in response to eliciting agents [14]. Preincubation of the cells with Ch or atorvastatin up-regulates the TGF-β1 production significantly. Since mevalonate treatment abolished Ch or atorvastatin-induced increase in TGF-β1 production, the latter is obviously owed to the inhibition of mevalonate pathway. Mevalonate did not abolish Ch or atorvastatin effects only, but also reduced the TGF-β1 levels to that significantly lower, than in non-pretreated Mf. Furthermore, the same holds true for mevalonate effect in control non-pretreated Mf. Assuming the plausible role of mevalonate pathway inhibition in TGF-β1 production and taking into account the presence of Ch-rich lipoproteins in peritoneal fluid [15] one cannot rule out the possibility that such an inhibition occurs during Mf recruitment into peritoneal cavity and promotes TGF-β1 production. In contrast to Mf preincubation with Ch or atorvastatin Mf preincubation with LXR agonist 25-OH-Ch or with farnesol down-regulated TGF-β1 production as compared to non-treated control, and mevalonate had minimal impact on their effect. Consequently, the induction of LXR suppressed production of both pro- and anti-inflammatory cytokines, and this effect (as well as effect of farnesol) is not dependent substantially on mevalonate pathway. Thus, it is logical to suggest that farnesyl pyrophosphate is one of the intermediates of mevalonate pathway, the deficiency of which can be responsible, at least in mice, for TGF-β1 production. 7-keto-Ch pretreatment did not reduce baseline TGF-β1 production level, most likely, since 7-keto-Ch is an oxysterol unable to induce LXR.

Thus, Ch induces formation of Mf phenotype with a decreased ability to produce TNF-α and IL-10 and with an enhanced potential to produce TGF-β1, whereas mevalonate prevent the formation of this phenotype. Since the exogenic Ch and mevalonate increase the intracellular Ch level, their opposite effects on cytokine production is a clear evidence of a fact that Ch-induced Mf phenotype formation depends on the deficiency of mevalonate pathway intermediates. Taking into account the inhibition of squalene synthase induced in Mf under inflammatory stimulation [16, 17] the candidate intermediates are evidently the non-sterol isoprenoids. As mevalonate is unable to change the effects of farnesol on cytokine production, one can assume that farnesol is one of such intermediates. At the same time, the major mechanisms underlying the effects of hydroxysterols seem to be essentially independent on HMG-CoA reductase inhibition.

The molecular mechanisms responsible for the Ch-induced Mf phenotype formation are vague. One of the possibilities is hypoprenylation of Rho, Ras, Raf and other small G-proteins normally involved in intracellular signal transduction in Mf proinflammatory response. There are reports confirming the link between hypoprenylation and the increase of TGF-β1 expression and secretion in endothelial cells [18], in embryonic heart cells [19] and in some other cell types [20, 21, 22], although the role of hypoprenylation in TGF-β1 secretion in Mf remains to be confirmed.

5. Conclusion

Due to our findings Ch induces anti-inflammatory and fibrogenic Mf response, which is connected to the deficiency of mevalonate pathway intermediates, particularly to the deficiency of farnesol, whereas LXR agonists produce tolerogenic, but not fibrogenic effect independently of

mevalonate pathway inhibition. So, it is possible to conclude that hypercholesterolemia associated with inflammatory processes can be involved in the formation of M2-like Mf phenotype. The link established between that Mf phenotype and the deficiency of mevalonate pathway intermediates allows searching for the new approaches of pharmacological correction of Mf responsiveness in a variety of inflammatory diseases.

Acknowledgments

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