Methotrexate effects on adenosine receptor expression in peripheral monocytes of persons with type 2 diabetes and cardiovascular disease

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ABSTRACT

The Cardiovascular Inflammation Reduction Trial (CIRT) was designed to assess whether low-dose methotrexate (LD-MTX) would reduce future cardiac events in patients with metabolic syndrome or type 2 diabetes (T2DM) who are post-myocardial infarction (MI) or have multivessel disease. Our previous work indicates that MTX confers atheroprotection via adenosine A2A receptor (A2AR) activation. In order for A2AR ligation to reduce cardiovascular events, A2AR levels would need to be preserved during MTX treatment. This study was conducted to determine whether LD-MTX alters peripheral blood mononuclear cell (PBMC) adenosine receptor expression in persons at risk for cardiovascular events. Post-MI T2DM CIRT patients were randomized to LD-MTX or placebo (n=10/group). PBMC isolated from blood drawn at enrollment and after 6 weeks were evaluated for expression of adenosine receptors and reverse cholesterol transporters by real-time PCR. Fold change between time points was calculated using factorial analyses of variance. Compared with placebo, the LD-MTX group exhibited a trend toward an increase in A2AR (p=0.06), while A3R expression was significantly decreased (p=0.01) after 6 weeks. Cholesterol efflux gene expression did not change significantly. Persistence of A2AR combined with A3R downregulation indicates that failure of MTX to be atheroprotective in CIRT was not due to loss of adenosine receptors on PBMC (ClinicalTrials.gov identifier: NCT01594333).

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Methotrexate (MTX) is an anti-inflammatory drug that acts, in part, by raising levels of the endogenous autocoid adenosine. It has cardioprotective effects in patients with autoimmune rheumatic diseases such as rheumatoid arthritis. Based on its anti-atherosclerotic benefits in the inflammatory setting of rheumatoid arthritis, the Cardiovascular Inflammation Reduction Trial (CIRT) was conceived to directly test whether low-dose MTX could lower risk of major cardiovascular (CV) adverse events in patients with stable atherosclerosis and diabetes mellitus (DM) or metabolic syndrome. MTX failed to show benefit in CIRT and the study was discontinued.

WHAT THIS STUDY ADDS

⇒ The anti-inflammatory properties of MTX were expected to be beneficial to CIRT participants, but MTX did not reduce CV risk in the CIRT population. We still do not know why it was ineffective. This CIRT substudy examined a possible explanation for the CIRT failure by evaluating whether 6 weeks of exposure to MTX would reduce adenosine A2A receptors (A2AR) on circulating monocytes, therefore causing resistance to its own anti-inflammatory actions. Our study found that A2AR continued to be present on monocytes of our small sample of T2DM CIRT patients, and thus, loss of these receptors is not the likely reason for MTX lack of atheroprotective efficacy. MTX did not enhance expression of cholesterol efflux genes on the peripheral blood monocytes and this is consistent with its inability to improve CV risk.

INTRODUCTION

Atherosclerosis is responsible for almost all cases of coronary heart disease and myocardial infarction (MI).[1] Atherosclerotic cardiovascular disease (ASCVD) is highly prevalent among patients with type 2 diabetes (T2DM). The basis of this relationship involves a set of ASCVD-promoting risk factors that occur with high frequency in persons with T2DM, including an abnormal lipid profile, vascular endothelial damage, obesity, and chronic inflammation.[2] Chronic inflammation is implicated in the pathogenesis of ASCVD and is hypothesized to be involved in the eventual plaque rupture (atherothrombosis) and resulting cardiovascular event (ie, myocardial infarction).[3]

This "inflammatory hypothesis" of atherothrombosis was tested by the Cardiac Inflammation Reduction Trial (CIRT, NCT01594333),

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ When a major clinical trial such as CIRT is stopped early for futility, there is important knowledge to be gained. There is much literature affirming a role for inflammation in atherothrombosis, but the translation to treatment via suppression of inflammatory pathways is proving difficult and complicated. It has been suggested that the CIRT study population may not have had a large enough inflammatory burden to be helped by MTX.
In our small patient sample, the adenosine-elevating properties of MTX did not cause decreased expression of the anti-inflammatory A2AR on monocytes. It is possible that further studies building on the current study could be important in elucidating if MTX would be effective in reducing CV risk in a cohort with a greater inflammatory load.

a randomized, double-blind, placebo controlled multisite clinical trial enlisting 7000 stable post-MI or multivessel disease patients with metabolic syndrome or T2DM to assess whether reduction of inflammation with low-dose methotrexate (LD-MTX, target dose 15–20 mg/week) would reduce the risk of a future cardiac event in these high-risk patients.[3] Enrollment in the CIRT started in 2013 and then the CIRT was terminated prematurely in 2018 due to a lack of efficacy on cardiovascular events.[4]

Research laboratories associated with hospitals that participated in the CIRT study had the unique opportunity to conduct CIRT ancillary studies. Among these was a substudy conducted by our group that was able to draw samples from CIRT patients to explore the molecular basis of MTX anti-inflammatory action in this highly relevant, high cardiac event risk population. MTX, an antineoplastic antimetabolite folate analog, has long been used as an anti-inflammatory agent in the treatment of rheumatoid arthritis (RA).[5] MTX exerts its anti-inflammatory effects through increasing extracellular adenosine concentrations. The elevated levels of adenosine then activate adenosine receptors, most prominently the anti-inflammatory A2A receptor (A2AR).[6] The expression of this receptor may be influenced by exposure to inflammatory cytokines.[7] Our group has shown that A2A activation with MTX not only shifts the profile of monocytes/macrophages toward an anti-inflammatory state, but may also improve their handling of cholesterol, augmenting their defenses against atheroma-promoting lipid overload and foam cell transformation.[8]

Given what is known about chronic inflammation and CVD in patients with T2DM, adenosine-mediated anti-inflammatory pathways, and abnormal lipid handling in atherosclerosis, this CIRT ancillary study evaluated the effects of 6 weeks of LD-MTX in post-MI T2DM CIRT patients on PBMC adenosine receptor expression.

MATERIALS AND METHODS

Patient enrollment

Twenty post-MI T2DM patients enrolled under a protocol approved by the Institutional Review Board at Winthrop University Hospital (study number s18-01679) for participation in the CIRT study were randomized to either a treatment group (6 weeks of LD-MTX, n=10) or a placebo group (6 weeks of placebo, n=10). Blood samples were drawn from all patients at two time points: enrollment and after 6 weeks of LD-MTX or placebo treatment.

Criteria for inclusion and exclusion were identical to those of the parent CIRT study.[9]

Briefly, they are as follows:

Inclusion criteria: Age 18 years and above; history of T2DM, metabolic syndrome, myocardial infarction, or multivessel coronary artery disease.

Exclusion criteria: Under age 18 years; history of malignancy, liver disease, kidney disease, chronic infection, or pre-existing rheumatologic disease.

Demographic data are shown in online supplemental table 1.

PBMC isolation, culture, and QRT-PCR

PBMCs were isolated from blood samples collected in EDTA-treated tubes by Ficoll hypaque gradient centrifugation.[10]

PBMC were cultured for 3 days with macrophage colony stimulating factor to achieve sufficient cell population. TRIzol reagent was used to extract RNA, and cDNA was copied from 1 µg of total RNA using M-MLV reverse transcriptase primed with oligo-dT. All cDNA from PBMC conditions described above was evaluated for expression of adenosine receptor subtypes (A2AR, A2BR, A3R) by QRT-PCR with a FastStart SYBR Green Reagents Kit using a Roche Lightcycler 480 real-time PCR system (online supplemental table 2).[11–13]

PBMC from conditions described above were also evaluated for the expression of the cholesterol efflux genes *ABCA1*, *ABCG1*, *27-hydroxylase*, and *LXR-α* by QRT-PCR with a FastStart SYBR Green Reagents Kit using a Roche Lightcycler 480 Real-Time PCR System with specific primers used previously by our group (online supplemental table 2).[14–16]

Statistical analyses

Change in PBMC expression between time point 1 (enrollment) and time point 2 (following 6 weeks of treatment or placebo) was calculated for each adenosine receptor gene and each cholesterol efflux gene in all patients. Average fold change between time point 1 and time point 2 was calculated and compared for the placebo control group (n=10) and the LD-MTX treatment group (n=10) using factorial analyses of variance.

RESULTS

An increase in adenosine A2AR expression in the LD-MTX group trended toward significance (p=0.06), while adenosine A3R expression was significantly decreased (p=0.01) after 6 weeks in the LD-MTX group versus placebo group (figure 1).

The LD-MTX group did not display any significant differences in gene expression after 6 weeks when compared with the placebo group for any of the following: ABCA1, ABCG1, 27-hydroxylase, or LXR-α (figure 2).

DISCUSSION

Our access to blood samples of CIRT patients drawn at different time points allowed us to evaluate the changes in

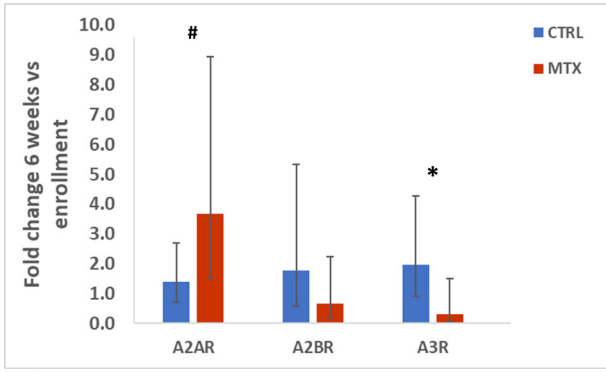

Figure 1 Real-time PCR analysis of A2AR, A2BR, and A3R expression in in human peripheral blood mononuclear cell (PBMC). PBMC were isolated from human blood samples of CIRT (Cardiovascular Inflammation Reduction Trial) study patients before and after a 6-week course of low-dose methotrexate (LD-MTX) (MTX, red bars) or placebo (CTRL, blue bars) (n=10 per group). Statistical significance between control and LD-MTX-treated subject PBMC is indicated by *p=0.01 or #p=0.06.

their PBMC cholesterol handling profile after 6 weeks of LD-MTX, compared with 6 weeks of placebo in an otherwise similar population. This was a unique opportunity as LD-MTX is not generally used in T2DM unless there is a co-occurring rheumatic disease.

ASCVD is highly accelerated in patients with T2DM.[17 18] Central to the pathogenesis of atherosclerosis is the interplay between inflammatory pathways and disrupted cholesterol metabolism and transport.[19] In atherosclerosis, inflammation has been linked with abnormal cholesterol handling characterized by increased cholesterol influx and impaired efflux capacity in macrophages. Macrophage lipid overload and foam cell formation is a hallmark of atherosclerosis. In addition to its anti-inflammatory properties, MTX has anti-atherogenic effects on lipid handling. Atheroprotective properties of MTX are well established in the setting of the

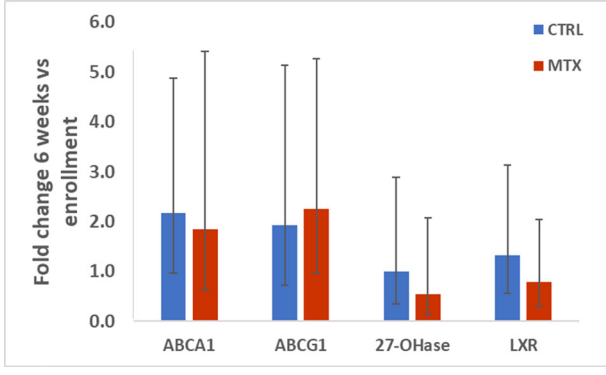

Figure 2 Effect of low-dose methotrexate (LD-MTX) versus placebo treatment on CIRT (Cardiovascular Inflammation Reduction Trial) patient's peripheral blood mononuclear cell (PBMC) cholesterol efflux gene expression. PBMC were isolated from human blood samples of CIRT study patients before and after a 6-week course of LD-MTX (MTX, red bars) or placebo (CTRL, blue bars) (n=10 per group).

autoimmune disorder rheumatoid arthritis where MTX reduces cardiovascular risk substantially.[20 21]

Our group and others have previously found that cholesterol efflux genes that code for proteins controlling lipid outflow from macrophages exhibit a proatherogenic pattern in populations at risk for ASCVD such as patients with RA.[22–25] Critical genes involved in cholesterol efflux includes the cytochrome P450 27-hydroxylase, ATP binding cassette transporter (ABC) A1, ABCG1, and liver X receptor (LXR)-α.[26 27] Dysregulated cholesterol handling in atherosclerosis is now a target for therapy that seeks to reverse the process and therefore resolve or attenuate plaque formation in ASCVD.[28] When analyzing these cholesterol transporter genes, there was no significant difference in PBMC expression of any of them between the LD-MTX group and the placebo group.

Our laboratory has described potent atheroprotective properties of MTX that influence separate, distinct points in cholesterol transport and metabolism.[10] Anti-inflammatory effects of MTX are ascribed to the release of the purine nucleoside adenosine.[29–31] We have shown that MTX potentiates cholesterol removal via adenosine acting at adenosine A2AR to enhance expression of genes involved in reverse cholesterol transport out of cells and back to the liver for catabolism.[5 11 32] Adenosine, via A2AR, may be responsible for the capacity of MTX to reduce death rates from ASCVD in RA.[33]

Atherosclerosis is recognized as a chronic inflammatory disease and T2DM has a strong inflammatory component as well.[34 35] Based on our understanding of the interaction of inflammation, T2DM, and ASCVD, the CIRT study was initiated with the hope that the anti-inflammatory, atheroprotective properties of MTX would be beneficial to persons with T2DM and high risk for cardiovascular events. It was postulated that LD-MTX would exert these effects via A2AR agonism.

Consistent with the overall CIRT study findings, our results did not support an atheroprotective effect of LD-MTX on PBMC. This is in contrast to its benefits in RA.[36 37] The difference in results may be related to the dissimilarities between RA and T2DM. RA is a more homogeneous disorder with a more marked and uniformly inflammatory milieu and greater ASCVD risk than that seen in T2DM.[38] Effects of LD-MTX were not diminished due to changes in the expression of adenosine receptors over a 6-week period of exposure to the drug as A2A receptors were not suppressed by LD-MTX treatment.

A strength of the present study is its prospective nature as well as the use of each subject prior to LD-MTX or placebo treatment as its own control for the effect after 6 weeks. As a substudy of CIRT, we had the unique and rare opportunity to determine the effect of LD-MTX on MTX-naïve subjects who do not have an autoimmune rheumatic disorder.

Limitations to our study include the small number of subjects and the measurement of gene expression in PBMC which have very low levels of cholesterol efflux genes at baseline.

CONCLUSIONS

The high prevalence of atherosclerosis in T2DM underscores the need for new and effective therapies. Our CIRT substudy sought to test the effectiveness of an MTX-based anti-inflammatory regimen in improving cholesterol

handling in PBMC. MTX has proven antiatherogenic properties in patients with RA, but it failed to demonstrate reduced cardiovascular events in the CIRT population.[4 39] In our small subgroup of T2DM CIRT patients, PBMC expression of key cholesterol transport and metabolism genes was not changed by 6 weeks of LD-MTX. The reasons for this are unclear, but are not due to downregulation of the expression of the adenosine A2AR, the receptor linked most closely to anti-inflammatory and atheroprotective effects of MTX. Desensitization and downregulation after prolonged exposure are common reasons for loss of drug potency.[40] Whether MTX may prove efficacious against ASCVD in other disease states may be elucidated in future studies. Specific A2AR agonists have been developed and may be considered for evaluation as cardiovascular drug candidates with less off-target effects than MTX.[41 42]

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