Indian Journal of Clinical Biochemistry, 2006 / 21 (2) 49-52

POSITIVE INFLUENCE OF METHOTREXATE-HYDROXYCHLOROQUINE COMBINATION ON THE EXPRESSION OF GM-CSF RECEPTOR ON NEUTROPHILS OF SYNOVIAL FLUID IN RHEUMATOID ARTHRITIS

Ananth. N Rao, Beena.V. Shetty[1] and D.M. Vasudevan

Department of Biochemistry, Amrita Institute of Medical Sciences, Kochi 682 026, Kerala and
[1]Kasturba Medical College, Mangalore 575 004

ABSTRACT

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) has been inducted as a mediator of inflammation in rheumatoid arthritis. Methotrexate combination therapy forms an important component of the treatment regimen in rheumatoid arthritis. The present study was undertaken to evaluate the influence of Methotrexate-Hydroxychloroquine (MTX-HCQ) combination and Sulfsalazine- Hydroxychloroquine (SSZ-HCQ) combination on the expression GM-CSFR in neutrophils isolated from synovial fluids. 15 cases of confirmed rheumatoid arthritis patients who presented at the hospital for surgical correction of joint deformities were selected for the study. Neutrophils isolated from the synovial fluids were used as the source of the receptor for quantitation on an enzyme immunoassay (EIA). The EIA was developed and standardized in our laboratory for quantification of the GM-CSF R. The findings are suggestive of the fact that the administration of MTX-HCQ combination has positive influence on the expression of the GM-CSF R on neutrophils as against SSZ-HCQ combination. The physiological basis of this increase needs further investigation.

KEY WORDS

Methotrexate, Rheumatoid arthritis, GM-CSF receptor, Synovial fluid

INTRODUCTION

Methotrexate, is probably the oldest in the list of drugs used in the second line treatment of Arthritis. The beneficial and the side effects of the drug had been a topic of study in the yesteryears and its dosage-related toxicity is established beyond doubts now. The drug has also been used with enough success in patients of rheumatoid and psoriatic arthritis. Methotrexate (MTX) has been used widely as a Disease modifying anti-rheumatoid drug (DMARD) with varying percentage of success (1). Combinations of DMARD's are used by rheumatologists all over the globe. The most commonly used combinations are Methotrexate-Hydroxychloroquine (MTX-HCQ), Methotrexate-Sulfsalazine (MTX-SSZ) and Sulfsalazine-Hydroxychloroquine (SSZ-HCQ). The most commonly used combination of Methotrexate - Hydroxychloroquine (MTX-HCQ) are prescribed by 99 % of rheumatologists in the United States (2). The role of cytokines in inflammation has been dealt with in detail and a conclusive hypothesis on their mode of action has been arrived at (3).

Address for Correspondence:

Dr. Ananth N Rao
Department of Biochemistry
Amrita Institute of Medical Sciences,
Cochin 682 026, Kerala
E-mail : drananthrao@medical.amrita.edu

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) is a cytokine involved in promotion of proliferation among haemopoeitic progenitors (4). The molecule has been used with varying degree of success as an adjuvant in the therapy of cancer to enhance the Absolute Neutrophil Count (ANC). (5) The molecule is known to mediate its effects through a distinct receptor known as the GM-CSF receptor (GM-CSF R) which occurs on monocytes neutrophils, eosinophils (6). The receptor, first isolated in 1989 was shown to be a 85 kDa molecule and a member of the family of receptors which includes IL-2, IL-4, IL-6, IL-7, erythropoeitin, growth hormone and prolactin (7). The high affinity receptor is composed of two subunits alpha and beta subunits. The alpha subunit is critical for high affinity binding and initiating ligand-receptor interactions (8).

The role of the GM-CSF receptor (CD 116) has not yet been

fully understood. The present study was taken up to study the expression of the receptors on neutrophils isolated from synovial fluids of patients on combination therapy for arthritis.

MATERIALS AND METHODS

All cases chosen for the study were known cases of arthritis. Subjects selected for the study were women aged between 40-45 years who were confirmed cases of seropositive Rheumatoid Arthritis.

Group I consisted of 15 individuals on MTX-HCQ therapy since the diagnosis of RA. They were on Methotrexate at 7.5 mg in three doses per week (i.e. 2.5 mg in three divided doses 12 hours apart for a week) and Hydroxychloroquine 500 mg daily.

The Group II consisted of 15 individuals who were also confirmed cases of seropositive rheumatoid arthritis and advised SSZ-HCQ combination (devoid of MTX) as their treatment regimen (Group II). They received 500 mg Hydroxychloroquine and 1 g Sulfsalazine daily.

Age & sex matched Normal controls were cases who presented for orthopedic surgery but not known cases of RA were also included in the study. (Group III).

Synovial fluid was drawn from subjects of all study groups during surgical correction of joint deformities in RA or surgery for any other cause excepting RA. The neutrophils were isolated from the synovial fluid by centrifugation (1500 rpm) at 4^0C on a REMI C 24 centrifuge for 20 minutes and stored frozen in Cell Resuspension Buffer (CRB) (9). Cell resuspension buffer (CRB) was a modified Dulbecco's Phosphate buffered saline (DPBS) containing 5 mM KCl, 147 mM NaCl, 1.9 mM KH_2PO_4, 1.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.1 % glucose and 0.1 % bovine serum albumin (pH 7.4).

The cell suspension was then thawed and cell count determined by method of turbidimetry and fixed at 10^2 cells per ml. The standardized cell suspensions were then subjected to membrane solubilization of the receptor. The cell pellet obtained therefore was suspended in DPBS. The resuspended pellet was subjected to osmotic lysis. The resulting lysate was subjected to centrifugation at 45,000 rpm and 4^0C. The pellets obtained were carefully transferred into a fresh sterile polypropylene tube, resuspended in Membrane resuspension Buffer (MRB) and stored frozen until further use. Membrane resuspension buffer (MRB) consisted of 100mM KCl, 10 mM NaCl, 10mM Hepes, 1mM EDTA, 0.1 mM Dithiothreitol, 1mM PMSF (pH 7.3).

After thawing on ice, the membrane pellets were treated with 1 ml of 5 M NaCl per 4 ml of MRB. The mixture was vigorously shaken and subjected to centrifugation at 1,00,000 g for 35 minutes. The supernatant was discarded and the pellet resuspended in MRB. Solubilisation was rendered complete by adjusting the protein concentration to 1-2 mg/ml with MRB and addition of Triton-X-100 to make a final detergent concentration of 2 % (w/v). The solution was vortexed, kept on ice for 30 minutes and centrifuged at 1,00,000 *g* for 30 minutes at 4^0C. The supernatant was then used for enzyme immunoassay (EIA) for quantification of GM-CSF R. The EIA was developed by us at our laboratory for the purpose using Monoclonal antibody against GM-CSF R (Serotec, UK). The microwell plates were procured from NUNC-Nalgene International. The developed EIA was standardized against a commercial kit procured from Immunotec, France. Specificity of the developed Enzyme immunoassay was determined to be 96.3 % and sensitivity was fixed at 92 %.

RESULTS

Results of the estimations are shown in Figure 1. Statistical analysis was performed using the Mann – Whitney "U" test. Our results indicate a positive influence of MTX-HCQ combination therapy on GM-CSF receptor expression on neutrophils isolated from the synovial fluids of RA patients in comparison with SSZ-HCQ treated group ($p < 0.01$) and Normal controls ($p < 0.01$).

DISCUSSION

One of the biggest challenges that most rheumatologists face when treating patients with Rheumatoid arthritis is selecting the best DMARD, or combination of DMARD's. (2,10).

TABLE 1
Baseline parameters of the study groups

Charactersitic	MTX-HCQ (n=15)	HCQ-SSZ (n=15)	Normals (n=15)
Age range (in years)	40-45	40-45	40-45
Sex	F	F	F
Duration of disease (in years)	8 ± 2	8 ± 2	–
RA factor	All positive	All positive	–
Hemoglobin (g/dL)	11.8 ± 2	12 ± 2	13.1 ± 2
ESR (mm/hour)	39 ± 23	47 ± 21	21 ± 8

However, many cannot arrive at a single best combination at once and frequent trials with several DMARD combinations have to be carried out before arriving at the best choice. Combinations of DMARD's are used by rheumatologists all over the globe. The most commonly used combinations of MTX-HCQ, MTX-SSZ and SSZ-HCQ are prescribed by 99%, 84% and 63% rheumatologists of United States respectively (2).

The increased uses of combinations were proposed owing to the fact that combinations could be safely used and with greater efficacy than mono-DMARD therapy. (11, 12, 13). The most commonly used combination of Methotrexate-Hydroxychloroquine (MTX-HCQ) is known to have the lowest discontinuation rate (14).

The destruction of joint tissue in RA is a result of a loss of the normal balances of the patient's inflammatory systems. The inflammatory response is under the control of a large family of cytokines, which act as "molecular messengers" during inflammatory reaction in RA. (15) The GM-CSF receptor has been implicated as a mediator of inflammation in RA. (16). An increase in the expression of the receptor has been reported on monocytes (17) in RA patients. However, no studies on the receptor expression in treated patients have been reported. We have evaluated the GM-CSF receptor concentration on neutrophils of the synovial fluid in patients undergoing MTX-HCQ and SSZ-HCQ combination therapy. Our results indicate an increase in the receptor concentration in patients on MTX-HCQ therapy. The finding is a paradox in its true nature since, most treatment protocols in arthritis aim at reduction or suppression of the inflammatory pathway.

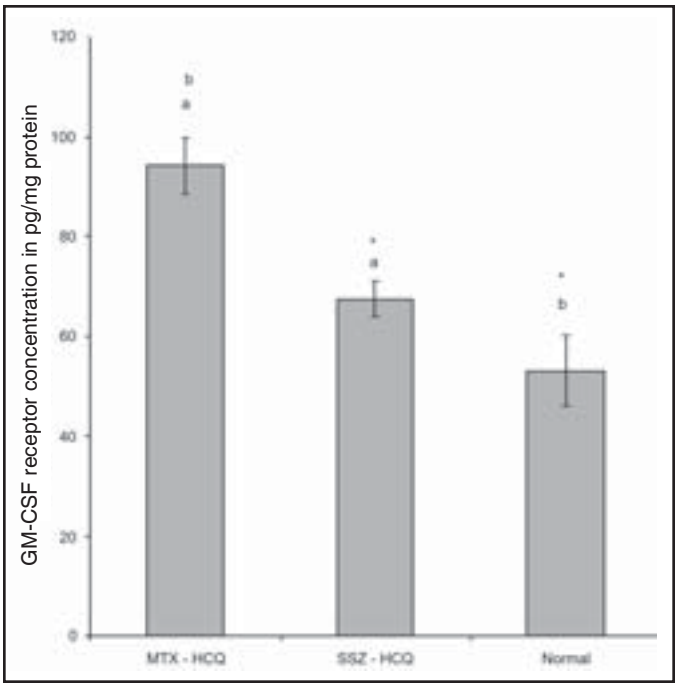
GM-CSF is a molecule that could mediate inflammation and the elevation in the concentration of the GM-CSF receptor concentrations present a situation, where the MTX-HCQ combination therapy may be pro-inflammatory. The contribution of such an elevation in the concentration of the receptor & its consequent role in the inflammatory processes of arthritis needs an isolated experiment. The other basal parameters viz. ESR, hemoglobin concentration and RA factor have been used for the clinical basis of the disease and show changes that have been well defined in literature.

TABLE 2

Mean values of concentration of GM-CSF receptor in pg/ml in various groups of the study.

Group I (MTC-HCQ) (n=15)	Group II (SSZ-HCQ) (n=15)	Group III (Normal) (n=15)
94.14 pg/mL	67.4 pg/mL	53.24 pg/mL

Fig 1: Comparison between treated groups and normals



δ Values Mean ± S.D

a - Highly Significant, comparison between MTX - HCQ and SSZ -HCQ. ($p < 0.01$)
b - Highly Significant, comparison between SSZ -HCQ and Normals. ($p < 0.01$)
* - Highly Significant, comparison between MTX -HCQ and Normals. ($p < 0.01$)

Gene amplification has been shown with respect to methotrexate and DHFR gene. (18) It needs to be elucidated whether a similar effect is the mechanism behind the current finding. Studies with affinity-purified receptor are being conducted for further ascertaining of the results. The sample size in the present study could not be increased owing to the rarity of RA patients consulting orthopedic surgeons for surgical correction of joint deformities. However, animal model studies with mono-DMARD administration could provide a greater basis for the explanation of our present finding thus allowing a closer approach to its molecular and physiological basis.

REFERENCES:

1. Weinblatt, M.E., Kaplan, H, Germain, B.F., et al.(1994) Methotrexate in rheumatoid arthritis: a five-year prospective multicenter study. Arthritis Rheum.37, 1492-1498.
2. O'Dell, J.R., Hairem, C.E., Erikson, N, et al. (1996) Treatment of rheumatoid arthritis with methotrexate alone, sulfasalazine and hydroxychloroquine, or a combination of all three medications. N. Engl. J. Med. 334, 1287-1291.

3. Hamilton, J.A. (1993) Rheumatoid arthritis: opposing actions of haemopoietic growth factors and slow acting anti-rheumatic drugs. Lancet 342,536-539.

4. Gasson, J.C. (1991) Molecular physiology of granulocyte macrophage colony simulating factor. Blood 77, 1131-45.

5. Janeway, C. A., Travers, P., Walport, M., Schlomchik, M. (2001) Immunobiology; 5th edn. Garland Publishing.

6. Di, Persio, J., Billing, P., Kaufman, S., Eghtesady, P., Williams, R.C. and Casson, J.C. (1988) Characterisation of the human granulocyte macrophage colony stimulating receptor. J. Biol. Chem. 263(4), 1834-41.

7. Stacchini, A., Fubini, L. and Aglietta, M. (1996) Flow cytometric detection and quantitative analysis of the GM-CSF receptor in human granulocytes and comparision with radioligand binding assay. Cytometry 24 (4), 374-81.

8. Di Persio, J.F., Golde, D.W. and Gasson, J.D. (1990) GM-CSF: receptor structure and transmembrane signaling. Int. J. Cell-Cloning 74(5), 63-74.

9. Sidney, P., Colowick and Nathan, O., Kaplan. (1984) Methods in Enzymology 105, 359-362.

10. Felson, D.T., Anderson, J.J. and Meenan, R.F. (1994) The efficacy and toxicity of combination therapy in rheumatoid arthritis, A meta-analysis. Arthritis Rheum. 37, 1487-1491.

11. Boers, M., Verhoeven, A.C., Markusse, H.M. et al (1997) Randomised comparison of combined step-down prednisolone, methotrexate and sulphsalazine alone in early rheumatoid arthritis. Lancet 350, 309-318.

12. O'Dell, J.R., Haire, C.E., Palmer, W. et al (1997) Treatment of early rheumatoid arthritis with minocycline or placebo. Arthritis Rheum. 40, 842-848.

13. Tugwell, P., Pincus, T., Yocum, D. et al (1995) Combination therapy with cyclosporine and methotrexate in severe rheumatoid arthritis. N. Eng. J. Med. 333, 137-141.

14. Alarcon, G.S., Tracy, I.C., Strand, G.M. et al (1995) Survival and drug discontinuation analyses in a large cohort of methotrexate-treated rheumatoid arthritis patients. Ann. Rhem. Dis. 54, 708-712.

15. Arend, W.P. (2001) Physiology of cytokine pathways in rheumatoid arthritis. Arthritis Rheum. 45, 101–6

16. Xu, W.D., Firestein, G.S., Taetle, A. et al (1989) Cytokines in chronic inflammatory arthritis. II granulocyte macrophage colony stimulating factor in rheumatoid synovial effusions. J. Clin. Invest. 83, 876-882.

17. Field, M. and Clinton, L. (1993) Expression of GM-CSF receptor in rheumatoid arthritis (Letter). Lancet 342, 1244.

18. Schimke, R.T. (1980) Gene amplification and drug resistance. Sci. Am. 243, 60-69.