Clinica Chimica Acta xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Methotrexate normalized keratinocyte activation cycle by overturning abnormal keratins as well as deregulated inflammatory mediators in psoriatic patients

Tamilselvi Elango [a,*], Anand Thirupathi [b], Swapna Subramanian [c], Haripriya Dayalan [d], Pushpa Gnanaraj [e]

[a] Department of Medical Research, SRM Medical College, Hospital and Research Centre, Kattankulathur 603203, Tamilnadu, India
[b] Laboratory of Physiology and Biochemistry of Exercise, PPGCS, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil
[c] Department of Biochemistry, SRM Medical College, Hospital and Research Centre, Kattankulathur 603203, Tamilnadu, India
[d] Department of Biotechnology, Rajalakshmi Engineering College, Thandalam, Chennai 602105, Tamilnadu, India
[e] Department of Dermatology, SRM Medical College, Hospital and Research Centre, Kattankulathur 603203, Tamilnadu, India

ARTICLE INFO

Article history:
Received 23 June 2015
Received in revised form 19 October 2015
Accepted 21 October 2015
Available online xxxx

Keywords:
Methotrexate
Keratin 16
Keratin 17
Interferon-γ
Transforming growth factor-β_1

ABSTRACT

Background: In psoriatic skin, epidermal keratinocytes undergo deregulated inflammatory response that leads to prolonged expression of inflammatory mediators as well as abnormal keratins. Methotrexate (MTX) is an immunosuppressive agent used as a standard drug to treat severe psoriasis. The aim of the study is to find the pharmacological effect of MTX on abnormal keratin and deregulated inflammatory mediators.
Methods: Fifty-eight psoriasis vulgaris patients were recruited for this study. Skin biopsies of psoriatic patients were collected and analyzed for activation signal such as TNF-α and IFN-γ and deactivation signal such as TGF-β_1. Also, protein and gene expression of normal keratins K14 and K10 and abnormal keratins K16 and K17 were analyzed in skin biopsies before (day 0) and after (at the end of 6 and 12 weeks) MTX treatment.
Results: Results show a significant decrease in tissue TNF-α and IFN-γ and increase in TGF-β_1 after MTX treatment when compared with before MTX treatment in psoriasis patients ($p < 0.001$). Protein and gene expression of K14, K16 and K17 decreased after MTX treatment, whereas the expression of differentiation marker K10 increased after MTX treatment.
Conclusion: MTX resolves deregulated inflammatory markers and maintains normal keratin phenotype on hyperproliferating KC, thereby controlling acanthosis in psoriasis patients.

1. Introduction

Psoriasis is a chronic inflammatory skin disease mainly characterized by acanthosis, abnormal differentiation and infiltration of leukocytes from the dermis. The factors that cause this disease are genetic, environmental and inflammatory mediators. About 80% of the epidermal skin is comprised of keratinocytes (KCs). KCs also play a major role in this chronic inflammatory disease [1].

To maintain epidermal integrity, growth of KCs is regulated by balance between cell survival and cell death. Due to immune and genetic factors, KCs get activated and cell balance gets disturbed. This activation is mainly due to deregulated inflammatory response. A vicious cycle of KC-immune response leads to psoriasis [2].

Basal KCs have two alternative pathways to end up. Normally, they can differentiate through the spinosus, granular and cornified layers. Differentiation is affected by calcium, retinoic acid, vitamin D3 and protein kinase C (PKC) activators. Basal KC express keratins 5 (K5) and 14 (K14), while K1 and K10 are expressed by differentiating KC [3].

The other alternative pathway for KC is to become activated by a hyperproliferative and migrating phenotype. Activation occurs after epidermal injury and in certain pathological conditions, such as psoriasis. A keratinocyte activation cycle has recently been proposed to exist. The activation cycle begins with interleukin-1 (IL-1) release from KC. Activation changes the keratin pattern from K5/14 to K6/16. The activated phenotype is maintained by tumor necrosis factor α (TNFα) and transforming growth factor α (TGFα). In the later stages of lesional healing, KC becomes contractile by an interferon-γ (IFNγ) signal and start to express keratin 17 (K17). Once the lesion has been healed, a transforming growth factor β (TGFβ) signal from dermal fibroblasts makes the KC phenotype basal and the cells start to express K5/14. This whole process is called Keratinocyte activation cycle [3].

In wound healing, KC activation cycle is completed and the activation of KCs is a rapid, self-limiting process. By contrast, the failure to

Abbreviations: MTX, methotrexate; K14, K10, K16, K17, keratin 14,10,16,17; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TGF-β1, transforming growth factor beta1; KCs, keratinocytes.

* Corresponding author.
E-mail address: tami24684@gmail.com (T. Elango).

http://dx.doi.org/10.1016/j.cca.2015.10.020
0009-8981/

resolve the deregulated inflammatory response in psoriasis leads to the persistent activation of keratinocytes, which is characterized by prolonged K17 expression [4].

Methotrexate (MTX) has been used in the treatment of psoriasis and considered as the gold-standard therapy for moderate to severe psoriasis. In psoriasis, MTX appears to exert its effects by acting as both immunomodulatory agent and antimetabolite [5–7]. Immunomodulatory effects of MTX can be explained by the decreasing T-cell-mediated inflammation at multiple steps [8]. MTX inhibits the growth of keratinocytes and also capable of downregulating endothelial expression of the cell adhesion molecules ICAM-1 and E-selectin [9]. In vitro studies have shown that MTX decreases the markers associated with proliferation in the skin biopsies of psoriatic patients [10]. Only few evidence based-studies have been conducted by treating psoriatic patients with MTX. Immunosuppressive action of MTX has been studied well, but its action on keratinocytes is not well defined. In most of the cutaneous diseases, mutation of keratin molecules has been found. So finding the pharmacological role of keratin genes in skin disease such as psoriasis is mandatory.

2. Materials and methods

2.1. Patient details

Patients with psoriasis vulgaris (n = 58) who visited Dermatology Department, SRM Hospital, Kattankulathur, were recruited in this study. The age of all patients ranged from 18 to 70 y (mean ± SD 46.4 ± 14.1 y), and there were 27 men and 31 women. Patients ≥18 y, who had >20% body surface area involvement and who had not received any topical or systemic therapy for at least a month were included in this study.

Exclusion criteria were as follows: children (<18 y), pregnant and lactating women, patients with unstable psoriasis, liver and renal impairment, infertility, anemia, excessive alcohol intake or any other systemic diseases such as diabetes, hypertension, rheumatoid arthritis, cardiovascular diseases and respiratory syndrome. The study protocol was approved by the institutional ethical committee. Informed consent documents were signed by all the patients. The informed consent contains all these details. Full history including personal history (name, age, sex, occupation, residence and contact number), history of the disease (age of onset, course and duration of the disease), exacerbating factors, history of previous treatment, family history of similar condition, history of other systemic infection, clinical examination including site and severity of skin involvement, distribution of the lesion, generalized or localized, nail, scalp and joint involvement, and extent of skin involvement.

2.2. Treatment regimen

Patients with psoriasis were treated with 7.5 mg of MTX per week for 12 weeks. Folic acid was given at 5 mg once daily except on the day of MTX for 12 weeks. During systemic treatment, no concomitant antipsoriatic therapy was permitted, with the exception of emollients. Clinical evaluations were performed by the same dermatologist at four intervals (i.e. days 0, 2, 6 and 12 weeks) until completion of the study. Scoring was based on the Psoriasis Area Severity Index (PASI) scoring system.

2.3. Collection of tissue samples

In all patients, 10 mm of lesional and nonlesional skin biopsies were taken after local anesthesia, lidocaine hydrochloride and adrenaline bitartrate IP were given intradermally. Lesional biopsies were taken before (day 0) and after (6 and 12 weeks) treatment with MTX. Nonlesional skin biopsy served as control, which was collected only once. Biopsies of psoriatic lesional skin were taken within a lesion, 1 cm from the edge of the plaque border. Biopsies of nonlesional skin were taken 2 cm beyond the plaque border. Skin biopsies were immediately immersed in protease inhibitor cocktail and RNA later solution (Qiagen) and finally stored at −20 °C until further use.

2.4. Histological examination

Formalin-fixed skin biopsy were embedded in paraffin and processed routinely. Hematoxylin eosin staining was used to examine the histological changes in psoriatic skin before and after MTX treatment.

2.5. Preparation of tissue for ELISA

The skin biopsy was weighed (50–100 mg) and was homogenized using a glass homogenizer with 1.5 ml of extraction buffer (10 mmol/l Tris, 150 mmol/l NaCl, 1% Triton X-100, pH 7.4) of tissue. Then, the homogenate was centrifuged at 13,000 ×*g* for 10 min at 4 °C and the supernatant was used for ELISA [11].

2.6. Measurement of TNF-α, IFN-γ and TGF-β_1 in skin biopsy by ELISA

The concentration of TNF-α, IFN-γ and TGF-β_1 in supernatant of skin biopsy was measured using Bender MedSystem Kits from Austria, Europe (catalog no: BMS223HS; BMS228TEN and BMS249/4TEN) according to manufacturer's instructions.

2.7. Western blot analysis

Total cell extracts were prepared from skin biopsy as described earlier [12]. Keratin 14, 10, 16 and 17 expressions were determined by Western blot analysis [13] using the primary antibody [Cytokeratin 14 (LL001); Cytokeratin 10(RKSE 60); Cytokeratin 16 (LL025); Cytokeratin 17(Ks17.E3)] purchased from Santa Cruz and β-actin-peroxidase clone Ac-15; Sigma-Aldrich). Band intensity was analyzed using ImageJ software (http://rsb.info.nih.gov/ij/).

2.8. Real-time quantitative PCR

Total RNA was isolated from lesional (before, during and after MTX treatments) and nonlesional skin biopsies from psoriasis patients (AxyPrep Multisource Total RNA Miniprep Kit, Applied Biosystems). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcriptase reactions. Primer sequence for keratin 14, 10, 16 and 17 and the housekeeping gene beta-actin is depicted in Table 1. cDNA was PCR-amplified under the following conditions: at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, 2 min at 501 °C and 10 min, with data collection in the last 30S. For all PCRs, Power SYBRGreen RT PCR Reagent Kit (Applied Biosystems) was used in the reaction. The amount of each mRNA was normalized to the amount of beta actin in the same sample. Relative mRNA expression levels of all examined genes were measured using the comparative $2^{-\Delta\Delta CT}$ [14].

Table 1
Primer pairs used for quantitative real-time PCR.

S.No	Gene	Gene name	Primer sequences
1	Human keratin 10	HK10 F	5' ctactcttcctcccgcagtg 3′
		HK10 R	5′ tccataactcccaccaaagc 3′
2	Human keratin 14	HK14 F	5′ ttctgaacgagatgcgtgac 3′
		HK14 R	5′ gcagctcaatctccaggttc 3′
3	Human keratin 16	HK16 F	5′ gcatgcagtagcggcctt t 3′
		HK16 R	5' tccaacagcgaactggtacaga 3′
4	Human keratin 17	HK17 F	5′ cggagacagagaaccgctac 3′
		HK17 R	5′ cacaatggtacgcacctgac 3′
5	Human beta-actin	H β –actin F	5′ gctcgtcgtcgacaacggctc 3′
		H β –actin R	5′-catgatctgggtcatcttctc-3′

Please cite this article as: T. Elango, et al., Methotrexate normalized keratinocyte activation cycle by overturning abnormal keratins as well as deregulated inflammatory mediators..., Clin Chim Acta (2015), http://dx.doi.org/10.1016/j.cca.2015.10.020