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Review

Methotrexate in Psoriasis: From A to Z

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Abstract

Background: For decades methotrexate has been used in the treatment of psoriasis with well-established efficacy. Although many theories regarding methotrexate's mechanism of action in psoriasis were suggested, the exact the mechanism of action is still not very well understood. This paper reviews the published literature on methotrexate mechanism of action in psoriasis. Articles published with English abstracts between January 1970 and December 2008 identified in MEDLINE were reviewed. It is likely that methotrexate interferes with the inflammatory pathways critical to psoriasis pathogenesis by multiple mechanisms. Current evidence suggests that methotrexate, as an immunomodulatory agent and anti-metabolite, decreases T-cell-mediated inflammation at multiple steps and that explains its efficacy in treating various inflammatory diseases including psoriasis.

Introduction

For more than three decades, methotrexate has been used in the treatment of psoriasis with well-established record of efficacy. Even with the era of novel biological agents, methotrexate remains commonly used for the treatment of moderate to severe psoriasis. Despite the long history, methotrexate's mechanism of action in psoriasis remains not entirely clear. Many theories for methotrexate's mechanism of action in psoriasis were suggested. When the chemotherapeutic agent methotrexate was found to be effective in treating psoriasis, it was assumed that its efficacy lay in its cytotoxic effects on hyperproliferating keratinocytes [**1, 2**]. However, as appreciation of psoriasis as a T-cell mediated inflammatory process grew, interest in methotrexate's potential role as an immunosuppressant has also increased. Several immunomodulatory and immunosuppressive effects have been ascribed to methotrexate in both in vitro and in vivo studies, and it is becoming more apparent that methotrexate may intercept several points in the inflammatory pathways that lead to psoriasis. This review was performed by searching MEDLINE for articles published between 1970 and 2008 containing the keywords methotrexate, psoriasis and mechanism. Studies investigating the mechanism of action of methotrexate in rheumatoid and psoriatic arthritis were included. We therefore provide a review of current knowledge on methotrexate mechanism of action in the treatment of psoriasis.

Pathogenesis of Psoriasis

Psoriasis is a chronic inflammatory disease characterized by T-cell-driven keratinocyte hyperproliferation and hypervascularity, which presents clinically as scaly, erythema-

tous plaques.[**3, 4**] The current model of psoriasis pathogenesis is that antigen-presenting cells (APCs) from the skin migrate to regional lymph nodes and activate T cells with an, as yet, unidentified antigen. This APC and T-cell interaction depends on cell surface molecules such as intercellular adhesion molecule-3 (ICAM-3). T-cell activation induces expression of surface molecules such as cutaneous lymphocyte associated antigen (CLA-1) and leukocyte function-associated antigen-1 (LFA-1). CLA-1 binds endothelial leukocyte adhesion molecule 1 (E-selectin), a surface molecule on activated endothelial cells that facilitates the migration of T cells to the inflammatory site via blood vessels. Once in close proximity to the inflammatory site, the T cells' LFA-1 binds to intercellular adhesion molecule-1 (ICAM-1), which is found on endothelial cells. This binding facilitates T-cell adhesion to endothelial cells and allows for subsequent T-cell diapedesis from the intravascular space into the dermis. In the dermis, activated T cells mediate inflammation characterized by the predominant involvement of TH-1 cytokines. This inflammation is believed to drive keratinocyte hyperproliferation. Each of these steps represents potential sites of methotrexate action.

Effects on Keratinocytes

Methotrexate's anti-metabolite activity initially led to theories that it worked by disrupting keratinocyte reproduction. Keratinocyte hyperproliferation can be disrupted by inducing cell death, inhibiting proliferation, or inducing maturation. Methotrexate's role in each of these processes has been investigated. Although keratinocyte hyperproliferation is a key feature of psoriasis, few studies have been published that explore methotrexate effects on keratinocytes.

Cell Death

In 1992, *Schwartz* et al explored methotrexate effects on keratinocyte cell viability [**5**]. The authors compared the levels of cell viability between human neonatal foreskin keratinocytes cultured with methotrexate 10-5 M to 10-6 M for 72 hours and control keratinocytes not exposed to methotrexate. Neutral red vital staining and the trypan blue exclusion assay were used to assess cell viability. Healthy cell membranes are impermeable to trypan blue, while dying cell membranes are permeable, resulting in positive staining. Neutral red will positively stain the nuclei of viable cells. The study reported similar amounts of staining between the two groups, suggesting that methotrexate did not reduce the viability of the human keratinocytes. Three years later, Jeffes et al provided evidence suggesting that lymphocytes in psoriatic lesions were significantly more vulnerable to methotrexate cytotoxic effects than keratinocyte and epithelial cells [**6**]. Proliferating lymphoid cells that included macrophages and T cells, normal human keratinocytes and epithelial cells, were cultured for 24 hours with methotrexate and the number of killed cells was counted. Methotrexate was employed at concentrations that would be achieved with once weekly low-dose methotrexate therapy. They reported that over 95% of the lymphoid cells were killed while less than 10% of the proliferating epidermal cells were affected by this methotrexate exposure. In 1998, Heenen et al investigated the hypothesis that low-dose methotrexate triggers keratinocyte apoptosis [**7**]. The keratinocyte model used consisted of normal skin epidermal explants cultivated for 10 days on dead de-epidermized dermis. This model was incubated with methotrexate 10-7 M to 10-6 M for 5 days. Cells exhibiting chromatin aggregation, separation from neighboring cells, and apoptotic bodies under light microscopy were deemed apoptotic. The TUNEL assay was used to detect fragmented DNA and the presence of p53, a transcription factor that can activate the apoptosis pathway in the presence of severe cellular stress resulting from DNA damage. Light microscopy revealed an increased percentage of apoptotic cells in the basal layer of the methotrexate-treated skin versus the control (1% ± 0.4 apoptotic in methotrexate treated versus 0.02% ± 0.01 apoptotic in the control). In the methotrexate-treated cultures, TUNEL was positive in some basal and para-basal cells. No controls were described for the TUNEL assay. Forty percent of the basal layer stained positive for p53 but no controls were conducted for the p53 assay. Heenen et al also used TUNEL and histology to examine punch biopsies from four psoriasis patients 24 hours after they completed an eight-week course of 12 mg to 15 mg/week methotrexate therapy. They reported three

apoptotic cells per 1,000 germinative cells, but did not describe a control group. Based on the findings from both the model and biopsies, the authors of the study concluded that low-dose methotrexate induces apoptosis in human keratinocytes.

Hyperproliferation

The most intuitive approach for discouraging keratinocyte hyperproliferation would be to inhibit keratinocyte reproduction. This approach was employed in several studies. In 1992, *Schwartz* et al incubated healthy human keratinocytes with methotrexate 10-5 M to 10^{-7} M for 72 hours in thymidine-free media [**5**]. An electronic cell count of keratinocytes revealed a 75% inhibition of growth in the methotrexate exposed cells compared to the controls, suggesting that methotrexate had a growth inhibitory effect on the keratinocytes. The authors, however, note that methotrexate's effects on proliferation were completely prevented by the addition of thymidine, the nucleotide whose synthesis is prevented when methotrexate acts as a folate anti-metabolite. Based on these observations, the authors concluded that methotrexate is capable of inhibiting keratinocyte growth. Furthermore, the mechanism by which keratinocyte growth is inhibited appears to be based upon methotrexate's actions as an anti-metabolite. In 2003, *Pol* et al used a 96-wellplate assay system to investigate the antiproliferative effects of several anti-psoriatic drugs, including methotrexate, on human keratinocytes [**8**] Keratinocytes were exposed to methotrexate 10^{-5} M to 10^{-7} M and their proliferation over four days was assessed using a sensitive DNA binding dye. Increased staining is directly correlated to increased cell mass and indicates cell proliferation. Cytotoxic effects were controlled for with lactate dehydrogenase surveillance. Lactate dehydrogenase is normally an intracellular enzyme; detection of an increased release of lactate dehydrogenase suggests a loss of cell viability. Methotrexate exposure resulted in a 20% inhibition of keratinocyte cell growth on day 2, which was not statistically significant. The study concluded that methotrexate did not significantly affect proliferation at therapeutically relevant concentrations. These results were similar to the earlier findings of the 1995 study by *Jeffes* et al, who also concluded that methotrexate did not inhibit the growth of normal human keratinocytes at concentrations achieved with once-weekly methotrexate therapy. In a 2005 study published by Yazici et al, 10 psoriasis patients provided lesional skin biopsies before and after a six-week course of methotrexate 10 mg to 25 mg/ week [**9**]. The biopsy specimens were assessed by immunohistochemistry for expression of surface molecule proliferating cell nuclear antigen (PCNA) and Ki-67. PCNA and Ki-67 are nuclear proteins associated with proliferation and have been found to be increased in psoriasis [**10, 11**]. They found that lesions expressed lower levels of both Ki-67 ($p<0.01$) and PCNA ($p<0.01$) following methotrexate treatment. This suggests that methotrexate therapy may result in decreased hyperplasia within lesional skin.

Keratinocyte Maturation and Differentiation

Inducing keratinocyte maturation and differentiation may also potentially slow keratinocyte turnover. An in vitro study published by *Schwartz* et al in 1992 examined methotrexate effects on keratinocyte proliferation and differentiation [**5**]. Human neonatal foreskin keratinocytes were cultured with methotrexate 10^{-5} M to 10^{-7} M for three to six days. After 72 hours of exposure, an electronic cell counter detected a mean cell volume increase of 225%, which suggested an increase in cell size associated with keratinocyte differentiation. Immunohistochemical staining for involucrin, a marker of terminal keratinocyte differentiation, was increased in the methotrexate-treated cells (1×105 methotrexate versus 5.5×104 control). Microscopic evaluation of these keratinocytes revealed larger, flatter cells with decreased nuclear-to-cytoplasm ratios, consistent with a more mature stage of differentiation. At five days of methotrexate 10^{-6} M exposure, scintillation counts and fluorography showed a 2.0 to 2.3 increase in radioactive amino acid incorporation, suggesting an increase in protein synthesis. After six days of methotrexate 10^{-6} M exposure, a several-fold increase of insoluble protein staining, thought to represent cornified envelope protein, was observed. No significant findings were reported for cultures exposed to methotrexate concentrations less than 10^{-6} M. The authors concluded that,

based on the morphological and biochemical findings, methotrexate induces maturation and terminal differentiation in keratinocytes.

Methotrexate's Effects on Endothelial Cells

Downregulation of Adhesion Molecules

T-cell migration from the intravascular space into the dermis is a crucial step in the pathogenesis of psoriasis, and this process is dependent on interactions between endothelial cells and T cells. Endothelial expression of appropriate adhesion ligands such as E-selectin and ICAM-1, are necessary for successful T-cell adhesion and migration [**12, 13**]. In 2003, Yamasaki et al investigated how therapeutic concentrations of methotrexate (10^{-6} to 10^{-7} M) affected the expression of ICAM-1 on human umbilical vein endothelial cells [**14**]. Immunohistochemistry revealed a decrease in ICAM-1 expression after exposure to 10^{-6} M of methotrexate. Furthermore, they found that endothelial cells exposed to methotrexate expressed lower levels of ICAM-1 mRNA as measured by PCR. Based on these results, it was concluded that methotrexate downregulates ICAM-1 on endothelial cells, and may do so by downregulating gene expression. The following year, Sigmundsdottir et al studied the effects of methotrexate on endothelial expression of E-selectin [**13**]. A psoriasis patient was treated with low-dose methotrexate for five weeks. At days 4, 11 and 16 post-therapy, punch biopsies of the skin lesions and immunohistochemistry were performed to detect E-selectin. Methotrexate was restarted, and two additional punch biopsies performed on days 21 and 32 were also stained for E-selectin. Two observers blinded to the timing of the biopsies evaluated the immunohistochemistry independently. They found that clinical exacerbation coincided with rising E-selectin levels and CLA+ influx into the dermis. Conversely, the resumption of methotrexate was followed by decreased E-selectin expression and decreased CLA+ T-cell infiltrate in the dermis. This chronologically correlated relationship between the level of E-selectin and the subsequent number of infiltrating CLA+ lymphocytes demonstrated the potential significance of E-selectin expression in the psoriasis pathogenesis pathway. Overall, *Sigmundsdottir* et al concluded that methotrexate decreases endothelial expression of E-selectin. Similar findings were reported in the study of methotrexate effects on endothelial activation markers in patients with bullous pemphigoid. A decrease in ICAM-1 and E-selectin expression was found in patients with bullous pemphigoid treated with methotrexate [**15**].

Angiogenesis Inhibition

Histologically, hypervascularity is noted in psoriatic skin, which contributes to the grossly observed erythema. When used at the high dosages necessary for chemotherapy, methotrexate is capable of inhibiting angiogenesis. When hypervascularity became appreciated as a feature of psoriasis, it was natural to ask whether methotrexate exerted therapeutic effects in psoriasis by inhibiting angiogenesis. In 1989 *Hirata* et al performed in vivo studies to examine whether methotrexate can inhibit angiogenesis [**16**]. Methotrexate 5×10^{-9} M was injected intramuscularly into rabbits and the degree of corneal neovascularization was assessed. This early in vivo study concluded that low-dose methotrexate inhibits angiogenesis. In 2003 *Yamasakai* et al examined methotrexate effects on endothelial growth [**14**]. Human umbilical vein endothelial cells were incubated with 10^{-8} M to 10^{-7} M of methotrexate for three, six and eight days. Cells were stained with Alamar blue dye and the number of cells was determined by measuring absorbance of cell culture media at 590 nm. They found that methotrexate had a dose-dependent inhibitory effect on human umbilical vein endothelial cell growth (culture treated with methotrexate 10^{-7} M: 2×102 OD at 590 nm versus control culture with no methotrexate exposure: 5×102 OD at 590 nm, $p < 0.001$). The authors concluded that these findings indicated that methotrexate had an inhibitory effect on endothelial cell growth. Two years later, in 2005, *Yazici* et al employed immunohistochemistry on lesional skin biopsies to study the effects of methotrexate on angiogenesis [**9**]. CD31 is a commonly used endothelial marker and increased levels suggest newly formed blood and lymphatic vessels [**17, 18**]. Ten psoriasis patients were treated with methotrexate 10 mg to 25 mg per week. Biopsies were performed before and six weeks after treatment. All biopsies were stained with CD31 antibodies.

The degree of CD31 staining was evaluated using an arbitrary four-point scale: (0) no staining, (1) weak staining limited to the papillary endothelium, (2) moderate diffuse endothelial staining, and (3) severe diffuse endothelial staining. A statistically significant reduction in CD31 staining ($p < 0.05$) was observed in the post-methotrexate skin biopsies. The authors concluded that methotrexate decreased the expression of the endothelial marker CD31, which suggests that methotrexate inhibited the formation of new blood vessels. In the same year, *Fiehn* et al reported conflicting findings [**18**]. They studied methotrexate effects on angiogenesis using a human placental angiogenesis assay and a murine matrigel model. The human placental assay exposed fresh placental tissue to 10 µg to 100 µg of methotrexate and measured angiogenesis by counting the number of new microvessels. They found that 100 µg of methotrexate failed to inhibit angiogenesis in the human placental assay. The murine matrigel model is used to study anti-angiogenic drugs. A matrigel containing basic fibroblast growth factor and heparin was injected intracutaneously into 24 mice. Twice weekly, 14 mice received methotrexate 35 mg/kg intraperitoneally; 12 control mice received the placebo. The mice were killed on day 10 and the matrigel matrix removed for analysis. The matrigel matrix was homo-genized and the hemoglobin content, which parallels the matrigel vessel content, was measured. No significant difference in matrigel hemoglobin content was found (1.61 ± 1.59 g/L). Based on the findings from both models, *Fiehn* et al concluded that methotrexate did not inhibit angiogenesis. They suggested that *Hirata* et al findings [**16**] may have differed because the aqueous humor may act as a compartment where methotrexate can accumulate in high concentrations for prolonged periods.

Antigen-Presenting Cells

The stimulation of antigen-presenting cells in the skin, also known as dendritic cells or Langerhans cells (LC) may be a key upstream event in psoriasis pathogenesis. Modulation of this process may significantly influence whether psoriatic lesions develop. A 1983 study by *Morhenn* et al at Stanford examined the inhibition of LC with various anti-psoriatic agents using the skin cell lymphocyte reaction [**19**]. This assay measures an agent ability to stimulate lymphocyte proliferation after LC is pre-incubated with the agent. Pre-incubation for 24 hours with methotrexate did not affect the LC's capacity to stimulate lymphocytes; however, co-incubation of LC and peripheral lymphocytes with methotrexate completely inhibited the lymphocytes ability to respond to stimuli. In 1997, *Liu* et al tested methotrexate effects on LC immunostimulatory effects and LC viability [**20**]. Mixed LC-lymphocyte reaction (MLCLR) was used to assess methotrexate effect on LC stimulation. LC was incubated with methotrexate, then washed and re-incubated with peripheral blood lymphocytes. To assess LC viability, LC was incubated with methotrexate and subsequently stained with trypan blue. Methotrexate had a modest effect on the MLCLR and no effect was seen above 1 µg/mL. The authors concluded that it was unlikely that methotrexate exerted its immunomodulatory effects via LC suppression. Methotrexate showed no effect on LC viability, even at very high pharmacological levels (1 mg/mL).

Methotrexate's Effects on T Cells

Evidence supports that activated T cells are key players in the immunopathogenesis of psoriasis. The following components involving T cells are considered crucial in the pathogenesis of psoriasis: T-cell migration into lesional tissue, the number of activated CLA+ T cells in lesional tissue, and T-cell cytokine production. How methotrexate affects each of these steps has been the focus of the studies listed below.

Downregulation of T-Cell Adhesion Molecules

In 2004, *Sigmundsdottir* et al studied methotrexate effects on peripheral T-cell CLA expression in 16 psoriasis patients who received methotrexate 5 mg to 25 mg per week [**13**]. Blood samples were collected three to four days after dosing; and isolated peripheral T cells were stained with CLA-specific monoclonal antibodies (mAbs) and analyzed by flow cytometry. A negative correlation ($r = -0.505$, $p = 0.046$) between methotrexate dosage and the percentage of cells staining positive for CLA among peripheral T cells was observed, suggesting that methotrexate reduced CLA

expression in a dose-dependent manner. In another branch of this study, one psoriasis patient treated with methotrexate 25 mg per week underwent similar evaluation during which blood was collected for five weeks. Peripheral T cells were isolated, stained and analyzed with flow cytometry for surface CLA. The percentage of peripheral T cells staining positive for CLA decreased in the first three to four days following methotrexate administration, and then increased steadily until the next methotrexate dose. Methotrexate also decreased the amount of CLA expressed per T cell. *Sigmundsdottir* et al concluded that a dose-dependent, inverse relationship exists between methotrexate administration and the frequency and intensity of CLA expression on T cells in psoriasis patients. Methotrexate treatment resulted in fewer peripheral T cells expressing CLA and fewer CLA molecules per cell on the T cells that continued to express CLA. *Johnston* et al in 2005 investigated the effects of low-dose methotrexate on the lymphocyte expression of several adhesion molecules, including CLA and ICAM-1 [**21**]. Peripheral T cells were stimulated with streptococcal antigen and then incubated with methotrexate 10^{-9} M to 10^{-5} M for five days. Adhesion molecule expression was assessed with immunohistochemical staining and flow cytometry. They concluded that fewer T cells expressed CLA or ICAM-1 following methotrexate incubation at concentrations greater than 10^{-7} M. Follow-up experiments revealed that methotrexate suppression of CLA expression could be reversed by folinic acid (leucovorin) supplementation. Folinic acid is commonly used to rescue cells from the antimetabolite effects of methotrexate by providing an alternate route of thymidylate synthesis. Folinic acid supplementation suggests that CLA suppression by methotrexate occurs via a folate-dependent pathway. In a 2005 study published by *Yazıcı* et al, 10 psoriasis patients provided lesional skin biopsies before and after a six-week regimen of methotrexate 10 mg to 25 mg per week [**9**.] The biopsy specimens were assessed for expression of surface molecule ICAM-3 by immunohistochemistry. ICAM-3 is associated with T-cell and APC interactions. ICAM-3 expression was evaluated using an arbitrary four-point scale. The authors concluded that methotrexate decreased the expression of ICAM-3 ($p < 0.01$).

T-Cell Cytolysis

Since the discovery of a positive correlation between the number of CLA+ T cells and disease severity in psoriasis, interest has escalated in methotrexate's cytolytic effect on activated T cells. As previously discussed, Jeffes et al suggested that methotrexate possessed substantial cytotoxic effects on lymphocytes, and that lymphocytes are 1,000 times more sensitive to methotrexate than epithelial cell lines. [6] Other studies have confirmed T-cell sensitivity to methotrexate [**22**], including a recent study by *Herman* et al [**23**]. In addition to verifying methotrexate ability to induce T-cell death, *Herman* et al investigated the mechanism by which cell death occurs. T cells were incubated with methotrexate 10^{-5} M to 10^{-9} M for 24 hours prior to assessment. They found that T cells stained positive for apoptotic cell markers at a rate three times greater than controls ($22.5 \pm 1.5\%$ versus $9.1 \pm 0.7\%$ respectively, $p \leq 0.05$). These findings suggest that methotrexate can induce apoptosis in T cells. Methotrexate may also induce cell death via free radical oxygen species. *Phillips* et al inhibited methotrexate induced T-cell death with the addition of the antioxidant glutathione and its precursor, N-acetylcysteine [**22**].

T-Cell TNF-α Production

Accumulating evidence suggests that methotrexate's anti-inflammatory qualities arise from its effects on T-cell cytokine production, specifically, by reducing inflammatory cytokine and increasing inhibitory cytokine production [**24, 25, 26, 27**]. As a key element in psoriasis pathogenesis, the cytokine TNF-α is found at higher levels in psoriasis plaques and the synovial fluid of patients with psoriatic arthritis [**27, 28, 29**]. The clinical efficacy of newer anti-psoriatic drugs that target TNF-α, for example, etanercept, infliximab and adalimumab, underscore the importance of TNF-α in psoriasis pathogenesis. Associations between methotrexate and TNF-α levels have been observed since the 1990s. A 1995 study by *Seitz* et al found that psoriasis patients who clinically improved while on methotrexate had reduced TNF-α production among their peripheral blood mononuclear cells (PBMCs), comprised of monocytes, T cells and B cells [**27**]. Two studies involving patients with rheumatoid arthritis reported reduced

TNF-α levels in the synovial fluids of patients undergoing methotrexate therapy [**30, 31**]. In 1999, two studies investigated the relationship between methotrexate and TNF-α production by activated T cells [**26, 33**]. In one study, Neurath et al used mice to study methotrexate effects on cytokine production by splenic T cells [**26**]. Splenic T cells were isolated from healthy mice and cultured. These T cells were activated by stimulation with various commonly used antigens. The cultures were exposed to methotrexate 10^{-7} M to 10^{-5} M. Serial ELISAs of the supernatant tracked the production of TNF-α for 50 hours. They found that methotrexate significantly reduced TNF-α production by activated T cells (500 pg/mL TNF-α, methotrexatetreated group, versus 1,800 pg/mL TNF-α, control group). The authors concluded that in T cells, methotrexate reduces TNF-α production in murine models, and suggested that TNF-α modulation contributes to methotrexate's efficacy in rheumatoid arthritis. In another study, *Hildner* et al used peripheral CD4+ T cells to study the relationship between methotrexate and TNF-α production [**33**]. They harvested CD4+ T cells from the peripheral blood of healthy volunteers. Primed T cells were stimulated and cultured with IL-2 for nine days in the presence or absence of methotrexate (0.1 to 10.0 μg/mL). These primed T cells were then restimulated with two additional days of anti-CD3 mAb and IL-2 stimulation, along with methotrexate (0.1 to 10.0 μg/mL). The concentration of TNF-α in the supernatant was analyzed by ELISA. A statistically significant reduction of TNF-α production was observed at all methotrexate dosages. At 0.1 μg/mL, TNF-α was reduced to < 10% ($p < 0.01$) of the levels observed in the control group. They concluded that methotrexate can inhibit TNF-α production by primed human T cells. In a follow-up study, *Gerards* et al compared the production of several cytokines by peripheral blood mononuclear cells collected from 20 healthy human volunteers [**32**]. Blood monocytes were collected from healthy volunteers and rheumatoid arthritis patients and stimulated with various antigens. The cells were cultured for four days with methotrexate concentrations between 2 μg/mL and 2 ng/mL. ELISA was used to measure the amount of cytokine present in the culture supernatant. They found that the concentration of methotrexate required for inhibition varied between donors: in the control group not exposed to methotrexate, TNF-α concentrations ranged from 470 pg/mL to 11,000 pg/mL. Due to the wide inter-individual variability, data from each donor was analyzed using his or her own control trials. A statistically significant reduction in TNF-α production was observed at methotrexate concentrations greater than 8 ng/mL. At a methotrexate concentration of 1 μg/mL, TNF-α levels were suppressed by greater than 80% compared to control culture concentrations. Interestingly, the addition of folinic acid or thymidine abrogates methotrexate inhibitory effects on TNF-α production. A more recent study by Lange et al echoed the findings of earlier studies, concluding that methotrexate does indeed reduce TNF-α production by activated T cells [**34**]. This study compared the levels of TNF-α, among others, in methotrexate-treated and untreated mice. Findings included a significant reduction in the amount of TNF-α produced.

Discussion

Methotrexate is a folate anti-metabolite originally introduced as a chemotherapeutic agent; thus, early research regarding methotrexate mechanism of action focused on its cytotoxic capabilities. However, since its introduction, methotrexate has been found effective in the treatment of a variety of inflammatory diseases such as psoriasis, rheumatoid arthritis and inflammatory bowel disease [**35**]. Recognition of methotrexate's therapeutic efficacy in inflammatory disorders redirected research efforts toward its potential role in immunomodulation. This review of available literature on methotrexate mechanism of action in psoriasis therapy revealed that methotrexate does possess immunomodulating capabilities. Moreover, we found a lack of evidence for earlier but still commonly held beliefs that methotrexate primarily acts by interfering with keratinocyte reproduction. The following is a discussion of what is known, what has been shown to be unlikely, and what remains to be substantiated regarding the components underlying methotrexate efficacy in psoriasis treatment.

ICAM-1 and E-Selectin Downregulation

Endothelium expression of adhesion molecules on dermal vessels comprises a critical step in the pathogenesis of psoriasis, as it facilitates T-cell localization to the inflammation site. Yamasaki et al provided in vitro evidence of ICAM-1 downregulation in endothelial cells following methotrexate exposure [**14**]. Their research suggested that methotrexate did this by suppressing gene expression. Concurrent immunohistochemical experiments demonstrated a significant reduction in the number of cell surface ICAM-1 molecules on endothelial cells exposed to therapeutic concentrations of methotrexate. Taken together, these findings suggest that methotrexate effectively reduces endothelial cell surface expression of ICAM-1 by suppressing ICAM-1 gene expression.

Another cell adhesion molecule elevated in psoriasis and correlated with disease severity is E-selectin. As previously mentioned, E-selectin allows T cells to enter the skin. Inhibition of this step inhibits psoriasis [**36, 37, 38, 39**]. *Sigmundsdottir* et al reported that successive skin biopsies on a psoriatic patient on low-dose methotrexate therapy revealed a progressive decrease in E-selectin expression with continued methotrexate administration [**13**]. This was followed by a marked reduction in the number of CLA+ leukocytes in the skin lesions. The reduction in E-selectin and CLA+ leukocyte infiltrate coincided with clinical improvement. This finding reiterates the importance of E-selectin in the disease process. Although these results were derived from a single subject, they are compelling nonetheless due to the distinct patterns and correlations between methotrexate administration, E-selectin levels, CLA+ leukocyte levels and clinical disease. Similar results of decreased adhesion molecules were seen in other disease models such as bullous pemphigoid [**15**].

These studies provide promising preliminary evidence that methotrexate is capable of downregulating endothelial expression of the cell adhesion molecules ICAM-1 and E-selectin. Given the significance of ICAM-1 and E-selectin to the pathogenesis of psoriasis, this likely represents a major mechanism by which methotrexate exerts its clinical efficacy in psoriasis.

CLA and ICAM-3 Downregulation

Several studies provide clear evidence that methotrexate therapy reduces T-cell expression of the adhesion molecules CLA and ICAM-3. These molecules play pivotal roles in T-cell localization to inflammation sites, T-cell diapedesis to inflamed tissue, and interactions with APCs, respectively. The 2004 study by Sigmundsdottir et al offers clear in vivo evidence that methotrexate reduces the percentage of peripheral T cells expressing CLA, and the intensity of CLA expression per cell. Frequent blood draws on psoriasis patients receiving methotrexate therapy provided data sufficient to reveal a clear pattern of decreasing CLA+ T-cell counts in response to each methotrexate administration. Furthermore, this group demonstrated a statistically significant negative correlation between methotrexate dosage and CLA+ T-cell frequency, which provides further support of methotrexate effect on T-cell expression of CLA. Two other studies reflect these findings, also reporting a decrease in CLA+ T cells following methotrexate exposure. [9,40] There is also some evidence of reduced ICAM-3 expression in lesional skin following methotrexate therapy. While the evidence presented by these studies provide interesting and rather promising ground work in the topic of T-cell adhesion molecules, further studies are necessary for corroboration.

T-Cell Death

In the 1990s, it was initially assumed that methotrexate achieved its therapeutic benefit by targeting keratinocytes; therefore, the discovery that T cells were more vulnerable to methotrexate cytotoxic effects were surprising. In a breakthrough study, *Jeffes* et al demonstrated that T cells were more than 1,000 times more sensitive to methotrexate cytotoxicity than epithelial cell lines [**6**]. Since then, many studies have yielded similar results, confirming that methotrexate can induce activated T-cell death [**41, 42, 43, 44**]. While it has been argued that methotrexate is unlikely to function via its cytotoxic effects since methotrexate is used at much lower doses in psoriasis than in chemotherapy, these studies employed methotrexate concentrations corresponding to the exposure achieved during low-dose methotrexate therapy. It is likely that the activated T cells vulnerability to

methotrexate toxicity allows for cytolysis at lower doses than necessary for other cell types. Together with the T cells demonstrated enhanced susceptibility to methotrexate, it is probable that T-cell cytolysis contributes in part to methotrexate efficacy in psoriasis. Current studies are now focused on elucidating the mechanism of cytotoxicity. Work by *Herman* et al suggested that apoptosis is the mechanism of cytotoxicity [**23**], while *Phillips* et al concluded that the generation of radical oxygen species plays a role [**22**]. Regardless of the mechanism by which methotrexate induces T-cell death, T cells are undoubtedly key players in psoriasis pathogenesis, and the induction of T-cell death likely contributes significantly to methotrexate clinical efficacy in psoriasis.

TNF–α Production by Activated T Cells

Accumulating evidence suggests that methotrexate alters T-cell production of several cytokines, including IL-1, IL-2, IL-4, IL-8, INF-γ and TNF-α [**25, 28, 32, 42**]. Although these cytokines have well-established clinical significance in psoriasis, the discussion of each is beyond the scope of this review, which focuses on methotrexate effects on TNF-α. Over a decade ago, it was observed that psoriasis and rheumatoid arthritis patients improving on methotrexate therapy had reduced concentrations of serum and synovial TNF-α [**24, 30, 31**]. This association was further elucidated by *Gerard* et al, who demonstrated that a single oral administration of low-dose methotrexate induced a significant drop in serum TNF-α levels in 2 hours [**32**]. Initial *in vitro* studies, however, failed to demonstrate a significant effect of methotrexate on TNF-α production, foreshadowing the complex relationship between TNF-α and psoriasis [**24, 45, 46, 47, 48, 49**]. The relationship between methotrexate and TNF-α depends on several factors including the stage and route of T-cell activation, the presence of folinic acid and/or thymidine, the duration of contact while in culture, and intrinsic differences between individuals [**32, 33**]. Recent studies considering these factors have presented evidence that methotrexate inhibits T-cell TNF-α production [**28, 32, 33**]. Accumulating evidence suggests that methotrexate can reduce TNF-α production by activated T cells; however, because TNF-α production exhibits interindividual variability, the clinical significance of this mechanism in the average patient remains unknown.

Keratinocyte Maturation and Differentiation

Methotrexate might have a role in inducing keratinocyte differentiation and maturation. In *Schartz* et al, keratinocytes exposed to methotrexate exhibited histological changes consistent with a more mature stage of development. Furthermore, contrary to intuition, protein synthesis increased after methotrexate exposure, producing insoluble protein, possibly representing cornified envelope protein. The most compelling evidence, however, was the increased intracellular staining of involucrin, a marker of terminal keratinocyte differentiation [**5**].

Keratinocyte Death

Methotrexate is an anti-metabolite that binds irreversibly to dihydrofolate reductase with a greater affinity than folic acid. This binding prevents the de novo synthesis of the precursor for the DNA nucleotide thymidine. Cells are less likely to enter the synthesis or S-phase with a reduced availability of precursors; cells already in the S-phase die. Researchers initially studied methotrexate effects on keratinocytes because it was thought that methotrexate exerted its greatest effect there. A search for evidence of methotrexate antiproliferative effect on keratinocytes revealed few studies investigating this topic. Available literature is inconclusive owing to the small number of studies and/or their poor design. Results diverged widely, reporting no increased cell death, some effect, and significant apoptosis. One study employing viability stains reported no difference in cell viability between methotrexate-treated and untreated keratinocytes [**5**]. Another reported that less than 10% of proliferating keratinocytes were killed at concentrations achieved with once-weekly low-dose methotrexate therapy [**6**]. A study by *Heenen* et al concluded that low-dose methotrexate induces significant apoptosis in keratinocytes [**7**]. This study, however, is limited by its design: The TUNEL assay was employed, which may not be a valid assay to assess keratinocyte apoptosis because positive results also could be attributed to other cell

states, such as increased cell turnover (i.e. keratinocyte proliferation) [**50, 51**]. The study was further limited by an absence of controls. Together these factors limit the interpretability of the study results. It should be noted that all of these studies used healthy keratinocytes. Keratinocytes within psoriatic plaques have been shown to possess increased resistance to apoptosis induction compared to healthy keratinocytes [**51**]. Because these studies used healthy keratinocytes, it remains to be seen whether these findings are applicable to psoriasis. Little conclusive data exists to support the common conception that methotrexate acts in psoriasis by inducing keratinocyte death. However, the idea that methotrexate acts in psoriasis by slowing skin turnover remains pervasive in lay literature. Additional studies are necessary to definitively identify methotrexate effects on keratinocytes.

Angiogenesis

Angiogenesis inhibition by methotrexate is still a relatively new area of research. Very few studies discussed this issue. Among available studies, no agreement exists regarding methotrexate inhibitory effects on angiogenesis during low-dose therapy. A 2003 in vitro study reported significant methotrexate inhibition of endothelial cell proliferation, suggesting that methotrexate may prevent angiogenesis *in vivo* [**14**]. Two years later, an ex vivo study substantiated the earlier in vitro studies, reporting a statistically significant decrease in the endothelial marker CD31 after treatment with methotrexate [**9**]. Unfortunately, the authors did not state whether blinding was employed during the quantification of CD31 staining. Conversely, that same year, a study employing two different angiogenesis assay systems reported that methotrexate had no effect on angiogenesis [**16**]. Results from both an in vitro human placental model and an in vivo murine matrigel angiogenesis assay showed no increase in blood vessel formation following methotrexate exposure. Based on currently available evidence, it is still not known whether methotrexate inhibition of angiogenesis translates to clinical efficacy during psoriasis therapy.

Inhibiting Keratinocyte Proliferation

Methotrexate beneficial effect on psoriasis is frequently ascribed to its presumed ability to limit keratinocyte hyperproliferation by inhibiting de novo nucleotide synthesis. *Schwartz* et al concluded in 1992 that methotrexate inhibited proliferation of keratinocytes, but also noted that these effects were completely prevented by thymidine [**5**]. It can be argued that thymidine supplementation rescues these keratinocytes from cell cycle suspension by providing the desired DNA precursors. Regardless of the molecular explanation for this observation, a mechanism dependent on the complete absence of thymidine is unlikely to exert much clinical effect because thymidine is present in vivo. Furthermore, the human body can circumvent the de novo pathway via a salvage pathway to supply DNA precursors. A 2003 in vitro study reported no statistically significant difference in keratinocyte growth following methotrexate exposure [**8**]. The study did not address the presence of thymidine or folate in the culture medium; therefore, the findings cannot be compared to those obtained by *Schwartz* et al. Finally, a recent *ex vivo* study reported a decrease in the markers associated with proliferation in the skin biopsies of psoriasis patients who had undergone methotrexate therapy [**9**]. The presence of these markers was measured by immunohistochemistry and graded using an arbitrary four-point scale. Each patient demonstrated marked disease improvement as measured by the psoriasis area and severity index (PASI) score. The finding of reduced markers of proliferation may suggest that the resolution of psoriasis is associated with reduced proliferation, though not necessarily a direct result of methotrexate exposure. Reports of clinical efficacy achieved with reformulated versions of topical methotrexate are emerging [**52, 53**]. The clinical efficacy of topical methotrexate may stimulate renewed interest in the effects of methotrexate on keratinocytes. However, based on evidence available today, the inhibition of keratinocyte hyperproliferation has not proven to be methotrexate primary mechanism of action in psoriasis.

Antigen-Presenting Cell Inhibition

The inhibition of a key upstream event in psoriasis pathogenesis would be an elegant mechanism of action. The published studies addressing methotrexate effect on Langerhans cells (LCs) confirm that methotrexate

has no effect on LC viability [**19, 20**]. Furthermore, the studies implicate peripheral lymphocytes as methotrexate target. *Liu* et al found only a modest reduction in LC's immunostimulatory capabilities after incubation with methotrexate [**20**]. They concluded, however, that it was unlikely for methotrexate to exert its efficacy via LC suppression due to the high levels of methotrexate required for the modest response.

Conclusion

Methotrexate decreases T-cell-mediated inflammation at multiple steps. In the treatment of psoriasis, methotrexate appears to exert its effects by acting as both an immunomodulatory agent and anti-metabolite [**6, 23, 35, 37, 54**]. Methotrexate's multiple mechanisms of action may explain its clinical efficacy in psoriasis therapy. However, when compared to the much studied mechanisms of action of the new biological agents, the mechanisms of action of methotrexate remains elusive. Previous studies of the mechanism of methotrexate action in psoriasis have been difficult to undertake due to the limitations in assay systems that study cellular responses and inter-individual variability in the pharmacokinetics and response to methotrexate [**55, 56**].

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