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Methotrexate inhibits the viability of human melanoma cell lines and enhances Fas/Fas-ligand expression, apoptosis and response to interferon-alpha: Rationale for its use in combination therapy

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Abstract

Melanoma, a highly aggressive form of cancer, is notoriously resistant to available therapies. Methotrexate (MTX), an antifolate, competitively inhibits DNA synthesis and is effective for several types of cancer. In cutaneous T-cell lymphoma (CTCL), MTX increases Fas death receptor by decreasing Fas promoter methylation by blocking the synthesis of SAM, the principal methyl donor for DNMTs, resulting in enhanced Fas-mediated apoptosis. The objective of this study was to explore the effects of MTX in human melanoma. MTX variably inhibited the survival of melanoma cells and induced apoptosis as evident by annexin V positivity and senescence associated β -galactosidase activity induction. Furthermore, MTX caused increased transcript and protein levels of extrinsic apoptotic pathway factors Fas and Fas-ligand, albeit at different levels in different cell lines. Our pyrosequencing studies showed that this increased expression of Fas was associated with Fas promoter demethylation. Overall, the ability of MTX to up-regulate Fas/FasL and enhance melanoma apoptosis through extrinsic as well as intrinsic pathways might make it a useful component of novel combination therapies designed to affect multiple melanoma targets simultaneously. In support of this concept, combination therapy with MTX and interferon-alpha (IFN α) induced significantly greater apoptosis in the aggressive A375 cell line than either agent alone.

Keywords

Melanoma; Methotrexate; Fas/FasL; Apoptosis; DNA methylation

Introduction

Advanced-stage melanomas are notorious for their high mortality and resistance to available forms of therapy. Even the newest kinase inhibitors and immune modulators have only transient benefit in most cases [1–3]. Therefore there is an unmet need for novel agents or novel uses of older agents to help combat this aggressive malignancy. Methotrexate (MTX) is an antimetabolic, antineoplastic compound that competitively inhibits dihydrofolate reductase which in turn inhibits DNA synthesis by impeding the conversion of dihydrofolate to the active tetrahydrofolate required for thymidine and purine synthesis [4]. Therefore, MTX arrests cells in the S-phase of the cell cycle [4]. MTX is an effective drug for several types of cancer including breast cancer, lymphomas and leukemias, gastric cancer, colorectal cancer, head and neck cancers, lung cancer [5–10].

A key regulator of apoptosis is the Fas death receptor/Fas ligand (Fas/FasL) system [11,12]. It is one of the major extrinsic apoptosis pathways and a key regulator of T-cell apoptosis [13]. Fas-apoptotic pathway is active in early- and intermediate-phase melanomas and is often altered in highly metastatic melanomas [14–16]. These include mutations within the Fas gene, downregulation of Fas transcription due to promoter silencing, promoter methylation and inhibition of Fas trafficking to the cell surface [17,18]. Human malignant melanomas that lack expression of Fas have a poor prognosis [19]. Comparative studies of Fas and FasL expression revealed downregulation of Fas and upregulation of FasL expression during melanoma progression [20].

Recently, we have shown that MTX also acts as a DNA methylation inhibitor by blocking synthesis of S-adenosylmethionine (SAM), the principal methyl donor for DNA methyltransferases [21]. In cutaneous T-cell lymphoma (CTCL), MTX increases Fas and FasL at least in part by decreasing Fas/FasL promoter methylation, resulting in enhanced Fas pathway apoptosis. In the current article, we explore the effects of MTX on Fas/FasL death signaling and promoter demethylation in melanoma and demonstrate that MTX can induce significant apoptosis in various human melanoma cell lines. We also show that combination therapy with MTX and IFN α induces apoptosis at significantly higher levels than either agent alone. Our findings provide preclinical evidence that MTX can be repurposed as a novel demethylating agent (as well as known S-phase inhibitor) for combination therapy regimens directed against melanoma.

Materials and methods

Cell lines and treatments

All melanoma cells were obtained from The American Type Culture Collection (ATCC; Manassas, VA) and were maintained at standard tissue culture conditions as recommended by the vendor. MTX was obtained from MP Biomedicals (Santa Ana, CA) and dissolved in DMSO and diluted in culture medium for treatments. IFN α 2b was obtained from Schering-Plough (Merck) and diluted in sterile water.

Cell growth and viability assay

Cellular growth and viability was performed using the CytoTox-Glo assay system (Promega Inc., Madison, WI) as per manufacturer's protocol. Briefly, 10 K cells in a total of 100 μ l volume were plated in a 96-well plate and treated with MTX or DMSO control ($n = 3$ or more) for 72 h. Thereafter cells were incubated for 15 min with AAF-Glo substrate (alanyl-alanylphenylalanyl-aminoluciferin), that measures a distinct intracellular protease activity associated with cytotoxicity (dead-cell protease) via a luminescent signal. Cell viability was calculated by subtracting the luminescent signal resulting from experimental cell death from total luminescent values following cell lysis. Data were represented as percent reduction in viable cells and percent increase in dead cells normalized to no treatment control.

Flow cytometry

Control and treated melanoma cells were collected, washed with PBS twice and cell pellets were resuspended in 50 μ l PBS containing Fas (BD Pharmingen, DX2, FITC)/FasL (Enzo, Mike-2) antibody (1:50) for 60 min at room temperature, followed by washing and tagging with AlexaFluor 488 conjugated secondary antibody and subjected to flow cytometry.

For Annexin V/PI stained apoptosis analysis, control and treated cells were collected, washed with PBS and then resuspended in 100 μ l binding buffer (0.1 M Hepes, pH 7.4; 1.4 M NaCl; 25 mM CaCl_2) and stained with FITC conjugated annexin V antibody and propidium iodide (BD Biosciences, CA) as per vendor's guidelines. After staining, flow cytometric analysis was performed with a FACScan benchtop cytometer (BD Biosciences, San Jose, CA) at the UWCCC Flow Cytometry Facility using FlowJo software (Treestar, Ashland, OR).

Senescence-associated β -galactosidase (SA- β -Gal)

Melanoma cells seeded in 6-well plates were treated with 5 μ M MTX for 48 h. After that, cells were rescued for 72 h in regular medium. Thereafter plates were rinsed with PBS, fixed with 2% formaldehyde containing 0.2% glutaraldehyde and incubated with freshly prepared senescence-associated β -Galactosidase (SA- β -Gal) staining solution (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl_2) and incubated at 37 $^{\circ}\text{C}$ overnight. Nuclei were counterstained with nuclear fast red stain. After washing fast red stain with PBS SA- β -Gal staining positive cells images, which had blue color sedimentation in their cytoplasm, were taken under the Evos microscope at 40 $\times$ magnification.

Real-time quantitative PCR (Q-RT/PCR)

For Q-RT/PCR, RNA was isolated with Trizol reagent (Invitrogen, CA), treated with DNase (Promega, WI) and first strand cDNA created with M-MLV reverse transcriptase (Promega, WI) according to vendor's protocol. Q-RT/PCR was performed, in triplicate, with SYBR Premix *Ex Taq* Perfect Real Time (Takara, WI) with first strand cDNA for each Fas, FasL, and GAPDH primers as below. Relative Fas/FasL mRNA expression was calculated using the C_T method using GAPDH as an endogenous control.

The primer sequences and procedures for real-time RT-PCR detection of FAS, FasL and GAPDH mRNA are as shown below:

Immunoblot analysis

Following treatments, cells were washed with ice-cold PBS and lysed with RIPA buffer. For Western blot analysis, 30 µg of protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membrane. Blots were exposed to anti-cleaved caspase-8 and 9 primary antibodies and HRP conjugated appropriate secondary antibodies (Cell Signaling, Inc., Danvers, MA), followed by enhanced chemiluminescent detection (Thermo Fisher Scientific, Inc., Rockford, IL). Loading control antibody was GAPDH. Represented blots are from three independent experiments with similar results.

Pyrosequencing

DNA extraction and bisulfite conversion for cytosine to uracil was done using EpiTect Plus DNA bisulfite kit (Qiagen, Valencia, CA). DNA fragments flanking the related CpG sites were amplified using HotStarTap polymerase (Qiagen, Valencia, CA) and pyrosequencing primers. Pyrosequencing using the primers specific to bisulfite-treated DNA was performed as described previously [21]. Pyro Q-CpG™ software was used to analyze each sequencing reaction and to calculate the corresponding percentage of methylation at each 11–18 CpG sites, which was later plotted as a line graph.

	Sequence (5'→3')	Length	Tm	Location
Fw Fas Primer	TCTGGTTCTTACGTCTGTTGC	21	60.0	27-47
Rev Fas Primer	CTGTGCAGTCCCTAGCTTTCC	21	62.4	223-203
Fw FasL Primer	TGCCTTGGTAGGATTGGGC	19	61.7	276-294
Rev FasL Primer	GCTGGTAGACTCTCGGAGTTC	21	61.3	357-337
Fw GAPDH Primer	TGTGGGCATCAATGGATTGG	21	60.9	231-251
Rev GAPDH Primer	ACACCATGTATTCCGGGTCAAT	22	61.4	346-325

Results

MTX reduces viability and induces apoptosis and senescence in human melanoma cell lines

We evaluated the effect of 1 µM MTX in several human melanoma cell lines at 72 h post-treatment. Melanoma cells showed growth inhibition and reduced cellular viability using a luminescence based Cyto-Tox-Glo assay system (Fig. 1A and B). Reduced cellular density and morphological changes at the dose of 5 µM MTX for 72 h are shown in Fig. 1C. In a dose dependent manner, MTX induces apoptosis at 48 h as assessed by annexin V binding assay (Fig. 1D). The magnitude of these responses varied among the different cell lines but was pronounced in several cell lines. MTX treatment also induced senescence in melanoma cells as evident by the induction of β-galactosidase activity with post 48 h MTX treatment at 5 µM, with an additional 72 h rescuing of cells in regular medium (Fig. 1E).

Apoptosis of melanoma cells induced by MTX involves both extrinsic and intrinsic pathways

As shown in Figs. 2 and 3, the Fas death receptor and its ligand (FasL) are generally expressed at relatively low baseline levels in melanoma cells. MTX enhanced the expression of both Fas and FasL. Fas was upregulated most in A375 and Hs-294T, to a lesser extent in G361, modestly in WM35 and marginally in SK-Mel-28 as shown in overlaid flow cytometry curves in Fig. 2. Similar to Fas upregulation, FasL was most upregulated in A375 and Hs-294T, and to a lesser extent in G361 and WM35, with least change in SK-Mel-28 with MTX treatment. However; Q-RT/PCR data showed fairly uniform upregulation of Fas transcript among the melanoma cells. FasL was most upregulated (~5 fold) in Hs-294T cells, and least modulated in SK-Mel-2 and SK-Mel-28 (1.5 fold). WM35 showed about 3 fold increase in FasL, and A375 and G361 had a 2 fold increase (Fig. 4).

Extrinsic (death receptor) pathway activation involves cleavage of caspase 8. As shown in Fig. 5, cleaved caspase 8 expression was induced by MTX in all six melanoma cell lines but was most pronounced in A375, Hs-294T and SK-Mel-2. Intrinsic pathway of apoptosis involves cleavage of caspase 9. Cleaved caspase 9 expression was induced by MTX in 4/6 melanoma cell lines. The induction was greatest in Hs-294T but generally less pronounced than that observed for cleaved caspase 8.

Of interest, though MTX treated SK-Mel-28 cells showed an increase in dead and apoptotic and senescent cell population, they did not exhibit profound change in Fas/FasL upregulation, cleaved caspase 8 and 9 induction and Fas promoter demethylation, suggesting a possible different mechanism for MTX action for these cells.

MTX reduces FAS promoter methylation in melanoma cell lines

The pyrosequencing analysis of the -1781 KB Fas promoter region immediately upstream of the first exon revealed that the level of methylation present at several CpG sites was decreased by MTX in several melanoma cell lines as shown in Fig. 6. The pyrogram profiles of melanoma cells showed 60% baseline promoter methylation at CpG site 16 in WM35 and Hs-294T that was reduced to 20%. Similarly, 80% promoter methylation at CpG site 16 in A375 was reduced to 50%. Hs-294T showed 90% promoter methylation at CpG site 14 that was reduced to 20%. In contrast, SK-Mel-28 cells exhibited a highly methylated basal Fas profile with minimal reduction in promoter methylation after MTX treatment for CpG site 11 and 12 only.

When combined with the increase in Fas and FasL transcript and protein noted in Figs. 2–4, these findings suggest that the increased Fas/FasL expression induced by MTX in melanoma cells is due at least in part to derepression of their promoters by demethylation.

Combination therapy with MTX and interferon α 2b (IFN α 2b) enhances apoptosis of melanoma cells

Our findings thus far suggested that, although not optimal as a single agent for the treatment of melanoma, MTX might be useful as part of a combination therapy regimen. IFN α 2b has shown clinical benefit in the treatment of melanoma. Previously, we showed that IFN α 2b

can enhance apoptosis induced by MTX in CTCL [21]. IFN α 2b increases Fas expression by a methylation-independent STAT1-mediated mechanism [22]. Therefore, we tested the combination of MTX and IFN α 2b in the rapidly growing A375 melanoma cell line. As shown in Fig. 7, combination therapy induced a significantly greater level of apoptosis (45%) than either agent alone (27%). This provides preclinical proof-of-principle that MTX might be able to enhance the clinical efficacy of combination therapeutic regimens for melanoma.

Discussion

Advanced stage melanomas are notoriously refractory to chemo and radiation therapy [23]. Melanomas derive some of their therapeutic resistance from the diversity of mutations that drive their proliferation. For example, only about half of all melanomas harbor BRAF¹ mutations. Although showing initial significant tumor responses, even newer agents such as BRAF inhibitors (e.g. vemurafenib, dabrafenib) and anti-CTLA4 antibodies (e.g. Ipilimumab) produce only transient benefits with tumor progression occurring in approximately six months [2,3]. Progression is secondary to reactivation of the MAPK pathway or other pathways such as PI3K-AKT-mTOR and VEGF [2]. Furthermore, these newer agents have major side effects that sometimes prove fatal. Clinical trials are underway with alternative agents that inhibit the MAPK pathway downstream of BRAF (such as the MEK inhibitor, trametinib) and with combinations of novel agents designed to inhibit MAPK signaling and enhance anti-tumor immune responses simultaneously [24,2].

However, it is evident that additional novel approaches are needed to combat melanoma. Rather than focusing on the inhibition of dysregulated tumor cell proliferation or enhancement of the host immune response, we were interested in augmenting the death of tumor cells directly through manipulation of their own apoptotic signaling. To achieve this, we repurposed an established folate antagonist, MTX, for novel use as a de-repressor of silenced tumor suppressor genes. Our prior work showed that MTX acts as a DNA methylation inhibitor by blocking the synthesis of S-adenosylmethionine (SAM), the principal methyl donor for DNA methyltransferases [21]. In cutaneous T-cell lymphoma (CTCL) cells, this results in demethylation of the Fas and FasL promoters and upregulation of Fas/FasL protein expression. As a result, apoptosis is enhanced significantly. The importance of Fas expression was established using the CTCL cell line SeAx, which lacks Fas expression due to gene deletion [22,25]. These cells are resistant to apoptosis induced by exogenous FasL unless they are first transfected with a Fas expression vector. Similarly, they are also resistant to apoptosis induced by upregulation of endogenous FasL whereas other CTCL cell lines that express adequate levels of Fas are quite sensitive [39].

Our current data show that although baseline Fas and FasL are relatively low in several melanoma cell lines, their expression can further be upregulated significantly at both the mRNA and protein levels following treatment with MTX. The Fas promoter demethylating effect observed in melanoma cell lines is similar to our observations in CTCL cells [21].

¹Abbreviations used: BRAF, v-Raf murine sarcoma viral oncogene homologue B1; CTCL, Cutaneous T-cell lymphoma; IFN, Interferon; MTX, Methotrexate; MITF, microphthalmia-associated transcription factor; Ras, rat sarcoma viral oncogene homolog; SAM, S-adenosylmethionine; TMECG, 3-O-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin.

Although we have yet to analyze the effects of MTX on the FasL promoter in melanomas, our unpublished studies of FasL in CTCL indicate that MTX also demethylates the FasL promoter. The ability of MTX to act as a DNA methylation inhibitor is a novel mechanism of action that allows the repurposing of an established folate antagonist as a demethylating agent with a clinical safety profile much better than traditional demethylating agents such as 5-azacytidine and decitabine. In addition, because of its distinct mechanism of action, MTX might also inhibit the methylation of key proteins such as Ras which requires methylation of its carboxyl terminus for proper intracellular transport and membrane localization [26].

The observed MTX-induced effects in melanoma cell lines were associated with reduced viability, enhanced apoptosis and cleavage of both caspase-8 and -9. Melanomas express several extrinsic death receptors like Fas, TNF and TRAIL (DR4 and DR5). All these receptors involve the generation of cleaved caspase 8 because they share a common pathway downstream of the cell membrane death receptor. Although our current work focused on the Fas pathway, it would not be surprising if MTX was able to up regulate other death receptors as well. MTX also induces the intrinsic pathway by inhibition of purine synthesis resulting in S-phase arrest [4]. These responses to MTX were significant but heterogeneous in magnitude. The biological diversity of melanomas might be due to a wide variety of factors such as somatically acquired tumor clone defects in extrinsic or intrinsic apoptotic pathway cascades. Changes at the genomic, transcriptional and post-translational levels of G-proteins, protein kinases (Ras, B-Raf) and their transcription factor effectors (c-Jun, ATF2, Stat3 and NF- κ B) can alter TNF, Fas and TRAIL (DR4 and DR5) receptors, resulting in increased resistance to apoptosis [17]. Additional factors include variable levels of pro- and anti-apoptotic regulators such as bcl-2 [18]; variations in the cellular transport, export and sequestration of MTX [27,28]; differential gene copy number of dihydrofolate reductase [29]; amplification of the multidrug resistance gene [30]; or expression of soluble as opposed to membrane-bound Fas [31].

The impact of MTX on Fas pathway apoptosis suggests that it might have value in novel combination therapy regimens for melanoma. In CTCL, the combination of MTX and IFN α 2b has proven highly effective clinically with a 74% complete response rate achieved at one year in a large series of patients with advanced stage disease [32]. IFN α 2b has protean effects on tumor cells; however, its combination with MTX might be particularly useful because we showed that IFN α 2b induces Fas upregulation by a STAT-1 mediated mechanism that is independent of DNA methylation [22]. Consistent with this view our current findings show that the combination of MTX and IFN α 2b induces significantly greater apoptotic response in aggressive amelanotic A375 melanoma cell line than either agent alone. This provides preclinical proof-of-principle that MTX might be significantly effective as part of a combination therapy regimen for advanced melanomas or even as adjuvant therapy to reduce the risk of recurrence in thick primary melanomas [33]. In fact, independent studies have already shown that MTX can enhance anti-tumor efficacy when used in combination with other agents [33]. Without providing a clear mechanism of action, other groups have shown that MTX potentiates the ability of photodynamic therapy to kill tumor cells [34] and that MTX upregulates MITF thereby increasing tyrosinase which converts a nontoxic pro-drug (TMEGC) into a potent cytotoxic agent selectively within melanoma cells [35–38]. We showed recently that the enhancement of photodynamic

therapy by MTX is likely due to its demethylating effects (and we suspect the same may be true for the effect of MTX on MITF expression [40]).

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Fig. 1.

MTX reduces viability and induces apoptosis and senescence in melanoma cell lines. Panels A and B show the effect on growth and viability of MTX at 1 μ M dose for 72 h. This was assessed using the luminescence based Cyto-Tox-Glo assay system. The Y-axes show relative luminescence units (RLU) of live and dead cells and the asterisk (*) represents statistical significance ($p < 0.05$) when compared with no treatment (NT) vehicle controls. Data represent mean $\pm$ standard deviation of three experiments with similar results. Panel C shows the morphological changes in controls versus 5 μ M MTX treatment. Cells were photographed using Nikon Eclipse Ti microscope and a high performance CCD camera at

20× magnification. Panel D shows that at 48 h post-treatment, MTX induces apoptosis in a dose dependent manner as assessed by annexin V-FITC/PI staining. Data represent total annexin V-positive cells (mean ± standard deviation of three experiments with similar results), with statistical significance ($p < 0.05$) when compared with NT (*), 1 μM MTX (!) and 5 μM MTX (^). Panel E shows that MTX induced senescence in melanoma lines at 48 h post-treatments as assessed by β-galactosidase staining (blue) and counter stained with nuclear fast red at 40× using Evos (AMG) microscope.

Fig. 2.

MTX upregulates Fas death receptor in melanoma cell lines. Panel A shows that MTX treatment upregulates Fas in melanoma cell lines as demonstrated by overlaid flow cytometry histograms. Filled grey curve is isotype control staining; dotted line is DMSO treated control; and heavy black line is MTX treated cells. Panel B shows the histograms of the mean fluorescence intensity (MFI) of Fas on Y-axis (as in Panel A). Data represent the mean $\pm$ standard deviation of three experiments with similar results when compared with vehicle treated control (* $p < 0.05$).

Fig. 3. MTX upregulates FasL in melanoma cell lines. Panel A shows upregulation of FasL in melanoma cell with MTX treatment by overlaid flow cytometry histograms as in Fig. 2A. Panel B shows the histogram of the MFI of FasL as in Fig. 2B. Data represented as mean $\pm$ standard deviation of three experiments with similar results compared with vehicle treated control (* $p < 0.05$).

Fig. 4.

MTX upregulates Fas/FasL transcripts in melanoma cell lines. Histogram of Q-RT/PCR data shows upregulation of Fas and FasL transcript among all the melanoma cell lines studied. Data represent mean $\pm$ standard deviation of three experiments with similar results relative to the baseline transcript of vehicle treated controls that were set as 1(* $p < 0.05$).

Fig. 5. MTX induces apoptosis through both the extrinsic and intrinsic pathways in melanoma cell lines. Immunoblots show that both cleaved caspase-8 and -9 are induced by MTX treatment. GAPDH is the loading control.

Fig. 6.

MTX demethylates the FAS promoter in melanoma cell lines. Line graph shows pyrographs of Fas promoter CpG sites in melanoma cell lines before and after MTX treatment. Y axis represents % methylation and X axis represents selected CpG sites (11–18).

Fig. 7.

MTX/IFN α 2b combination treatment enhances apoptosis of A375 melanoma cells significantly more than either agent alone. Methotrexate functions as a demethylating agent and together with interferon- α 2b, induces superior apoptotic response in A375 melanoma cells. Histogram shows % apoptotic cells (total annexin V-positive) on the Y axis. Overlaid flow cytometry plots show the distribution of annexin V positivity. Filled grey filled histogram represents NT control; dashed line is MTX treated cells; dotted line is IFN treated cells; and heavy black line is MTX + IFN treated cells. Data represent mean $\pm$ standard deviation of three experiments with similar results (* $p < 0.05$) when compared with NT (*), 5 μ M MTX (^) and 1000 U/ml IFN α 2b (!).