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Photobiological Implications of Folate Depletion and Repletion in Cultured Human Keratinocytes

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Abstract

Folate nutrition is critical in humans and a high dietary folate intake is associated with a diminished risk of many types of cancer. Both synthetic folic acid and the most biologically abundant extracellular reduced folate, 5-methyltetrahydrofolate, are degraded under conditions of ultraviolet radiation (UVR) exposure. Skin is a proliferative tissue with increased folate nutrient demands due to a dependence upon continuous epidermal cell proliferation and differentiation to maintain homeostasis. Regions of skin are also chronically exposed to UVR, which penetrates to the actively dividing basal layer of the epidermis, increasing the folate nutrient demands in order to replace folate species degraded by UVR exposure and to supply the folate cofactors required for repair of photo-damaged DNA. Localized folate deficiencies of skin are a likely consequence of UVR exposure. We report here a cultured keratinocyte model of folate deficiency that has been applied to examine possible effects of folate nutritional deficiencies in skin. Utilizing this model, we were able to quantify the concentrations of key intracellular folate species during folate depletion and repletion. We investigated the hypotheses that the genomic instability observed under conditions of folate deficiency in other cell types extends to skin, adversely effecting cellular capacity to handle UVR insult and that optimizing folate levels in skin is beneficial in preventing or repairing the pro-carcinogenic effects of UVR exposure. Folate restriction leads to rapid depletion of intracellular reduced folates resulting in S-phase growth arrest, increased levels of inherent DNA damage, and increased uracil misincorporation into DNA, without a significant losses in overall cellular viability. Folate depleted keratinocytes were sensitized toward UVR induced apoptosis and displayed a diminished capacity to remove DNA breaks resulting from both photo and oxidative DNA damage. Thus, folate deficiency creates a permissive environment for genomic instability, an early event in the process of skin carcinogenesis. The effects of folate restriction, even in severely depleted, growth-arrested keratinocytes, were reversible by repletion with folic acid. Overall, these results indicate that skin health can be positively influenced by optimal folate nutriture.

Keywords

Folate; Keratinocytes; Skin Biology; Solar Simulated Light; DNA Damage; Cancer

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Conflicts of Interest

Myron K. Jacobson serves as a consultant to Niadyne, Inc.

1. Introduction

A large and evolving body of evidence shows that folate nutrition plays prominently in human health and disease [1,2]. The folates are a family of structurally similar, water-soluble, B vitamins. Folic acid refers specifically to the synthetic, oxidized, chemically stable form of the vitamin utilized to fortify foods and as a dietary supplement while the biological reduced folate species are designated by their proper chemical names. In human studies, high dietary folate intake has been associated with a diminished risk of many types of cancer including colon, breast, esophagus, stomach, and blood [1,3–5]. The inverse association between folate status and cancer risk is proposed to be due to the roles that intracellular, reduced folates serve in one-carbon metabolism. Folates are necessary in the synthesis of purine and pyrimidine nucleotides required for DNA replication and repair, and in the conversion of homocysteine to methionine in the synthesis of S-adenosylmethionine, the primary donor for intracellular methylation reactions including those involved in the epigenetic regulation of gene expression [1,6–8].

Low dietary folate intake, defects in folate absorption and other environmental factors including exposure to ultraviolet radiation (UVR), may result in folate deficiency [9,10]. Folic acid has an absorption peak at 350 nm within the range of UV-A radiation (315–400 nm) prevalent at the Earth's surface, while the most biologically abundant reduced folate, 5-methyltetrahydrofolate (5-methylTHF), has an absorption maximum at 290 nm in the UV-B range (280–315 nm). Folic acid in solutions exposed to biologically relevant levels of UV-A is converted to the breakdown products *p*-aminobenzoyl-L-glutamic acid and pterin-6-carboxylic acid, while exposure of 5-methylTHF to UV-B results in a mixture of oxidation to 5-methyldihydrofolate and C9-N10 bond cleavage, which is increased in the presence of photosensitizers [11–14].

Folate deficiency results in reduced levels of thymine containing nucleotides that leads to the misincorporation of uracil into DNA with subsequent formation of single and double stranded DNA breaks in Chinese hamster ovary cells, murine intestinal epithelial cells, and human lymphocytes [15–18]. Folate depletion also leads to DNA breaks, chromosomal instability, altered proliferation, and sensitivity toward DNA damage in primary human lymphocytes and colonic epithelial cells [19–21]. Dietary folate deficiencies promote DNA strand breaks in the livers of rats and micropigs [22,23]. Folate deficiency has been shown to inhibit DNA repair in cells subjected to pro-mutagenic oxidative or alkylative damage [15,24]. Folate depletion has been shown to cause global genomic hypomethylation both *in vivo* and *in vitro* while both hypo- and hyper- methylation associated changes in expression are observed at the level of specific genes [18,19,24,25]. In general, folate deficiency has been observed to promote genetic instability and alter epigenetic gene regulation, both of which are established risk factors for the development of cancer.

Humans are unable to synthesize folate *de novo* and are thus dependent upon dietary sources to meet nutrient requirements. Folate nutritional status is determined by dietary intake, bioavailability, excretion rate, polyglutamylation mediated retention, and catabolism [26,27]. Cellular reduced folates differ in their stability, with the 5-substituted forms of reduced folates, 5-formyltetrahydrofolate (5-formylTHF), 5-methylTHF, being the most stable and the un- and 10-substituted reduced folates, tetrahydrofolate (THF), and 10-formyltetrahydrofolate, being more susceptible to degradation *in vitro* [28,29]. Localized, tissue specific folate deficiency has been observed in cancer patients in the absence of whole body folate deficiency [30,31]. This phenomenon, attributed to localized variation in the rates of folate catabolism, indicates that folate nutritional status is dependent upon the cellular environment.

Skin, like other tissues proposed to have high folate requirements, is a proliferative tissue that depends upon continuous epidermal cell proliferation and differentiation to maintain homeostasis. However, in contrast to other tissues, regions of skin are chronically exposed to UVR, a complete carcinogen known to penetrate to the actively dividing basal layer of the epidermis. Most current studies on folate and UVR photolysis have focused on the role of UVR exposure in the degradation of folates in the blood with the consequence of overall folate deficiencies; however, UVR does not readily penetrate through the skin making localized folate deficiencies of the skin tissue itself a more likely consequence of UVR exposure. It is also likely that folate stores in skin are subjected to increased degradation by reactive oxygen species (ROS) such as H₂O₂ which are generated as deeper penetrating, longer wavelengths of light interact with the abundant photosensitizers that exist in skin tissue [32,33]. Overall micronutrient levels are also lower in the skin due to the fact that the epidermal layer is poorly vascularized and situated last in line for the delivery of dietary nutrients. The nutrient deficiencies of skin are exacerbated in aging skin and the skin aging process is accelerated by exposure to UVR [34,35].

Elucidating the mechanisms that regulate folate content of individual tissues is critical to understanding the relationships between folate nutrient status, folate metabolism, and complex disease processes such as carcinogenesis. In order to address the dearth of knowledge about folate metabolism in skin, we report here a cultured keratinocyte model of folate deficiency that has been applied to examine possible effects of folate nutritional deficiencies in skin. The HaCaT cell line, despite immortalization, retains many of the normal characteristics of adult human keratinocytes with full epidermal differentiation capacity. While HaCaT cells have a transformed phenotype *in vitro*, they remain nontumorigenic upon transplantation on to athymic nude mice [36]. However, HaCaT cells exhibit UV-B type-specific mutations of the p53 tumor suppressor gene and thus may be considered to represent an early stage of precancerous or pre-initiated skin cell [37]. Utilizing this model, we are able for the first time to quantify the intracellular concentrations of key tetrahydrofolate species during folate restriction as well as to gauge the feasibility of modulating intracellular folate concentrations by tissue specific folic acid supplementation. Characterization of this model has allowed us to investigate the hypotheses that the genomic instability observed under conditions of folate deficiency in other cell types will extend to skin, adversely effecting cellular capacity to handle UVR insult and that optimizing folate levels in skin is beneficial in preventing or repairing the pro-carcinogenic effects of UVR exposure.

2. Materials and Methods

2.1 Cell culture

The established cell line of spontaneously immortalized human epidermal keratinocytes (HaCaT cells), a gift from Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg Germany), was routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and kept in a humidified atmosphere containing 5% CO₂ at 37° C. For folate modulation, cells were cultured in special DMEM (Gibco BRL) in which folic acid was absent, supplemented with 10% dialyzed FBS (Gibco BRL). Folate concentrations in the growth medium were controlled by addition of folic acid to the desired concentrations between 9100 nM (regular DMEM) and no folic acid for complete folate restriction. Cell numbers were determined after washing and trypsin detachment using a Z1 Coulter counter (Beckman Coulter). Only cells that were adherent to the culture flask were counted.

2.2 Cell cycle analysis

Cell cycle analysis was performed as described by Benavente and Jacobson [38]. Briefly, cells were harvested, washed and resuspended in phosphate buffered saline (PBS) at a final

concentration of approx 1×10^6 cells/mL. Cells were permeabilized and fixed using 3 volumes of cold absolute ethanol and incubated for 1 hr at 4°C. Cells were washed twice with PBS and stained with propidium iodide at a final concentration of 50 µg/mL. RNase A was added to a final concentration of 500 ng/mL followed by incubation for 1 hr at 4°C. Samples were stored at 4°C until flow cytometry analysis was performed on a FACScan analyzer (BD Biosciences).

2.3 Folate extraction and quantification of intracellular folate concentrations

Folic acid, 5-methylTHF, 5-formylTHF, and THF were extracted from cultured cells by homogenization in buffered, anti-oxidant conditions (0.1 M potassium phosphate buffer, pH 7.0, 50 mM Ascorbic Acid, 10 mM 2-mercaptoethanol, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% v/v Triton $\times$ 100). Homogenization was performed on a FastPrep®-24 homogenizer (MP Biomedicals) in 2 mL sample tubes containing 1.4 mm ceramic spheres (Lysing Matrix D, MP Biomedicals) at speeds of 4.0–6.0 m/s for cycles of 30–60 sec. After homogenization, methotrexate (Mtx) was added as an internal standard (40 pmol per sample). Samples were subjected to centrifugation for 10 min (15,000g at 4°C) and the supernatant transferred to a clean sample tube while the remaining pellet was frozen for subsequent protein quantification (BCA Protein Assay Kit, Pierce). The intracellular folates were converted to homogenous monoglutamate populations by the enzymatic activity of folate conjugase contained in 200 µL of rat serum (Innovative Research). The folate conjugase step was validated using pteroyltri- γ -L-glutamic acid (Schircks Laboratories) as a substrate in extraction buffer and monitored for each quantification assay as described by Patring *et al.* [39]. Serum proteins were denatured by heating to 99°C for 10 min to release all bound folates and removed from the sample by precipitation on ice for 10 min followed by centrifugation for 10 min (15,000g at 4°C). Folates were acidified by addition of 2 mL 0.1 M HCl and isolated by reversed-phase affinity chromatography on a pre-conditioned (2 mL methanol followed by 2 mL water) 1 mL C-18 SepPak cartridge (Alltech). Folates were eluted with 5 volumes of elution solution (70:30 v/v Acetonitrile:Aqueous buffer) and concentrated by lyophilization. Lyophilized samples were reconstituted in a small volume of 0.01 M formic acid and were amenable to short-term storage at –80°C or immediate HPLC-MS-MS analysis.

HPLC-MS-MS retention times and transition masses of the four folates analyzed, folic acid, THF, 5-formylTHF, and 5-methylTHF occurred at 8.2 min (295 m/z), 4.2 min (299 m/z), 7.0 min (327 m/z), and 4.3 min (313 m/z), respectively. The retention time and transition mass for the internal standard Mtx was 12.4 min (308 m/z). Chromatographic separation was achieved using a Waters X-Terra MS 3.9 $\times$ 100 mm C-18 column with a particle size of 3.5 µm. A mobile phase of 90% 10 mM formic acid in water and 10% acetonitrile (pre-mixed) was handled by a Finnigan Surveyor MS Pump Plus (Thermo Electron) at a flow rate of 0.3 mL/min. Sample handling was done by a Finnigan Surveyor Autosampler Plus (Thermo Electron). The injection volume was 50 µL and the sample tray was maintained at 4°C. Analytes were ionized by electrospray ionization (ESI) operating in positive polarity. Final sample quantification was achieved by external standard methods with correction for both internal standard recovery and addition of endogenous folate from serum. Methods are adapted from [39–42].

2.4 Cell viability analysis

Cell viability was determined by Annexin-V-fluorescein isothiocyanate /propidium iodide dual staining of cells followed by flow cytometric analysis. Cell staining was performed using an apoptosis detection kit according to manufacturer's specifications (APO-AF; Sigma-Aldrich). Flow cytometry analysis was performed on a FACScan analyzer (BD Biosciences) with results shown in a standard 4 quadrant display in which the lower left quadrant (AnnexinV –, PI –) represents viable cells, the lower right (AnnexinV +, PI –) represents early apoptosis, and the upper right quadrant (AnnexinV +, PI +) represents either late apoptotic or necrotic, non-viable cells.

2.5 Comet assay and uracil misincorporation analysis

Cells were removed from dishes by trypsinization and analyzed by alkaline single cell gel electrophoreses (comet assay) based on the method of Singh *et al.* [43]. Briefly, 100 μ L of cells (100,000 cells/mL) suspended in PBS were mixed with 100 μ L of 0.5% low melting point agarose (Sigma) and layered on CometSlides (Trevigen). The mixture was allowed to solidify at 4°C for 15 min. Cells were exposed for 1 hr at 4°C to freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% Triton $\times$ 100, and 10 mM Tris, adjusted to pH 10 with NaOH). Following cell lysis, the slides were incubated with freshly prepared alkali solution at room temperature for 40 min for DNA denaturation and unwinding. Slides were then placed in a horizontal electrophoresis box in chilled alkali solution (300 mM NaOH, 1 mM EDTA, pH >13) and electrophoresis was conducted at 25 V for 30 min. Following electrophoresis, the slides were neutralized with three washes of 0.4 M Tris-HCl pH 7.4 before fixation with 100% ethanol. Slides were stored in the dark at 4°C until analysis.

Uracil misincorporation was assessed by slight modifications to the comet assay as described by Duthie *et al.* [15]. Briefly, after cell lysis, the slides were washed three times for 5 min each in uracil DNA glycosylase (UDG) buffer (60 mM Tris-HCl, 1 mM EDTA, 0.1 mg/mL BSA, pH 8.0) (Fermentas). The agarose gel was then covered with 50 μ L of buffer with or without UDG (0.1 unit/gel). Slides were incubated in a humid atmosphere at 37°C for 1 hr and placed into alkali unwinding buffer.

Comet slides were hydrated and stained by exposure to 1 mg/mL ethidium bromide overnight. Comets were imaged using a fluorescence based digital imaging system. Images were analyzed and % tail DNA was calculated for a minimum of 100 comets per condition using Comet Assay Software Project imaging software.

2.6 DNA damage treatments

Irradiation with solar simulated light (SSL) was conducted utilizing a kilowatt large area light source solar simulator (model 91293, Oriel Corporation) equipped with a 1000 W Xenon arc lamp power supply, model 68920, and a VIS-IR bandpass blocking filter combined with an atmospheric attenuation filter (output 290–400 nm plus residual 650–800 nm). The output was quantified using a dosimeter from International Light Inc., model IL1700, with an SED240 detector for UV-B (range 265–315 nm, peak 285 nm), or a SED033 detector for UV-A (range 315–400 nm, peak 365 nm), at a distance of 345 cm from the source, which was used for all experiments. At 345 cm from the source, the SSL dose was 675 mJ/cm²/min UV-A and 34.4 mJ/cm²/min UV-B radiation. Growth media was removed and replaced with an equal volume of PBS for both control and SSL irradiated HaCaT cells. For treatment, cells were irradiated for the indicated time while control cells were returned to the dark in a humidified atmosphere containing 5% CO₂ at 37°C. After treatment, the specified growth media was returned to both treated and control cells that were then replaced in a humidified atmosphere containing 5% CO₂ at 37°C for the specified time.

Treatments of HaCaT cells with H₂O₂ were conducted at a concentration of 100 μ M. A 100 $\times$ H₂O₂ stock solution was initially prepared and fresh aliquots were diluted prior to each experiment. The H₂O₂ stock concentrations were verified prior to each use utilizing spectrophotometric analysis. Growth media was spiked with minimal volumes of H₂O₂ in order to achieve 100 μ M treatment conditions while an equal volume of PBS was added to the growth media of control cells. Both treatment and control cells were returned to the dark in a humidified atmosphere containing 5% CO₂ at 37°C for the course of the 30 min experiment. After treatment, the specified growth media was returned to both treated and control cells which were then replaced in a humidified atmosphere containing 5% CO₂ at 37°C for the specified time.

2.7 Statistics

All comparative statistics were done using a two-tailed, unpaired students t test with a confidence interval of 95%.

3. Results

3.1. Folate restriction of HaCaT cells results in proliferation arrest primarily in the S phase of the cell cycle

HaCaT cells cultured in folic acid free media grew comparably to folate sufficient cells for approximately 2 population doublings before the proliferation rate began to slow. Folate restricted cells eventually ceased division after approximately 4 population doublings, between days 8 and 10, while cells grown in sufficient folate continued to divide until reaching confluence (Fig. 1A left panel). During exponential proliferation a high percentage of cells were in the S and G2 phases of the cell cycle under both folate restricted and sufficient conditions. As cells grown in sufficient folate began to reach confluence, the percentage of S/G2 cells dropped as the majority of the cell population halted in G1 (Fig. 1A right panel). As the proliferation rate of the folate restricted cells began to slow, and subsequently stop, a high percentage of cells (about 40%) remained in S or G2 phase (Fig. 1A right panel). The combination of S and G2 phase cells was plotted due to ambiguities in separating the two populations within the analysis software; however, the majority of cells in folate restricted media were present in the S phase as shown in supplemental Fig. 1.

Cells that had been folate restricted for 10 days, exhibiting both proliferation and S phase arrest, were switched to growth medium in which folic acid had been added at varying concentrations up to 9100 nM. Upon restoration of folic acid, cell proliferation resumed in a concentration dependent manner, with cells under continued folate restriction remaining in a non-proliferative yet viable state (Fig. 1B left panel). Upon restoration of folic acid, the percentage of cells in the S and G2 phases of the cell cycle diminished as the concentration of folic acid increased. The percentage of S/G2 cells under continued folate restriction continued to rise while the percentages of the 9100 nM supplemented cells returned to near non-depleted levels (Fig. 1B right panel).

3.2. Folate restriction results in rapid depletion of intracellular folate levels that are reversed upon folate repletion

Intracellular levels of 5-methylTHF, 5-formylTHF, THF, and folic acid were quantified by HPLC-MS-MS analysis (Fig. 2). Measurements after 5 and 10 days of folate restricted culture showed that intracellular levels of 5-methylTHF, 5-formylTHF, and THF dropped to 4.1%, 6.4%, and 5.4% after 5 days and 2.8%, 5.4%, and 1.9% after 10 days, respectively. Intracellular folate concentrations were measured 24, 48, 72, and 120 hours after returning cells that had been cultured under folate deficiency for 10 days to growth media in which folic acid had been added at a concentration of 9100 nM. Levels of both 5-methylTHF and 5-formylTHF recovered to near 80% of non-depleted controls after 48 hours with a full recovery observed after 120 hours of nutrient restoration. The level of THF fully recovered by 24 hours after which it was slightly but significantly elevated until returning to the level of non-depleted controls by 120 hours. Intracellular levels of folic acid were below the 0.7 pmole per mg protein level of quantification for all conditions except after 24 hours of nutrient restoration where levels were slightly elevated to 1.59 ± 0.73 pmole per mg protein as shown in supplemental Fig. 2.

3.3. Folate restriction results in only a minimal loss of cellular viability

Cells cultured in folic acid free media exhibited no significant loss in viability as they continued to proliferate for 5 days and exhibited a small, yet significant, 9.1% loss of viability even after

10 days of folate restriction as assessed by Annexin V/PI staining (Fig. 3A). When folic acid was returned to the growth medium, the viability loss exhibited by 10 day depleted cells was completely reversed as viability increased significantly, returning to the level of non-depleted control cells after 120 hours of nutrient replenishment (Fig. 3B).

3.4. HaCaT cells show progressive DNA instability and uracil misincorporation under folate deficient culture conditions

Inherent DNA instability, as indicated by the amount of DNA in the comet tails of cells increased significantly from 5.3% to 7.5% and 9.4% after 5 and 10 days of folate restricted culture, respectively. The amount of uracil misincorporated in DNA, as indicated by the difference between the amount of DNA in the comet tails of cells treated with UDG and cells treated with UDG free buffer, also increased significantly from 0.9% to 1.0% and 4.9% (Fig. 4). The increased DNA instability and uracil misincorporation caused by folate restriction was reversed when folic acid was added back to the growth media of 10 day depleted cells in a concentration dependent manner. Continued culture in folate free conditions resulted in a further increase in both inherent DNA instability as well as uracil misincorporation (Fig. 5A). Low-level repletion with 9 nM folic acid slowed but did not stop the increases observed in folate free conditions (Fig. 5B). Folic acid repletion at a 45 nM level effectively halted but did not diminish DNA instability as the % tail DNA after 3 and 5 days of repletion was not significantly different than that observed in 10 day folate depleted cells. Folic acid repletion at a 45 nM level was observed to decrease the amount of uracil misincorporated into DNA significantly after 5 days of repletion (Fig. 5C). Folic acid repletion of either 91 or 9100 nM significantly reduced both inherent DNA instability and uracil misincorporation observable after 3 days of nutrient restoration (Fig. 5D and 5E).

3.5. Folate restriction sensitizes cells to SSL exposure

Non-depleted control cells exhibited a 10% reduction in viability 24 hours after exposure to SSL (4 J/cm² UV-A and 206 mJ/cm² UV-B) as assessed by Annexin V/PI staining. Cells cultured in folic acid free media for 5 days exhibited a 12% loss in viability 24 hours post SSL, which was not significantly different from controls. After 10 days of culture in folic acid free media, cells exhibited a 20% loss in viability, which was significantly higher when compared to non-depleted controls (Fig. 6A). The sensitization toward SSL induced apoptosis was reversed when folic acid was returned to the growth media. The sensitization of 10 day depleted cells was completely reversed after 72 hours of nutrient restoration (Fig. 6B).

3.6. HaCaT cells grown in folate deficient conditions show a diminished capacity to remove DNA strand breaks induced by exposure to skin specific insults

After exposure to SSL (4 J/cm² UV-A and 206 mJ/cm² UV-B), non-depleted control cells exhibited a significant increase in DNA strand breaks 4 hours post irradiation. The SSL induced strand breaks were significantly diminished at both 8 and 24 hours post irradiation when DNA breaks were almost completely removed (Fig. 7, solid bars). In addition to an initial increase in inherent DNA instability, cells cultured for 10 days in folic acid free media also exhibited a significant increase in DNA strand breaks 4 hours post SSL exposure but the level of breaks were not observed to diminish at either 8 or 24 hours post SSL exposure, indicating a deficiency in DNA repair in folate depleted cells (Fig. 7, open bars). The deficiencies in DNA strand break removal induced by folate restriction were reversible in a time and concentration dependent manner when folic acid was added back to the growth media of 10 day depleted cells. Continued culture in folate free conditions resulted in a further sensitization toward SSL induced DNA damage and a continued presence of DNA strand breaks (Fig. 8A). Repletion with 9 nM folic acid was observed to significantly reactivate the removal of DNA strand breaks only after 120 hours of folate recovery and only at 24 hours post SSL exposure (Fig. 8B), while repletion

with 45 nM folic acid was observed to significantly reactivate DNA strand break removal after 72 hours of nutrient restoration (Fig. 8C). Higher-level folic acid repletion of either 91 or 9100 nM showed a significant return of DNA strand break removal after 48 hours of nutrient restoration at the 24 hour post SSL measurement and a response to DNA damage similar to that of non-depleted control cells after 72 and 120 hours of repletion (Fig. 8D and 8E).

HaCaT cells grown in folate deficient conditions show a diminished capacity to repair DNA damage induced by exposure to ROS. After a 30 min exposure to 100 μ M H₂O₂, non-depleted control cells exhibited a significant increase in DNA strand breaks 0.5 hours post treatment. The H₂O₂ induced strand breaks were significantly removed after 24 hours of recovery. In contrast, cells cultured for 10 days in folic acid free media exhibited a significantly reduced DNA strand break response 0.5 hours post treatment when compared to their non-depleted counterparts. Although diminished, folate depleted cells did exhibit an increase in DNA strand breaks 0.5 hours post treatment, these strand breaks were not removed after 24 hours of recovery (Fig. 9A). Again, the deficiencies in DNA strand break removal induced by folate restriction were reversible in a concentration dependent manner when folic acid was added back to the growth media of 10 day depleted cells for 72 hours. Response to H₂O₂ treatment, as indicated by increased DNA strand breaks immediately after damage, was directly related to the concentration of folic acid restored to the growth media with continued folate depletion exhibiting no significant response and all levels of nutrient restoration exhibiting a significant increase in DNA strand breaks. When measured 24 hours post treatment, the remaining H₂O₂ induced DNA strand breaks were inversely related to the concentration of folic acid restored to the growth media with continued folate depletion exhibiting no removal and 9100 nM nutrient restoration exhibiting strand break removal similar to that of non-depleted controls (Fig. 9B).

4. Discussion

Previous studies have examined the role of folate in cells where optimum nutrition is proposed to promote health and prevent carcinogenesis such as colonic epithelium and lymphocytes [15,19,24], but there has been little investigation into the roles that folates play in the health of skin. In this work we establish a cell culture model of folate deficiency in skin to examine the effects that deficiencies in folate nutritional status have on skin cells and to determine if optimizing folate levels in skin may be beneficial in preventing or repairing the effects of UVR exposure.

Folate depletion, via removal of folic acid from the culture medium, results in a dramatic drop in intracellular concentrations of all measured reduced folates with levels decreasing quickly to under 5% of non-depleted control cells in only 10 days (Fig. 2). These results indicate the sensitivity of cellular folates to depletion, demonstrate the validity of the model as one of folate deficiency, and for the first time show the inability of proliferating skin cells to conserve intracellular folate stores. This inability to conserve folates leads to a reduction, then a complete halt in proliferation and an accumulation of cells in the S-phase of the cell cycle (Fig. 1A). This observation is consistent with observations made in lymphocytes and colonic epithelial cells [19,44].

Both reduced proliferation and elongated S-phase are particularly concerning in skin where maintenance and renewal of barrier function depends upon continued epidermal cell renewal and S-phase DNA is particularly vulnerable to the accumulation of pro-carcinogenic mutations [45]. S-phase accumulation may result either from activation of cell cycle checkpoint proteins induced by the accumulation of folate-depletion-induced DNA damage, likely uracil incorporation into newly synthesized DNA, or simply through the depletion of metabolites necessary for continued DNA synthesis. We observe that folate depletion in keratinocytes leads

to an increase in both inherent DNA damage and DNA uracil misincorporation. Both inherent DNA strand breaks and single or double strand breaks formed through the excision of uracil residues contribute to genomic instability, an established risk factor for the development of cancer [46,47]. Exacerbating the concerns related to proliferation, cell cycle, and genomic instability is the observation that HaCaT cells subjected to folate depletion are not greatly sensitized toward apoptosis (Fig. 3A). Almost complete reduction of intracellular folate levels after 10 days of folate depletion results in only a 10% reduction in overall cell viability, suggesting that these cells are conditioned to survive folate depletion despite the potential adverse genetic consequences.

The potentially detrimental effects of folate depletion are reversible when folic acid is restored to the culture media. Intracellular concentrations of reduced folates are reestablished significantly by 48 hours and completely by 120 hours after folate is restored to 10 day depleted cells (Fig. 2), demonstrating that folic acid is a suitable supplement for the optimization of folate levels in epidermal cells. Of interest is that intracellular concentrations of folic acid were below the limit of quantification at all time points with the exception of 24 hours after nutrient restoration. This indicates that while folic acid serves as an adequate folate source, it is rapidly reduced upon uptake and not accumulated intracellularly. The elevated levels of THF, the product of folic acid reduction, which are observed 48 and 72 hours after nutrient restoration, further support this observation. Supplementation of skin cells with folic acid appears beneficial as the recovery of intracellular folate pools corresponds to recovery of cellular viability as well as a reversal of both proliferation and S-phase cell cycle arrest. Nutrient repletion also restores the ability of epidermal cells to repair DNA and to remove misincorporated uracil. Interestingly, both proliferation and DNA repair capacity are restored in a folate dose dependent manner. These results indicate that additional benefit to skin may be derived from folic acid supplementation as the processes of cellular turnover and DNA damage repair, critical to the maintenance of healthy skin, will operate at a diminished capacity when folate nutrition levels are not optimal.

An established cell culture model of folate depletion in skin allowed for an examination of the consequences that reduced intracellular folate concentrations have upon the cellular response of keratinocytes to UVR. To test the effects of ultraviolet radiation, HaCaT cells were exposed to a physiologically relevant dose of SSL (4 J/cm² UVA and 206 mJ/cm² UVB). This corresponds to 1–2 minimum erythral doses in fair skinned individuals, a dose readily achievable through normal environmental exposure, although the effects of SSL exposure on cultured cells are likely intensified when compared to whole skin as the model does not account for the light filtering ability of an intact stratum corneum [48]. Not surprisingly, folate depletion sensitizes keratinocytes toward apoptosis when they are exposed to SSL. Folate depleted cells exhibit a greater than 20% loss in viability as compared to their non-depleted, SSL exposed counterparts. Apoptosis is an essential process for the maintenance of skin homeostasis through the elimination of damaged or mutated cells [49]. However, an abnormal propensity of keratinocytes toward apoptosis under generally recoverable levels of stress would upset the normal processes of upward migration and differentiation ultimately, compromising the skin's ability to protect itself against environmental insult.

HaCaT cells harbor mutant p53 alleles, which in combination with defective NF-κB signaling have been proposed to sensitize these cells toward UV-B-induced apoptosis [50,51]. Thus the increased sensitivity toward SSL-induced apoptosis observed in folate deficient cells, when compared to non-depleted controls, is not thought to be p53-mediated. However, as the observed sensitivity is specifically induced by the depletion of folate, the increase in SSL-induced apoptosis may be in response to unrepaired DNA damage by a p53 independent mechanism. Indeed, folate deficiency significantly potentiates the damaging effects of SSL by inhibiting DNA repair. Keratinocytes grown in folate free media for 10 days were unable to

repair DNA damage, as indicated by the persistence of DNA strand breaks after SSL exposure. In contrast, non-depleted control keratinocytes exposed to the same SSL dose show a near complete removal of DNA strand breaks to the level of non-exposed control cells after 24 hours. The DNA repair capacity of 10 day depleted keratinocytes can be restored when folic acid is returned in a concentration dependent manner. Optimization of DNA repair activity is a major potential benefit of skin specific folic acid supplementation.

In addition to direct photodamage mediated by exposure to UV-B, much of the cellular damage induced by ultraviolet radiation is mediated by longer wavelength, deeper penetrating UV-A radiation via the excitation of cellular photosensitizers in the photochemical generation of ROS [52]. Similar to the results seen with SSL, folate deficiency also significantly potentiates the damaging effects of H₂O₂. In contrast to the results seen with SSL, folate deficient keratinocytes exhibit a diminished increase in DNA strand breaks when measured shortly after H₂O₂ treatment. SSL is a high energy insult capable of causing direct strand breaks while H₂O₂ insult results in oxidative DNA damage which is converted to DNA strand breaks only upon the initiation of repair. As DNA repair capacity is restored to folate deficient cells in an incremental fashion utilizing increasing concentrations of folic acid supplementation, the immediate increase in DNA strand breaks after H₂O₂ treatment increases in direct relation. This increased initial strand break response translates to more effective DNA repair when measured after 24 hours of recovery.

Recent results have raised concerns that folic acid supplementation may be linked to increased cancer incidence in some populations [2,53,54]. These safety concerns, raised under conditions of general oral folic acid supplementation, serve to further underscore the need to better understand the dual modality of nutrient modulation before the tissue specific impacts of therapy may be appreciated. Previous studies have demonstrated that folate deficiency inhibits the repair of endogenous, as well as radiation damaged DNA in Chinese hamster ovary cells, colon epithelial cells, and primary lymphocytes [9,16,24,44]. The results from previous studies are supportive of the current work and collectively provide evidence that early intervention in tissues exposed to chronic genotoxic insult may achieve the most benefit from folate supplementation at the lowest risk.

In skin, a continuously proliferating tissue, efficient DNA repair is critical in the maintenance of genetic stability as genomic insult in the form of UVR is chronic. Thymine dimers are the major DNA photoproducts observed after solar UV-B exposure and are also observed when comparably erythematous UV-A doses are received [55]. Animal and human studies have confirmed a central role of DNA photoproducts in the development of skin cancer, in which these photoproducts lead to highly specific CC→TT and C→T substitutions known as UVR fingerprint mutations [56,57]. Folate deficiency decreases the cellular levels of 5,10-methylenetetrahydrofolate thus inhibiting synthesis of thymidylate from deoxyuridylate ultimately resulting in an imbalance of deoxyribonucleotides similar to that seen when thymidylate synthase is inhibited by 5-fluorouracil. This nucleotide imbalance has been shown to promote uracil misincorporation into DNA, inhibit DNA synthesis, and reduce the fidelity of DNA replication [58]. The repair of thymine dimers and thus the ability of skin to protect against the procarcinogenic effects of UVR exposure may be particularly sensitive to folate deficiency induced alterations in DNA repair efficiency. In addition, intracellular folates must be present in their reduced forms for biological activity. It has been proposed that a portion of the intracellular pools of reduced folates such as 5-methylTHF act in a sacrificial, antioxidant capacity to quench the excited state of photosensitizers and scavenge singlet oxygen species [59]. Therefore, the amounts of reduced folates lost to oxidative decay would be higher in both proliferating tissues generating endogenous ROS and in tissues exposed to UV-A radiation with high levels of ROS due to photosensitization reactions. It can therefore be surmised that the unique physiologic environment, the chronic and specific type of DNA damage to which

skin is subjected, and the cellular regeneration required to maintain homeostasis will combine to make optimal folate nutrient levels in skin higher than those of other tissues.

5. Conclusions

Folate depletion as examined by nutrient restriction in HaCaT cells occurs rapidly in dividing cell populations. This rapid depletion spans all of the reduced intracellular folates examined and ultimately results in S-phase proliferation arrest and increased levels of inherent DNA damage without significant losses in overall cellular viability. Indeed, the consequences of folate deficiency observed in other cell types extend to and may be exacerbated in skin cells. Folate depleted keratinocytes are sensitized toward UVR induced apoptosis and, most alarmingly, display a diminished capacity to repair both photo and oxidative DNA damage. Taken as a whole, the consequences of folate depletion create a permissive environment for genomic instability, with the potential to increase the probability of events early in the process of skin carcinogenesis. The effects of folate restriction, even in severely depleted, growth-arrested keratinocytes, are reversible with folic acid supplementation. Folate supplementation restores the resistance of HaCaT cells to apoptosis after SSL exposure by reestablishing cellular capacity for DNA damage repair. The dose and time dependence of the ability of folic acid to restore both cell proliferation and DNA damage repair indicate that these processes may operate at diminished capacity under sub-optimal levels of folate nutrition. Overall, these results indicate that skin health can be positively influenced by folic acid supplementation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

5-methylTHF	5-methyltetrahydrofolate
5-formylTHF	5-formyltetrahydrofolate
THF	tetrahydrofolate
Mtx	methotrexate
UVR	ultraviolet radiation
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
PBS	phosphate buffered saline
ESI	electrospray ionization
UDG	uracil DNA glycosylase
SSL	solar simulated light
ROS	reactive oxygen species

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Figure 1.

Effects of folate modulation on HaCaT cell proliferation and cell cycle distribution during (A) folate depletion over the course of 10 days and (B) differential folate repletion of 10 day depleted cells. The left panels show the number of population doublings as a function of time and the right panels show the percentage of cells in the S/G2 phase of the cell cycle. Results are mean $\pm$ SD.

Figure 2.

Effects of folate depletion and repletion on intracellular concentrations of 5-methylTHF (A), 5-formylTHF (B), and THF (C) during culture in folic acid free media for 5 days (F- D5) or 10 days (F- D10), and repletion of 10 day depleted cells with media containing 9100 nM folic acid for 24 (R24), 48 (R48), 72 (R72), or 120 (R120) hrs. Results are mean $\pm$ SD. *** $P < 0.0005$, * $P < 0.05$, and n.s. non significant $P > 0.05$ as compared to (F+) non-depleted control cells.

Figure 3.

Effects of folate restriction and repletion on cell viability as measured by Annexin V/PI flow cytometry. (A) Non-depleted control cells (F+), 5 day folate depletion (F- D5), and 10 day folate depletion (F- D10). (B) Viability of 5 and 10 day folate depleted cells and 10 day depleted cells after 9100 nM folate repletion for 24 (R24), 48 (R48), 72 (R72), and 120 (R120) hrs shown as a % of non-depleted controls. Results are mean $\pm$ SD. *** $P < 0.0005$ and n.s. non significant $P > 0.05$ as compared to (F+) non-depleted control cells unless otherwise indicated.

Figure 4.

Effects of folate restriction on inherent DNA damage and uracil misincorporation as measured by comet assay with (solid bars) or without (open bars) uracil DNA glycosylase (UDG) treatment. Results are mean $\pm$ SEM. *** $P < 0.0005$ and n.s. non significant $P > 0.05$ where P values refer to difference compared to Day 0 inherent DNA damage (- UDG); ‡‡ $P < 0.005$ and n.s. non significant $P > 0.05$ where P values refer to difference to Day 0 uracil misincorporation quantified as the difference between (+UDG) and (- UDG).

Figure 5.

Effects of differential folate repletion on inherent DNA damage and uracil misincorporation in 10 day folate depleted HaCaT cells as measured by comet assay with (solid bars) or without (open bars) UD treatment. Cells cultured for 10 days in folate free media were further folate depleted with (A) 0 nM, or replenished with (B) 9 nM, (C) 45 nM, (D) 91 nM, or (E) 9100 nM folic acid added to the growth media for 5 additional days of culture. Results are mean $\pm$ SEM. *** $P < 0.0005$, ** $P < 0.005$, and n.s. non significant $P > 0.05$ where P values refer to difference to Day 0 (10 day folate depleted) inherent DNA damage (open bars, -UDG); ‡‡‡ $P < 0.0005$, ‡ $P < 0.05$, and n.s. non significant $P > 0.05$ where P values refer to difference to Day 0 (10 day folate depleted) uracil misincorporation (closed bars, +UDG).

Figure 6.

Effects of folate restriction and repletion on cell sensitivity to apoptosis induced by SSL exposure as measured by Annexin V/PI flow cytometry. (A) Non-depleted control cells (F+), 5 day folate depletion (F-D5), and 10 day folate depletion (F-D10) prior to and 24 hours after SSL exposure. (B) Viability of SSL exposed 5 and 10 day folate depleted cells and 10 day depleted cells after 9100 nM folate repletion for 24 (R24), 48 (R48), 72 (R72), and 120 (R120) hrs shown as a % of non-depleted controls. Results are mean ± SD. ** $P < 0.005$ and n.s. non significant $P > 0.05$ as compared to (F+) non-depleted control cells unless otherwise indicated.

Figure 7.

Effects of folate restriction on DNA damage induced by SSL exposure and DNA damage repair kinetics as measured by comet assay in control (solid bars) or 10 day folate depleted (open bars) HaCaT cells. Results are mean $\pm$ SEM. % Tail DNA increased significantly 4 hour post SSL exposure in both non-depleted control and 10 day folate depleted cells ($P < 0.0001$ for both). The % tail DNA decreased significantly from the 4 hour observation at both 8 and 24 hours in non-depleted control cells ($P < 0.0001$ for both). The % tail DNA did not decrease from the 4 hour observation at either 8 ($P = 0.21$) or 24 ($P = 0.67$) hours in 10 day folate depleted cells.

Figure 8.

Effects of differential folate replenition on DNA damage induced by SSL exposure and DNA damage repair kinetics in 10 day folate depleted HaCaT cells as measured by comet assay. Cells depleted of folate for 10 days (F-) were replenished for (24), (48), (72), or (120) hrs with (A) 0 nM, (B) 9 nM, (C) 45 nM, (D) 91 nM, or (E) 9100 nM folic acid prior to SSL exposure with the three bars at each replenishment time point corresponding to measurements taken 4, 8, and 24 hrs post SSL exposure. Results are mean $\pm$ SEM. *** $P < 0.0005$, ** $P < 0.005$, and * $P < 0.05$ where P values refer to DNA repair examined as the difference to the % tail DNA observed in F- cells (10 day folate depleted) 4 hours post SSL exposure. All non significant, $P > 0.05$, values are not indicated.

Figure 9.

Panel A. Effects of folate restriction on DNA damage induced by H₂O₂ exposure and DNA damage repair kinetics as measured by comet assays. Non-depleted control (solid bars) as compared to 10 day folate depleted (open bars). Results are mean $\pm$ SEM. The % tail DNA increased significantly 0.5 hour post H₂O₂ exposure in both non-depleted control and 10 day folate depleted cells ($P < 0.0001$ for both). The increase in % tail DNA 0.5 post H₂O₂ exposure in 10 day folate depleted cells was $2.7 \pm 0.83\%$ which is significantly lower than the $11.3 \pm 0.59\%$ increase observed in non-depleted control cells ($P < 0.001$). The % tail DNA decreased significantly from the 0.5 hour observation after 24 hours of recovery in non-depleted control

cells ($P < 0.0001$). The % tail DNA did not decrease from the 0.5 hour observation after 24 hours of recovery in 10 day folate depleted cells ($P = 0.33$).

Panel B. Effects of differential folate repletion on DNA damage induced by H_2O_2 exposure and DNA damage repair kinetics. Cells depleted of folate for 10 days that had been differentially replenished with folic acid for 72 hrs prior to H_2O_2 exposure and recovery. Results are mean $\pm$ SEM. *** $P < 0.0005$ and n.s. non significant $P > 0.05$ where P values refer to difference to the % tail DNA of non- H_2O_2 exposed cells with the same level of folate repletion. ††† $P < 0.0005$ and n.s. non significant $P > 0.05$ where P values refer to difference to the % tail DNA 0.5 hours post H_2O_2 exposure in cells with the same level of folate repletion.