FOLIC ACID ROLE IN MUTAGENESIS, CARCINOGENESIS, PREVENTION AND TREATMENT OF CANCER

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ABSTRACT

In 1987 we have published a hypothesis for the role of folic acid in the cancer prevention. Many research data, concerning the involvement of folate in the aetiology of cancer, accumulated since 1987 till now, support our hypothesis. Nowadays, the important role of folic acid in the normal cell metabolism, especially in the DNA synthesis and gene expression, and the role of folate deficiency in the development of some pathological processes, including cancer, are considered to be proved. In the present article we show the suppressive effect of folic and tetrahydrofolic acid on the DNA alkylation with a spin-labeled (containing nitroxyl radical) hydrazine mustard antitumor agent, by the electron spin resonance (ESR) method in vitro. We follow briefly the normal biological functions of folic acid and the established molecular effects of folate deficiency. Basing on our and other authors studies, including quantum biochemical ones, on the folic acid and other low molecular weight nucleophilic compounds in the cell we connect the folate depletion with the strong nucleophilic properties of hydrogenated folates, especially tetrahydrofolate, that determinate it as one of the first targets of the electrophilic attack of agents with carcinogenic potential, including the reactive oxygen species. We consider that an unified theory for mutagenesis, carcinogenesis, cancer prevention and therapy putting the folate and tetrahydrofolate in the center of the events, has been created.

Introduction

It is clear now that initiators of carcinogenesis fall into three broad groups: radiant energy, chemical compounds and viruses. In general these act by causing mutation or by introducing novel genes into cells (e.g. by viruses).

Apart from direct damaging effects on DNA the ionizing radiation cause free radicals to form in tissues. The resultants reactive oxygen species (ROS) – superoxide (O₂), hydrogen peroxide (H₂O₂), hydroxyl radical (⋅OH) and other radicals can interact with DNA and other cell macromolecules leading to their damage (34). The oxidative DNA damage may lead either to activation of proto-oncogenes or to inactivation of tumor suppressor genes and in this way – to malignant cell transformation (1).

It is estimated that approximately 80% of human cancers are caused by environ-
mental factors, principally chemicals. Exposure to such compounds can occur because of a person’s occupation, diet, lifestyle (cigarette smoking, alcohol) or in other ways (certain drugs, including anti-tumor ones can be carcinogenic).

Carcinogens are highly reactive and usually are electrophiles (i.e. molecules deficient of electrons), that readily attack nucleophilic (electron-rich) groups in DNA, RNA and proteins forming covalently bound adducts. Carcinogens have been found to interact with purine, pyrimidine, or phosphodiester group of DNA and despite of existence of repair systems certain DNA modifications are of special importance in generating mutations critical to carcinogenesis. Mutations, caused by chemical carcinogens or irradiation, can affect the specific DNA sequences either in proto-oncogenes and their becoming oncogenes, or in tumor-suppressor genes and their inactivation.

DNA is the main target molecule for the action of the ionizing radiation, ROS and chemical carcinogens that appear electrophilic agents. But, the complex molecular organization of DNA in the nucleus makes it difficult of access for a carcinogenic attack. The DNA precursors in the nucleotide pool, used in both DNA synthesis and DNA repair, are far easier accessible for agents with carcinogenic potential. Except the free nucleotides, other low molecular weight nucleophiles (LMN) in the cell are pteridines – folic acid (FA), dihydrofolic acid (DHFA) and tetrahydrofolic acid (THFA). The last plays an important role in the synthesis of purine and pyrimidine nucleotides and in this way in DNA synthesis and DNA repair.

In the present article we promote our previous concept for the important role of LMN, as the first targets of carcinogenic electrophilic attack, in mutagenesis, carcinogenesis and also – in prevention and treatment of cancer (23). The accumulated epidemiological and experimental data connecting the folate deficiency with cancer risk put the folic acid in the centre of our consideration.

**Materials and Methods**

**Chemicals**

Calf thymus DNA, folic acid, tetrahydrofolic acid and deoxyribonuclease I (DNase I) were obtained from Sigma. The deoxyribonucleotides – dGMP, dAMP, dCMP and TMP were obtained from Aldrich. The hydrazine mustard spin-label 3-[N,N-bis(2-chloroethyl)carbohydrazide]-2,2,5,5-tetramethylpyroli dine-1-oxyl (HMSL) is synthesized in our laboratory by a method described elsewhere (24).

**Spin-labeled DNA assay**

The interaction of calf thymus DNA with HMSL (spin-labeling of DNA) was carried out by incubation in 0.01 x SSC, pH 7.0 of DNA (1 mg/ml) with HMSL (10 mg/ml) at 37°C for 24 h. The unbound spin-label was removed from spin-labeled DNA by hydroxyapatite chromatography.

The electron spin resonance spectrum (ESR spectrum) of spin-labeled DNA was recorded on a Bruker ESR. The amount of spin-label covalently bound to DNA was determined by measuring the area under the ESR spectrum recorded after treatment of DNA sample with DNase I. The effect of the low molecular weight nucleophils (LMN), FA, THFA, dGMP, dAMP, dCMP and TMP on the alkylation of DNA with HMSL was determined by spin labeling of DNA in the presence of 10M excess (in respect to DNA) of one of the above mentioned substances. All experiments with the participation of THFA were carried out under nitrogen atmosphere. The LMN was removed from DNA by hydroxyapatite chromatography and the purified spin-labeled DNA was hydrolyzed with DNase I. The ESR spectra were measured and the areas under the spectra were related to a constant amount of DNA (arbitrary units).

**Results and Discussion**

Before the representation of a discussion on our and other authors results, confirm-
ing our view on the reasons for folic acid deficiency, we follow briefly the biological functions of folic acid and the established molecular effect of folate deficiency.

**Biological functions of the folic acid**

It is well known that the tetrahydrofolic acid (THFA), which is produced in human cells by reduction of the vitamin folic acid (FA), transfers one-carbon groups (methyl, methylene, methenyl and formyl) from one compound to another. The biological functions of FA, respectively of its coenzyme form THFA, are shown in **Fig. 1**.

The nucleotide pool, filled in with folic acid participation, provides not only for the DNA replication, but for DNA repair. S-adenosylmethionine (SAM) is the principal methyl donor in methylation of nucleotides, especially cytosine in DNA. Approximately 4% of cytosine residue are modified post synthetically to 5-methyl cytosine (5mC) (27). Genes that are methylated at specific location in the DNA molecule are either not transcribed or are transcribed at reduced rate. In this way site-specific DNA methylation controls gene expression.

From the brief review of the FA biological function it is clear that FA, respectively THFA, is required for the normal growth and division of the cell. In view of important role of FA, it is not surprising that a folate deficiency has been implicated in a wide variety of health disorders like heart diseases (coronary heart disease, heart attacks, strokes), mediated through elevated levels of the endothelial cell toxin homocysteine (19); depression 2); Alzheimer's disease (10), and cancer.

**Folic acid deficiency and cancer**

There are accumulated data implicating folic acid deficiency in the development of cancer, notably of the cervix, lung, breast, brain, colorectum, etc.

There appear to be two principal mechanisms through which low folate status may increase the risk of malignancy. FA deficiency, by reducing intracellular SAM, can alter cytosine methylation in DNA, leading to inappropriate activation of proto-oncogenes and induction of malignant transformation. Folate deficiency may cause an imbalance in DNA precursors, uracil misincorporation into DNA and chromosome breakage (8).

A brief description of the evidence from cellular, animal and human studies that folic acid can modulate DNA by such mechanisms is present below in the article.

**a) Folic acid deficiency and DNA methylation**

There is considerable evidence that aberrant DNA methylation plays an integral role in oncogenesis. A decreased level of genomic methylation is a nearly universal finding in tumorigenesis: this has been observed in cancer of colon, stomach, uterine cervix, prostate, thyroid and breast (28). Several studies have shown that specific human genes (growth hormone and proto-oncogenes) from tumor tissue (lung and colon) are substantially less methylated than genes from adjacent normal tissue (27). The liver from methyl-deficient rats (fed diet deficient in methionine, choline, folic acid and vit. B12) expressed elevated levels of mRNA for the proto-oncogenes c-myc, c-fos, and c-tta-ras; normal levels are restored when a control diet is fed (31).

Folate depletion has been shown to induce hypomethylation on the coding region of the p53 tumor suppressor gene (16).

The findings of a relationship between folate status and methylation patterns in individuals already diagnosed with cancer and in healthy normal individuals provide credible evidence for a mechanism through which folate may modify DNA methylation and alter cancer risk (see **Fig. 2**).

**b) Folic acid deficiency, DNA integrity and repair**

It has been sworn from some years that folate deficiency induces breaks in chromosomes and that such breaks are associated with an increased risk of cancer in humans. A mechanism by which folate de-
Fig. 1. Biological functions of FA, respectively THFA. The THFA is required for the formation of thymine, which is used for DNA synthesis, and for the synthesis of purines, which are necessary for both DNA and RNA synthesis. THFA participate in the methionine synthesis which via SAM provides methyl group for the cytosine in DNA.

Folic acid deficiency might create such breaks is the misincorporation of uracil into DNA. Folate deficiency by blocking the methylation of dUMP to TMP can disrupt the balance of DNA precursors leading to accumulation of excess dUMP in the nucleotide pool. This may result in dUMP being misincorporated into DNA in place of thymi-
Fig. 2. Reasons for THFA, respectively FA deficiency and its molecular effects leading to cancer risk. Ultimate electrophils, obtained during biotransformation of chemical carcinogens and ROS, which increased production (oxidative stress) might be due to radiation, inflammation, chemical carcinogens and drug biotransformation, low antioxidant status, etc. are the main reasons for THFA depletion. As one of the strongest nucleophils in the cell THFA combines readily with electrophilic agents and gets inactive.
dine as DNA polymerases can not distinguish between dUMP and dTTP. Uracil DNA glycosylase removes any misincorporated uracil from DNA molecule and in the process a transient single-strand break develops in the DNA (3). Simultaneous removal and repair of two adjacent uracil residues on opposite strands can result in a double-strand DNA breaks. If thymidine is continually limited under condition of folate deficiency, uracil misincorporation and repair may occur repeatedly in what has termed a „catastrophic” repair cycle (25). Strand breaks, as intermediates in excision repair, may destabilize the DNA molecule, leading to chromosome aberrations and malignant transformation. In those instances where cancers are enhanced by particular viruses, the phenomena of hypomethylation and strand breaks may have additional significance.

Folat deficiency disrupt DNA repair. As was mentioned above, folate deficiency induced dNTP pool imbalance and uracil misincorporation into DNA. Although much of this uracil can be removed by DNA repair enzymes, the lack of available dTTP blocks the step of DNA repair catalyzed by DNA polymerase; thus, base-excision repair, one of DNA repair mechanisms, is disrupted (6). The results are DNA strand breaks and blockage of normal DNA replication. Site-specific DNA hypomethylation, induced by folate deficiency, might affect the methyl-directed mismatch repair- the other major cellular DNA repair system (18) (see Fig. 2).

Alteration in DNA methylation (hypomethylation), disruption of DNA integrity, caused by increased uracil misincorporation, and DNA strand breaks, and disruption of DNA repair are related phenomena that can each be induced by folate depletion and are believed to enhance carcinogenesis by altering the expression of critical genes. Moreover, folate supplementation can reduce DNA instability in folate-deficient subjects. In this connection, it is important to reveal the causes for folate depletion.

**Reasons for folic acid deficiency**

Some of the reasons for the tissue folate deficiency could be: inadequate dietary intake of folate; vitamin B12 deficiency leading to secondary folate deficiency because the most of the folate in the body is irreversibly “trapped” as its N5-methyl-THFA (Fig. 1) and adequate supply of free THFA is not available; drugs, that interfere with folate absorption and metabolism.

In reviewed articles concerning the folate status and cancer risk, the lower folate levels in cancer patients than healthy volunteers and the consequences of folate deficiency have been discussed mainly (9). There was no discussion about the question what is (are) the reason(s) for the folic acid depletion. We consider that we can give the answer of this question. As THFA is one of the strongest low molecular nucleophiles in the cell, probably it is one of the first target of electrophilic attack of agents with carcinogenic potential (chemical carcinogens, free radicals, esp. ROS). The combination of electrophilic agents with THFA leads to depletion of the free THFA and therefore to FA deficiency.

Quantum biochemical studies of Pulman and Pulman on the purine and pyrimidine bases, FA, DHFA and THFA showed that the most powerful nucleophils among them are guanine and hydrogenated folates – DHFA and THFA (21) (Table 1).

We have investigated the interaction of FA and THFA in vitro with nitrosourea antimutor drugs (Lomustine - CCNU, Carmustine - BCNU), appearing alkylation
TABLE 1
Quantum biochemical characteristics of pyrimidines, purines and pteridines according to Pulman and Pulman

<table>
<thead>
<tr>
<th>Pyrimidines, Purines, Pteridines</th>
<th>Energy of the highest occupied molecular orbit</th>
<th>Energy of the lowest free molecular orbit</th>
</tr>
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<tbody>
<tr>
<td>Cytidine</td>
<td>0.60</td>
<td>-0.80</td>
</tr>
<tr>
<td>Thymidine</td>
<td>0.51</td>
<td>-0.96</td>
</tr>
<tr>
<td>Adenine</td>
<td>0.49</td>
<td>-0.87</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.31</td>
<td>-1.05</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.49</td>
<td>-0.65</td>
</tr>
<tr>
<td>7,8-Dihydrofolic acid</td>
<td>0.29</td>
<td>-0.75</td>
</tr>
<tr>
<td>Tetrahydrofolic acid</td>
<td>0.05</td>
<td>-1.07</td>
</tr>
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TABLE 2
Competitive effect of LMNs on the alkylation of DNA with HMSL

<table>
<thead>
<tr>
<th>LMNs</th>
<th>Area under the spectrum of the hydrolized spin-labeled DNA (arbitrary units)</th>
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<tbody>
<tr>
<td>THFA</td>
<td>2.8</td>
</tr>
<tr>
<td>dGMP</td>
<td>4.6</td>
</tr>
<tr>
<td>FA</td>
<td>11.8</td>
</tr>
<tr>
<td>dAMP</td>
<td>12.3</td>
</tr>
<tr>
<td>dCMP</td>
<td>23.3</td>
</tr>
<tr>
<td>TMP</td>
<td>57.3</td>
</tr>
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</table>

agents that decompose (in vitro and in vivo) forming electrophilic carbocations (23). The results obtained indicate that FA and THFA interact with the electrophilic agents and that the reactivity of THFA is higher (THFA is alkylated more quickly than FA).

Recently we studied the effect of LMNs – FA and THFA, and four deoxyribonucleoside-monophosphates (dGMP, dAMP, dCMP and TMP) on the alkylation of DNA with hydrazine mustard spin-label (HMSL), a bifunctional alkylating cytostatic (see Materials and Methods). According to their competitive effect on DNA alkylation the LMNs can be ordered in the following way:

THFA>dGMP>FA>dAMP>dCMP>TMP (Table 2). These results show that the alkylating agents combines preferably with THFA and dGMP, and respectively in lower extend with the other free nucleotides, than with the DNA.

In CHO cell study it was established that folate deficiency acted synergistically with alkylating agents to increase somatic mutations, and with γ-irradiation to promote DNA strand breaks by limiting DNA repair (4). These results could be interpreted by our hypothesis for the role of THFA as a “outpost” in the defense against DNA damage. When the FA deficiency is already available, the amount of the “trapped” by THFA ultimate electrophils (obtained from alkylating agents or free radicals obtained by γ-irradiation) is low and the DNA damage increases by the action of the rest considerable amount of electrophils.

Branda and co-workers found that folate supplementation decreased the frequency of the “common deletion” in liver mitochondrial DNA from rats, treated with cyclophosphamide, an alkylating anticancer drug (5). This result also could find its explanation by our hypothesis. The folate supplementation decreases, by THFA, the amount of ultimate electrophil, obtained from cyclophosphamide, and in this way decreases its DNA impairing effect.

It is known that nitrosamines - dimethyl- and diethyl-nitrosamine (DENA) are powerful chemical carcinogens forming an electrophilic ultimate carcinogen during their metabolic activation. We found that the administration of folic acid in rats before or during their treatment with DENA reduces the damaging effect of DENA on liver DNA (13). This was the first study, confirmed our hypothesis for the protective effect of THFA against a carcinogenic attack.

Several reports indicate that cigarette
smoke may cause localized folate deficiency in the bronchial epithelium. Serum folate levels are lower in smokers than in non-smokers and also in smokers with premalignant metaplasia (20). Supplementation with folate and vitamin B₁₂ reduces the severity of smoking-induced bronchial squamous metaplasia in humans and in chemical-induced metaplasia in rats (15). There were identified more than 40 components of cigarette smoke (among them – polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines, etc.) which carry out its genotoxic action by ultimate electrophils, formed during their metabolic activation. It was proved that many chemical carcinogens especially polycyclic hydrocarbons, act also through free radical intermediates that could generate secondary reactive oxygen species (ROS) (7). It was established that ROS themselves are components of cigarette smoke. Many studies prove the share of ROS in the pathogenesis of cancer – breast, stomach, colon, liver, lung, etc. (22).

Having in mind that all oxygen centre free radicals are electron acceptors (electrophils), a direct interaction between them and THFA is fully possible. There are studies supporting this our consideration. The in vitro studies with folic acid, aminopterin, and methotrexate have suggested that these inhibit oxidation of hypoxanthine into uric acid by xanthine oxidase (XO), one of the major sources of ROS (O₂⁻) in biological systems (26). It was established that XO is more active in radiolitically damaged tissue where, by the generation of free radicals, XO probably potentiates the effect of radiation. The folate supplementation in Swiss albino mice before γ-irradiation diminishes the levels of oxidative damage of the liver (29). In our opinion the results obtained are due to the interaction of THFA with ROS originated from both XO catalitic reaction and γ-radiation action.

Some authors suggest that cellular immune activation and oxidative stress, both estimated by increased neopterin concentration, could be involved in the development of hyperhomocysteinemia. Because tetrahydrofolate is very susceptible to oxidation, an increased oxidative degradation of tetrahydrofolates may become relevant under oxidative stress conditions and leads to hyperhomocysteinemia. In this way folate deficiency may develop despite of normal dietary intake of the vitamin (32). The conclusion of these authors gets close to our hypothesis for the reasons for FA, resp. THFA deficiency.

Folate deficiency has been considered as an important factor in alcohol-related carcinogenesis (colon cancer in men) because alcohol alters normal folate metabolism in a number of ways (11). It is known that during the alcohol catabolism to acetate free radical intermediates are generated – hydroxyethyl radical (CH₃CH₂O⁻) by the alcohol-dehydrogenase catalitic action and ROS by the xanthine oxidase or aldehyde oxidase action. As it was already mentioned, the folic acid inhibits the xanthine oxidase that, to our opinion, is due to the interaction of THFA with ROS normally formed during the catalitic reaction. This interaction leads to THFA, respectively FA depletion observed in the alcoholists.

We consider that FA deficiency (resp. THFA deficiency), established in conditions of increased ROS production is due to the interaction of THFA with ROS and the result of that is THFA inactivation and depletion. We also consider that the antioxidants, such as vitamin C, vitamin E and carotenoids, that eliminate ROS decrease the THFA inactivation. Moreover, to our
opinion the main role of the antioxidant vitamins and other natural antioxidants such as flavonoids, is to keep the folic acid in its active reduced (hydrogenated) form, e.g. – as THFA.

Nearly all the epidemiological studies to date have used dietary and/or blood folate levels as the index of folate status; such measures do not necessarily reflect the folate concentrations in the tissue of interest. This is potentially of considerable importance since some studies suggest that the susceptibility of folate depletion varies widely between tissues (30). For example, cigarette smoke may caused localized folate deficiency in the bronchial epithelium (14). We consider that the local FA, resp. THFA depletion, could be represented by a factor $F = \frac{N}{E}$, where $N$ is the nucleophilic pool and $E$ is the amount of the electrophils in the cell compartments. The low $F$ values should correlate with a risk of malignant transformation of the normal cell and tumorogenesis in certain tissue.

Our view for the reasons, leading to THFA, respectively FA depletion in the cells of certain tissue, and the molecular effects of FA deficiency, known from research data, are summarized in Fig. 2.

Molecular epidemiological studies in humans indicate that folate deficiency leads to an increase of DNA damage, while folate supplementation can reduce DNA instability in folate-deficient subjects (17). In this connection, it is advisable the folate supplementation in oncological patients, especially after irradiation and antitumor drugs therapy because of FA depletion during such treatments. The increased folate intake is necessary for the cancer risk groups of population such as smokers, alcoholists and people working in polluted environment. As the role of folate deficiency in atherosclerosis, cardiovascular disease, neurological and neuropsychiatric disorders, has become better understood, folate has been recognized as having great potential to prevent these many disorders through folate supplementation. As the oxidative stress plays an important role in the pathogenesis of chronic inflammatory diseases (33) and hematological disorders such as beta-thalassemia (12), we would recommend the intervention with folic acid of the patients suffering from these diseases.

Conclusions

An expanding body of epidemiological animal and human studies suggests that folate status modulates the risk of developing cancers in several tissues - folate depletion appears to enhance carcinogenesis while folate supplementation conveys a protective effect. There are two principal mechanisms where-by folate is thought to modulate DNA stability and cancer incidence. According to first mechanism the folate deficiency causes DNA hypomethylation and proto-oncogene activation. The second mechanism is that folate deficiency induces continuous uracil misincorporation during DNA synthesis leading to a catastrophic DNA repair cycle, DNA stand breakage and chromosome damage.

Having in mind the following considerations: the quantum biochemical studies proving that the hydrogenated folates – DHFA and THFA, together with guanine nucleotides, are the most powerful LMN in the cell, that the ultimate active metabolites of chemical carcinogens and some groups of drugs, including antitumor ones, as well free radicals, including ROS, appear to be electrophilic molecules, our and other authors studies proving the interaction of folates (FA, DHFA and THFA) and nucleotides (especially dGMP) with alkyla-
ting antitumor agents, as well the free radicals scavenging properties of the folates (FA and THFA), we speak out the assertion that LMN in the cell are the first targets under the attack of agents with carcinogenic potential, and in this way LMN appear to be a defense barrier against the gene damage. THFA, as the strongest nucleophile, plays the central role of a “preventive umbrella”. But, its active interaction with the electrophilic agents leads to the THFA inactivation and therefore – to THFA depletion in the cell. This is valid especially at permanent or continued carcinogenic effect that is available in individuals from the high risk groups like smokers, alcoholists, people working in a polluted environment, etc. Probably, when the tumorigenesis is already unlocked the FA deficiency will increase additionally because of acceleration of the synthesis of purine nucleotides, TTP and DNA replication in the tumor cell.

Considering that we are revealed the reason for THFA depletion and respectively for FA deficiency, we could recommend the application of folic acid in pharmacological doses during and after antitumor therapy of cancer patients, for cancer prevention of individuals from the high risk groups and for treatment of all disorders in which arising the oxidative stress is implicated.

REFERENCES