

## **Comparing the Genotoxic Effects Induced by Folate Deficiency to Those Induced By Ionizing Radiation in Cultured Human Lymphocytes**

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### **ABSTRACT**

DNA double-strand breaks (DSB) which is the primary cause of chromosomal aberrations and cancer and the most serious DNA lesion caused by ionizing radiation, are also caused by several common vitamin or mineral deficiencies such as folate, B6, or B12. In the current study, cultured human lymphocytes were either irradiated at low and high doses or cultured at different levels of folate deficiency to assess: cell proliferation, apoptosis, cell cycle, DNA single-strand (SSB) and double strand (DSB) breaks. Radiation and folate deficiency, decreased cell proliferation and induced DNA breaks, apoptosis, and cell cycle arrest. Cells irradiated with 1 Gy had proliferation rate ~50% that of unirradiated cells; 5 Gy completely abolished lymphocyte proliferation. The proliferation rate of cells cultured in 12 nM folate was 46% that of control cells and lymphocytes cultured in 0 nM folate did not proliferate. Irradiated lymphocytes showed cell cycle arrest in G<sub>2</sub>/M phase in a dose dependant manner. The G<sub>2</sub>/M arrest was still evident 72 h after irradiation. In contrast to radiation, folate deficiency caused an arrest in the S phase of the cell cycle in an apparently dose-dependent manner. DNA breaks as measured by the alkaline Comet assay were increased for radiation doses of 1 Gy and higher, although statistical significance was achieved only at 5 Gy. Similarly, cells cultured in low folate showed a dose-dependent increase in DNA breaks. For the conditions used in this study, physiological concentrations of folate deficiency (12 nM) induced as much DNA damage as did 1 Gy of radiation, a relatively high dose. Results suggest that physiological levels of folate deficiency cause as much DNA damage as relatively high levels of ionizing radiation, but with a different cellular response. The results also suggest that low doses of radiation can induce radio adaptive response, protecting the cells from damage induced by subsequent irradiation. This adaptive response has not been observed for folate deficiency.

**Keywords:** *DNA damage, Nutrition, Ionizing radiation, Cancer risk, Comet assay*

### **INTRODUCTION**

DNA damage can result from exposure to both external and endogenous sources of stress<sup>(1)</sup>. Though a comparison of DNA damage and cancer risk from different stresses is difficult when different kinds of DNA damage are produced, comparison can be made if a common endpoint is used such as the induction of DNA double-strand breaks (DSB). Both ionizing radiation and cellular deficiencies of vitamins and minerals can cause DSB, which is the primary cause of chromosomal

aberrations and cancer<sup>(2, 3)</sup>. Although radiation and vitamin deficiency both cause DSB, different cellular response and different levels of DNA damage may ensue<sup>(4)</sup>.

Cellular response to radiation has been extensively studied. Linear energy transfer (LET) radiation produces an estimated 25.40 DSB per cell per Gy, following acute exposure<sup>(5)</sup>. Radiation also produces reactive oxygen species, base lesions, and DNA single-strand breaks (SSB), which can be converted to DSB when damages are clustered<sup>(6)</sup>. Ionizing radiation induces arrests in the G<sub>1</sub>, S, and G<sub>2</sub> phases of the cell cycle; the ataxia telangiectasia mutated protein (ATM) appears to be involved in the initiation of these three checkpoint pathways<sup>(7, 8)</sup>.

Relatively high doses of radiation (between 0.5 and 10 Gy) are usually used in studies measuring the effects of ionizing radiation, mainly because acute biological effects are not easily detected following exposure to low dose of radiation. Typical population exposure, however, from occupational or background radiation ( $\sim 1 \times 10^4$  Gy per year, resulting in  $1 \times 10^5$ ) DSB per cell per day) falls in the low-dose range<sup>(9)</sup>. The risks of low dose exposure, such as DNA damage and cancer, have historically been linearly extrapolated from high dose studies. Whether it is meaningful to do this has been debated. New models have been proposed in which DNA damage occurs only above a certain threshold of radiation<sup>(10)</sup>. In addition, low doses of radiation have been shown to induce an adaptive response, reducing damage induced by subsequent irradiation<sup>(11-13)</sup>. It seems likely that the amount of DNA damage resulting from background radiation is much less than that occurring from endogenous oxidative metabolism, which results in  $\sim 1 \times 10^1$  DSB per cell per day<sup>(14)</sup>. Nutritional deficiencies in vitamins, minerals, and antioxidants, which are common in the population, could also contribute significantly to DNA damage and cancer risks<sup>(2-5)</sup>.

Folate is an essential vitamin that is involved in one-carbon transfer reactions and in the formation of purines and pyrimidines for DNA and RNA synthesis<sup>(15)</sup>. Folate deficiency has been linked to increased risk for several types of cancer<sup>(16)</sup>. Folate concentration of 12 nM is in the range found in plasma of individuals having low consumption of fruits and vegetables. Lower plasma folate (from 5 to 12 nM) has also been measured in folate-deficient individuals. Folate levels of individuals having a better diet are  $\sim 30$  nM, although it may not be the optimal folate level, as folic acid supplementation can raise plasma folate to 50 nM<sup>(17)</sup>. The most likely mechanism for folate-related enhancement of carcinogenesis is the misincorporation of uracil during DNA synthesis. Folate is required for the synthesis of deoxythymidylate (dTTP) from deoxyuridylate (dUMP) and hence for maintaining a proper ratio of dTTP/dUMP<sup>(18)</sup>. Removal of uracil by uracil-DNA glycosylase causes transient SSB; two nearby opposing lesions, such as uracil opposite an oxidized base, can cause DSB. Oxidized bases in DNA are common<sup>(19, 20)</sup>. Folate-deficient individuals have millions of uracil bases misincorporated per cell, as well as increased chromosomal breakage<sup>(15-20)</sup>. Vitamin B12 deficiency, which is common in the population, also causes uracil misincorporation into DNA and DSB by the same mechanism as folate deficiency<sup>(19)</sup>. In addition, DNA excision repair and mismatch repair may be impaired in folate-deficient cells. Excessive uracil misincorporation and defective DNA repair may explain why folate-deficient cells are more sensitive to the DNA-damaging effects of a second external stress<sup>(20)</sup>.

Normal human T lymphocytes can proliferate in culture for few weeks when stimulated with PHA and IL2. This study investigated whether radiation or folate deficiency affects this proliferation. In the present study, the effects of folate deficiency on DNA damage were compared with the effects caused by ionizing radiation. DSB formation, apoptosis and cell cycle variation after irradiation and during folate deficiency, were measured using normal human T lymphocytes in primary culture.

## MATERIALS AND METHODS

### Cell culture and irradiation

Normal human T lymphocytes were obtained from single donor, after being isolated from heparinized blood sample using Histopaque-1077 gradients (Sigma). Isolated lymphocytes were cultured in folate-free RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% dialyzed FBS (Invitrogen) and antibiotics. Folate (pteroyl-L-glutamic acid, Sigma, St. Louis, MO) was added to the folate-free medium to a final concentration of 0, 6, 12, 30, or 3000 nM folate. Phytohemagglutinin (PHA) (22.5 µg/ml, Sigma) and interleukin-2 (IL2) (10,000 U/ml, Sigma) were added on the first day of culture to ensure proliferation of T lymphocytes. Every 3 days, the cells were spun down and resuspended at  $0.5 \times 10^6$  cells/ml in fresh medium supplemented with IL2, for a total of 10 days of culture. For radiation, lymphocytes were cultured in 3000 nM folate-containing medium for a week and then irradiated with  $\gamma$ -rays from a Shepherd Cesium source ( $^{137}\text{Cs}$ ) at a dose rate of 0.25 Gy/min, for a total dose of 0, 0.1, 0.25, 1, or 5 Gy.

### Proliferation and cell cycle analysis

For the radiation studies, lymphocytes were cultured in folate-sufficient medium for 7 days and then irradiated at 0, 0.1, 0.25, 1, or 5 Gy. The total number of cells under each condition was counted for 3 consecutive days (until day 10 of culture) after irradiation. For folate deficiency studies, lymphocytes were cultured in 0, 6, 12, 30, 100 or 3000 nM folate for 10 days. Cell culture medium containing 3000 nM folate was used as the control because it is the concentration in standard RPMI 1640 medium. Proliferation kinetics were measured at 24-h intervals after irradiation or during folate deficiency by counting cell density with a Coulter counter, and cell viability was assessed with Trypan blue exclusion on a hemocytometer.

Cell cycle arrest is an early marker of induction of cell cycle checkpoints, DNA damage, and initiation of repair mechanisms. Flow cytometry of propidium iodide-stained cells was used to study how radiation and folate deficiency affect cell cycle distribution.

For cell cycle analysis, cells were harvested at 24-h intervals after irradiation or during folate deficiency. Cells ( $0.5 \times 10^6$ ) were washed with PBS, permeabilized in 0.3% saponin, treated with 100 µg/ml RNase A, and stained with 10 µg/ml propidium iodide. After 20 min incubation at room temperature, the distribution of DNA content was measured using a FACSort flowcytometer (Becton Dickinson, San Jose, CA). The percentage of cells in the G1, S, or G2/M phases of the cell cycle was determined using the ModFit software (Becton Dickinson).

### Detection of apoptosis

Because the growth curves of lymphocytes were lowered by both radiation and folate deficiency. Apoptosis was measured using the annexin-V binding assay (Roche Molecular Biochemicals, Indianapolis, IN) that allows distinction between viable, apoptotic, and necrotic cells. Cells ( $0.5 \times 10^6$ ) were harvested 24 h after irradiation or at day 8 of different folate-deficient cultures. Cells were washed in PBS and incubated in binding buffer containing annexin-V-fluorescein and propidium iodide for 15 min at room temperature before analysis using FACSort flowcytometer.

### Single cell electrophoresis

DNA breaks were measured using the alkaline comet assay, which is a method that allows detection of both SSB and DSB<sup>(21,22)</sup>. Single cell electrophoresis (Comet assay) was performed in alkaline condition to evaluate SSB and DSB, using Trevigen Kit (Comet Assay™ ES, Catalog # 4250-050-ES) according to Instruction manual. Cells were embedded in agarose on Comet slides before irradiation and kept at 4°C to stop DNA repair, following irradiation. The unirradiated, folate-deficient

cells were harvested on day 9 of culture. Cells were washed in PBS and resuspended in PBS and low melting point agarose at a final concentration of 0.6% agarose, and 100,000 cells/ml. Cell suspension was pipetted onto a Comet slide and lysed at 4°C in the dark for 1 h in Trevigen lysing solution. DNA was allowed to unwind for 1 h at room temperature in alkaline buffer (0.3 M NaOH, 1 mM EDTA) and subjected to electrophoresis in TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA) for 30 min at 25 V. The slides were removed and stained with SYBR Green (Molecular Probes, Eugene, OR), and comet tails from 50 cells were blindly scored on a scale of 0 (low damage) to 4 (high damage) using fluorescent microscope.

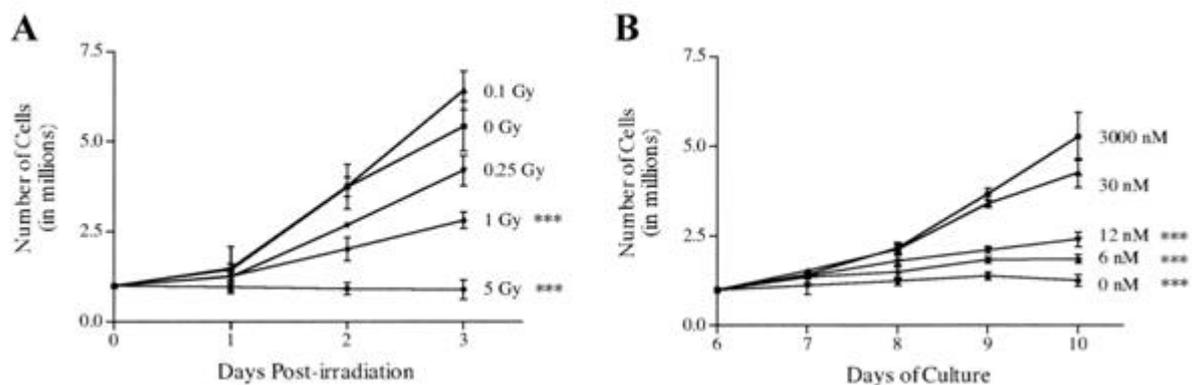
### Statistics

Each experiment was repeated three times using lymphocytes from a different donor. Mean values and SE were calculated. Linear regression and ANOVA were used to compare data within and between datasets.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Folate deficiency and high doses, but not low doses, of radiation inhibit growth of primary human lymphocytes

The growth curves for lymphocytes exposed to 1 Gy or 5 Gy were significantly lower than those exposed to 0 Gy, but the growth curves for lymphocytes exposed to the lower doses of 0.1 Gy and 0.25 Gy were not statistically different from the growth curve of unirradiated cells. Cells irradiated with 1 Gy had a proliferation rate of ~50% that of unirradiated cells ( $P < 0.001$ ); 5 Gy completely abolished lymphocyte proliferation ( $P < 0.001$ ) (Fig. 1A). Physiological concentrations of folate in human plasma are ~30 nM, whereas 12 nM is generally considered to be in the deficient range. Cells cultured in 12, 6, or 0 nM folate had decreased growth curve, relative to the growth curve of cells cultured in 3000 nM folate. The proliferation rate of cells cultured in 12 nM folate was 46% that of control cells ( $P < 0.001$ ) and 35% for cells cultured in 6 nM ( $P < 0.001$ ). Lymphocytes cultured in 0 nM folate did not proliferate ( $P < 0.001$ ) (Fig. 1B). Folate deficiency does not interfere with PHA activation; PHA-activated cells cultured in 0 nM folate for a week resume proliferating when folate is added back.



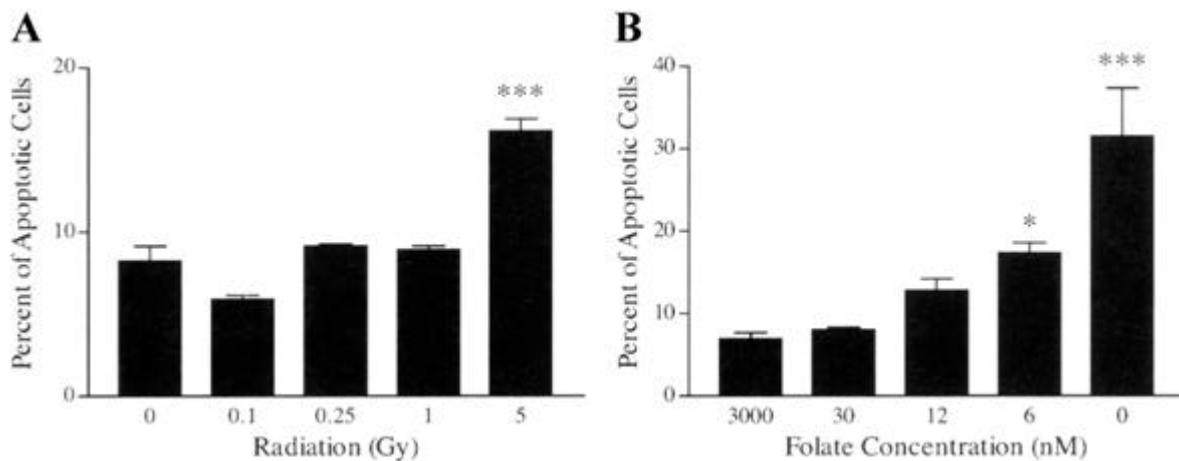
**Figure 1. Growth curves for primary human lymphocytes subjected to ionizing radiation (A) or cultured in varying folate concentrations (B).**

Culture and irradiation were carried out as described in Materials and Methods; viable cells were counted at 24 h intervals. Data are mean  $\pm$  SE of three separate experiments. \* $P < 0.05$  compared with control.

\*\*\* $P < 0.001$  compared with control; control is 0 Gy (A) and 3000 nM folate (B).

### Apoptosis is increased by both folate deficiency and high doses, but not low doses, of ionizing radiation

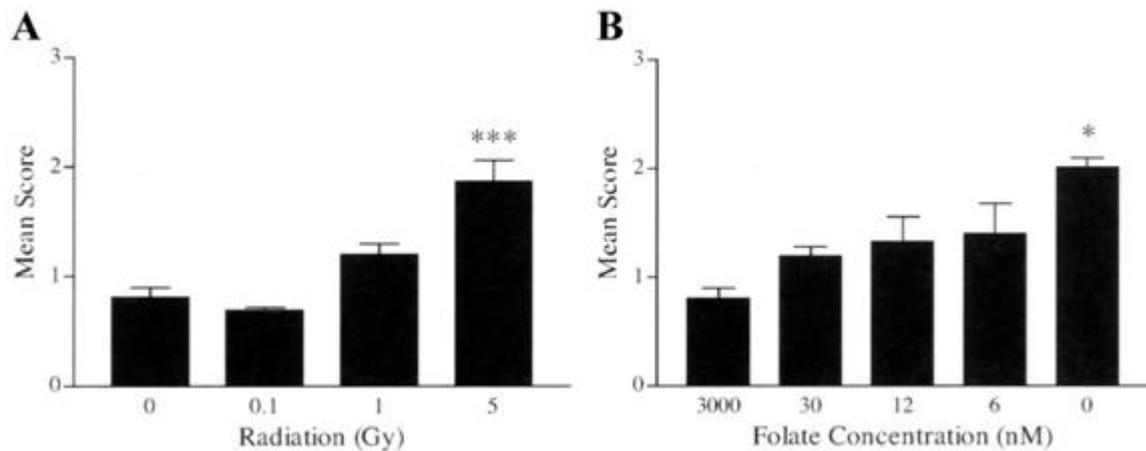
For radiation, significant increase in apoptosis was observed only at the highest dose, 5 Gy, where 16% of the cells were apoptotic, compared with 8% for unirradiated cells ( $P < 0.001$ ). Cells irradiated with 0.1, 0.25, or 1 Gy had a similar percentage of apoptotic cells as unirradiated cells. Although folate-deficient cells only showed statistically significant increases in apoptosis at 6 nM ( $P < 0.05$ ) and 0 nM ( $P < 0.001$ ), compared with non-folate-deficient cells (Fig. 2), linear regression analysis showed statistically significant dose-dependent increase in apoptosis in the folate-deficient cells relative to the folate-non deficient cells ( $P < 0.05$ ). The percentage of apoptotic cells was 7% for lymphocytes cultured in 3000 nM folate, 8% in 30 nM, 13% in 12 nM, 17% in 6 nM, and 31% in 0 nM. In this study, physiological condition of folate deficiency induced a level of apoptosis similar to that induced by 5 Gy of radiation.



**Figure 2. Induction of apoptosis 24 h after irradiation (A) or after 8 days of culture in varying folate concentrations (B).** The percentage of apoptotic cells was determined using the annexin-V binding assay and flowcytometry. Data are mean  $\pm$  SE of three separate experiments. \* $P < 0.05$  compared with control. \*\*\* $P < 0.001$  compared with control; control is 0 Gy (A) and 3000 nM folate (B).

### Both folate deficiency and ionizing radiation at relatively high doses cause DNA breaks, as measured by the Comet assay

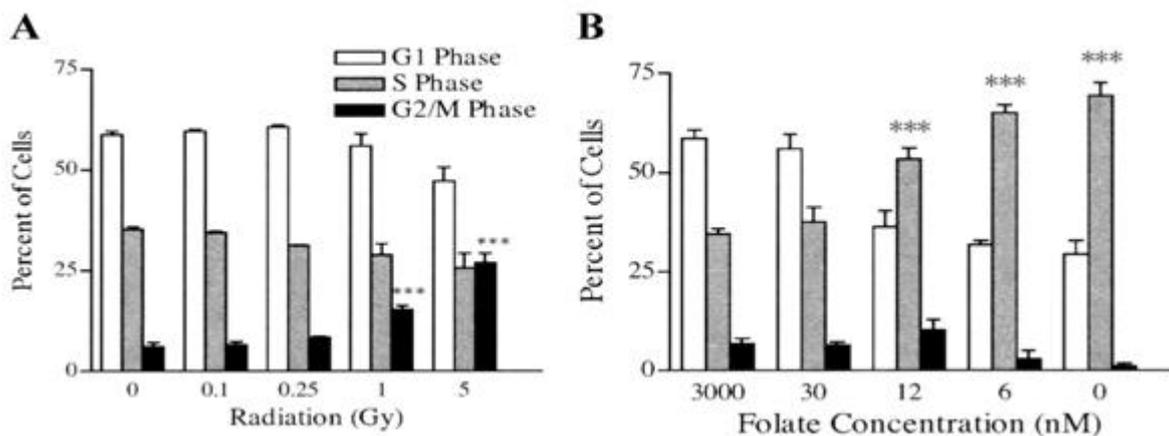
DNA damage was increased for radiation doses  $\geq 1$  Gy, although statistical significance was achieved only at 5 Gy where the Comet score was 1.87, compared with 0.81 for unirradiated cells ( $P < 0.001$ ) (Fig. 3A). A Comet score of 1.20 was obtained when lymphocytes were irradiated with 1 Gy. Similarly, cells cultured for 8 days in low folate showed an increase in DNA damage. Though complete folate deficiency (0 nM) was required to reach statistical significance ( $P < 0.05$ ) (Fig. 3B), linear regression analysis showed statistically dose-dependent trend in DNA damage ( $P < 0.05$ ). The Comet score was 0.81 for control cells (3000 nM), 1.33 for 12 nM, and 2.01 for 0 nM. For the conditions used in this study, physiological concentrations of folate deficiency induced as much DNA damage as did 1 Gy of radiation.



**Figure 3. DNA damage score from the alkaline Comet assay as described in Materials and Methods.** *A*) Cells were embedded in agarose, and then irradiated. *B*) Cells were harvested after 8 days of culture in varying folate concentrations. Data are mean  $\pm$  SE of three separate experiments. \* $P < 0.05$  compared with control \*\*\* $P < 0.001$  compared with control; control is 0 Gy (*A*) and 3000 nM folate (*B*).

**Ionizing radiation and folate deficiency differentially affect the cell cycle of lymphocytes**

Radiation doses =1 Gy caused significant arrest in the G2/M phase of the cell cycle (Fig. 4A). Unirradiated lymphocytes had 6.0% of their cells in G2/M, whereas it was 15.3% after 1 Gy of radiation ( $P < 0.001$ ) and 26.8% after 5 Gy ( $P < 0.001$ ). The G2/M arrest was still evident 72 h after irradiation. In contrast to radiation, folate deficiency caused an arrest in the S phase of the cell cycle in an apparently dose-dependent manner (Fig. 4B). The percentage of cells into the S phase was 34.5% for control cells, 53.4% for 12 nM ( $P < 0.001$ ), and 69.4% for 0 nM ( $P < 0.001$ ).



**Figure 4. Cell cycle distribution was measured by propidium iodine staining and flow cytometry.** *A*) Cells were harvested 24 h after irradiation. \*\*\* $P < 0.001$  for the G2/M phase compared with 0 Gy. *B*) Cells were harvested after 8 days of culture in varying folate concentrations. \*\*\* $P < 0.001$  for the S phase compared with 3000 nM. Data are mean  $\pm$  SE of three separate experiments.

## DISCUSSION

In the current study, it was sought to compare the ability to damage DNA of an exogenous source of stress (ionizing radiation) and an endogenous source (folate deficiency). The results suggest that physiologically relevant levels of folate deficiency cause more DNA breaks, induce more apoptosis, and halt the proliferation of normal human lymphocytes in primary culture more than acute exposure to relatively high doses of radiation.

The population is generally exposed to only very low doses of radiation. Because risk estimates for low doses are hard to assess, they are often extrapolated from effects observed at higher doses. The usefulness of this linear model has been debated because it may over estimate cancer risks<sup>(15, 16, 20)</sup>. Other models have been proposed in which low-dose radiation is harmless under certain threshold, or may even induce a radio-adaptive response<sup>(14,16, 18)</sup>. On the other hand, vitamin and mineral deficiencies are common in the population and may contribute to much of preventable cancer<sup>(4)</sup>. Folate deficiency leads to decreased thymidine and purine availability, which may inhibit DNA synthesis and consequently cell proliferation<sup>(19,20)</sup>.

Ionizing radiation and folate deficiency can both produce DSB by different mechanisms. Ionizing radiation can form DSB either directly or indirectly by forming clusters of oxidative damage, which can be converted to DNA breaks during repair<sup>(2, 6)</sup>. Folate deficiency, however, causes an imbalance in the thymidine/uracil ratio, which results in uracil being incorporated into DNA during synthesis. The repair of two nearby opposing lesions can cause DSB<sup>(18-20)</sup>. DNA damage after irradiation and during folate deficiency was measured using the Comet assay, an assay that has been widely used for both radiation exposure and nutritional studies<sup>(21, 22)</sup>. Because radiation and folate deficiency cause DNA breaks in a different fashion, the alkaline Comet assay<sup>(21)</sup> was used to simultaneously measure SSB and DSB, therefore allowing one comparison between the two risks. It would also be of interest to measure only DSB. DSB are often considered to be the most dangerous DNA lesions, although SSB can be converted to DSB when locations of damage (SSB and/or base lesions) are clustered. Lymphocytes that were irradiated with 5 Gy were virtually all damaged, as were lymphocytes cultured in 0 nM folate, and both Comet scores were increased by ~2.5-fold compared with control cells. These results are similar to those of experiments in which lymphocytes were either irradiated<sup>(23)</sup> or cultured for 8 days in folate-deficient medium<sup>(24)</sup>.

The cells cultured in a physiological level of folate deficiency (12 nM) sustained damage equivalent to that of lymphocytes irradiated with 1 Gy, which is a relatively high dose of radiation. Similar results were obtained using other techniques to measure DNA breaks. Fenech and Crott<sup>(25)</sup> have also investigated DNA damage resulting from folate deficiency and ionizing radiation in human lymphocytes using the cytokinesis-block micronuclei assay to measure DSB. and found that folate deficiency (12 nM) caused a fourfold increase in micronuclei frequency, as did 0.4 Gy of radiation, although the two independent experiments may not be directly comparable. Branda et al.<sup>(20)</sup> used another cell type and the alkaline filter elution assay to measure SSB and DSB. They found that folate-deficient Chinese hamster ovary cells manifested strand breaks equivalent to a dose of 0.3 Gy.

In addition to increased DNA damage, radiation and folate deficiency had other cellular effects. The proliferation rate was reduced to ~50% by 1 Gy and to nearly 0% by 5 Gy of radiation. Proliferation rate was not reduced by 0.25 Gy and was slightly increased by 0.1 Gy of radiation. Activation of proliferation after low dose of radiation has been observed for some cancer cells during radiotherapy and also, but more rarely, for normal cells<sup>(23-26)</sup>. Similarly for folate deficiency, the proliferation rate was reduced to 46% when cells were cultured in 12 nM folate, whereas cells cultured in the absence of folate totally stopped proliferation. The lower proliferation rate after irradiation and during folate deficiency was due to both increased apoptosis and cell cycle arrest. Irradiated cells are known to stop in G<sub>2</sub>/M because DNA damage will activate this checkpoint and prevent cells from undergoing mitosis<sup>(23)</sup>. In this study, significant arrest in G<sub>2</sub>/M was observed after 1 and 5 Gy of

radiation; the arrest lasted up to 72 h post-irradiation (data not shown). In contrast, folate deficiency induced cell cycle arrest in the S phase. A prominent arrest in S phase was observed using physiological level (12 nM) of folate deficiency. When lymphocytes were cultured in the absence of folate (0 nM), most cells were arrested in the S phase, and no cells were detected in the mitotic phase (G2/M). The S-phase arrest during folate deficiency has been observed with various cell types<sup>(14,24-26)</sup> and is likely due to impaired DNA synthesis, because folate is required for both purine and thymidylate synthesis. The S-phase arrest is reversible, and cell proliferation quickly resumes when folate (or a nucleotide mix) was added back to the folate-deficient cells<sup>(19)</sup>.

The proportion of lymphocytes undergoing apoptosis increased with the radiation dose and the level of folate deficiency. Cells irradiated with 5 Gy were highly damaged; therefore, apoptosis pathway was probably activated because the cells were unable to repair their DNA. Similar proportion of apoptotic cells were measured when lymphocytes were cultured in low concentrations of folate (6, 12 nM). In absence of folate (0 nM), almost one third of the lymphocytes were apoptotic. Huang et al. has suggested that folate deficiency may block DNA replication and/or mitosis, which may be involved in apoptosis induction<sup>(26)</sup>. In support of this hypothesis, two anti-folates, 5-fluorouracil (inhibitor of thymidylate synthase) and methotrexate (inhibitor of dihydrofolate reductase), have been shown to induce apoptosis in association with inhibition of DNA synthesis<sup>(27)</sup>.

Results of the present study showed that physiological levels of folate deficiency caused more DNA damage than low-dose radiation (=0.1 Gy) in primary T lymphocytes. Because this study was done using cultured cells, results cannot be directly extrapolated to estimate cancer risks. Human exposure to chronic low doses of radiation is likely to have different outcome than acute cell exposure. Interestingly, low doses of radiation can induce radio-adaptive response, therefore protecting the cells from damage induced by subsequent irradiation<sup>(11,12,28)</sup>. This adaptive response has not been observed for folate deficiency. Folate deficiency is known to cause DSB<sup>(16, 18, 19)</sup>, but DSB recognition and repair genes are not activated. Moreover, folate deficiency has been shown to impair DNA excision repair and mismatch repair<sup>(15, 18, 28)</sup>. This further suggests that folate deficiency may be more detrimental than low doses of radiation. In addition, dietary folate deficiency is systemic and also causes damage to sperm DNA<sup>(29)</sup>. Note that the present study did not address the issue of whether low doses of radiation are harmful to human health (for a review of low-dose radiation effects<sup>(see ref 30)</sup>); rather, the current study found that low doses of radiation had no measurable effects in comparison with conditions of folate deficiency. Although further investigation is needed, these findings suggest that exposure to very low doses of radiation from background or occupational exposure may pose a smaller cancer risk than consuming poor diet. Many vitamins and minerals are important for DNA integrity. Additional studies comparing the gene Expression changes and different cellular responses of ionizing radiation with deficiency in a vitamin such as folate may better elucidate the relative contributions of these two DNA damage exposures, and put both in perspective for radiation exposure standards, and for the determination of optimal vitamin requirements for DNA integrity.

## CONCLUSION

Results suggest that physiological levels of folate deficiency cause as much DNA damage as relatively high levels of ionizing radiation, but with a different cellular response. More importantly, these results suggest that research on the biological effects of low-dose radiation, in humans, should take into account the nutritional status of the subjects, because folate (and other vitamins and minerals) deficiency could confound the effects of low-dose radiation or could even have a synergistic effect and increase the sensitivity of cells to radiation .

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