Apoptosis of Late-Stage Erythroblasts in Megaloblastic Anemia: Association With DNA Damage and Macrocyte Production

By Mark J. Koury, Donald W. Horne, Zoe A. Brown, Jennifer A. Pietenpol, Benjamin C. Blount, Bruce N. Ames, Robert Hard, and Stephen T. Koury

Megaloblastic anemia is caused by deficiency of folate or cobalamin (vitamin B12). The peripheral blood of patients with megaloblastic anemia is characterized by pancytopenia and an increased erythrocyte mean corpuscular volume (MCV). The bone marrow has a prevalence of large, early stage hematopoietic precursor cells. Cytogenetic studies have shown increased chromosomal breakage in the bone marrow cells of patients with megaloblastic anemia. Increased bilirubin and lactate dehydrogenase in the serum results from the destruction of erythroid precursor cells in the bone marrow. A murine form of megaloblastic anemia can be induced by feeding mice a folate-free, amino acid-based diet. An in vitro system of folate-deficient erythropoiesis was developed by culturing proerythroblasts isolated from the spleens of mice that were fed the folate-free diet during the acute erythroblastosis phase of the disease caused by Friend leukemia virus. In folate-deficient medium, these erythroblasts undergo apoptosis, whereas in medium containing folic acid, they survive and differentiate into reticulocytes within 2 days. Thymidine added to the folate-deficient medium inhibits the apoptosis. Therefore, in the folate-deficient cells, we hypothesize that decreased levels of the coenzyme 5,10-methylene tetrahydrofolate inhibits the conversion of deoxyuridylate (dUMP) to thymidylate (dTMP). The resultant increased ratio of dUMP to dTMP leads to an increased rate of uracil misincorporation into DNA. This misincorporated uracil can be removed and the DNA repaired. However, the repair of misincorporated uracil residues that are located near each other on opposing strands of DNA has the potential to produce double-stranded breakage of the DNA.

DNA damage induced by irradiation and chemotherapeutic agents has been associated with accumulation of p53. A transcriptional factor with tumor-suppressor activity. When p53 is induced by DNA-damaging agents, the protein product of one of its target genes, p21CIP1/WAF1 (p21), also accumulates. p21 inhibits cell cycle progression and is the major effector for the arrest of cell cycle that has been associated with p53 accumulation. Although this inhibition of the cell cycle by p53 has been thought to contribute to its tumor-suppressor function, accumulation of p53 in hematopoietic cells has also been associated with death by apoptosis. We show here that (1) erythroblasts undergoing apoptosis due to intracellular folate deficiency have increased uracil misincorporation into DNA and increased p53 and p21 proteins and (2) the erythroblasts that survive this apoptotic process give rise to larger than normal reticulocytes.
same diet with the addition of 2 mg folic acid per kilogram of diet. After 2 weeks on the respective diets, the mice were infected with 10^3 spleen focus-forming units of the anemia-inducing strain of Friend leukemia virus as previously described. After 2 weeks of infection and 4 weeks of each respective diet, the mice were killed and their spleens, which had been enlarged by the virus-induced erythrophagocytosis, were removed. For the in vivo determination of macrocytosis induced by folate deficiency, 8-month-old female CD1mice were fed either control or folate-deficiency diet for a period of 9 weeks. Blood samples taken at 0, 5, 7, and 9 weeks were diluted in phosphate-buffered saline (PBS) with 2 mM EDTA as described previously. The blood samples were then analyzed with a Technicon H-1 automated blood cell counter (Bayer Corp., Tarrytown, NY) that was programmed with software to count murine cells. After 9 weeks, mice from each group were killed and the total folate content of their livers was determined.

**In vitro system of folate-deficient erythropoiesis.** Single-cell suspensions of erythroblasts were prepared by staining the splenic contents through nylon mesh. Purified populations of proerythroblasts from both folate-deficient and control mice were isolated by velocity sedimentation at unit gravity, as described previously. Amide gel electrophoresis (SDS-PAGE) using 10% gels for p53 and p21 proteins from each group were killed and the total folate content of Calbiochem-Novabiochem, La Jolla, CA). Cell lysates were centrifuged at 13,000g for 15 minutes. The supernatant was collected and protein concentration determined by the method of Bradford. Fifty micrograms of cellular protein was mixed with an equal volume of 2× Laemmli sample buffer and boiled at 100°C for 8 minutes. Cellular proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels for p53 and 12% gels for p21 and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with a 1:100 dilution of either anti-p53 Ab-3 or anti-p21 WAF1 Ab-2 (Oncogene Research Products, Calbiochem, Cambridge, MA), followed by incubation with a 1:20,000 dilution of secondary antibody, goat antimouse IgG horseradish peroxidase-conjugate (Pierce, Rockford, IL). Detection was enhanced by chemiluminescence (ECL Western blotting detection system; Amersham, Arlington Heights, IL).

**Determination of number and size of reticulocytes formed in vitro.** Erythroblasts were harvested at 8-hour intervals beginning at 16 hours of culture. Cells from triplicate cultures were cytocentrifuged and stained with 3,3′-diaminobenzidine and hematoxylin, coded, and counted in a blinded manner for reticulocytes. Thus, cultured erythroblasts could be classified into four groups depending on the diet of the mice from which they were isolated and the folate content of their culture medium. These groups were (1) control cells in control medium, (2) control cells in folate-deficient medium, (3) folate-deficient cells in control medium, and (4) folate-deficient cells in folate-deficient medium. At various times of culture, the erythroblasts were collected and analyzed for folate content, uracil misincorporation into DNA, accumulation of wild-type p53 and p21 proteins, and the number and size of newly formed reticulocytes.

**Determination of folate content and coenzyme distributions.** The harvested cells were washed twice in ice-cold PBS and the number of viable cells was determined by trypan blue dye exclusion. The pelleted erythroblasts were lysed by heating to 100°C for 5 minutes in folate coenzyme extraction buffer [0.2 mol/L β-mercaptoethanol, 2% (wt/vol) sodium ascorbate, 50 mmol/L HEPES, 50 mmol/L 2-(N-cyclohexylamino) ethanesulfonic acid [pH 7.85]] and treated with rat plasma conjugase to hydrolyze folylpolyglutamates. The extracted folate coenzymes were separated on a Beckman Ultrasphere (Beckman Instruments Inc., Fullerton, CA) C18 I.P. column. The folate content of unseparated samples of each of the separated fractions was determined with a Lactobacillus casei microbiologic assay as described previously. For determination of total folate content in livers, the hepatic tissue was homogenized in the same extraction buffer and assayed with the same microbiologic assay, and results expressed as nanomoles of folate per gram of liver.

**Determination of uracil content of DNA.** The harvested erythroblasts were pelleted and lysed in DNA extraction buffer (10 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 8.0], 20 mmol/L EDTA, 0.5% sodium dodecyl sulfate) with 200 μg proteinase K/mL. After incubation at 37°C for 24 hours, the DNA was extracted with phenol-chloroform and purified as described previously. The purified DNA was precipitated with ethanol and resuspended in 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA. The DNA was quantitated by measuring fluorescence of an aliquot stained with ethidium bromide. The uracil content was determined as described previously. Briefly, uracil residues were removed from the DNA with uracil-DNA glycosylase, derivatized with 3,5-bis(trifluoromethyl)benzyl bromide, and analyzed by gas chromatography-mass spectrometry.

**Determination of p53 and p21 protein accumulations.** The harvested erythroblasts were washed twice with ice-cold PBS and then lysed on ice for 30 minutes in a 0.5% Nonidet P-40 lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and protease inhibitors antipain (10 μg/mL), leupeptin (10 μg/mL), peptatin A (10 μg/mL), and chymostatin (10 μg/mL; Sigma, St Louis, MO) and 4-(2-aminoethyl)-benzenesulfonyl fluoride (200 μg/mL; Calbiochem-Novabiochem, La Jolla, CA). Cell lysates were centrifuged at 13,000g for 15 minutes. The supernatant was collected and protein content determined by the method of Bradford. Fifty micrograms of cellular protein was mixed with an equal volume of 2× Laemmli sample buffer and boiled at 100°C for 8 minutes. Cellular proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels for p53 and 12% gels for p21 and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with a 1:100 dilution of either anti-p53 Ab-3 or anti-p21 WAF1 Ab-2 (Oncogene Research Products, Calbiochem, Cambridge, MA), followed by incubation with a 1:20,000 dilution of secondary antibody, goat antimouse IgG horseradish peroxidase-conjugate (Pierce, Rockford, IL). Detection was enhanced by chemiluminescence (ECL Western blotting detection system; Amersham, Arlington Heights, IL).

**Determination of number and size of reticulocytes formed in vitro.** Erythroblasts were harvested at 8-hour intervals beginning at 16 hours of culture. Cells from triplicate cultures were cytocentrifuged and stained with 3,3′-diaminobenzidine and hematoxylin, coded, and counted in a blinded manner for reticulocytes, free nuclei, and nucleated cells as described previously. All counts were performed on 300 consecutive nuclei encountered in successive 100× microscopic fields. The same slides were then analyzed for sizes of reticulocytes by computer-assisted microscopic analysis of reticulocyte profile areas. Thirty consecutive reticulocytes were analyzed for each slide.

Statistical comparisons were performed using GraphPAD (Graph-PAD Software Inc, San Diego, CA) InStat using the Student’s t-test or one-way ANOVA with Tukey’s Multiple Comparison Test. P < .05 was considered significant.

**RESULTS**

At various times of culture, the total folate content (Fig 1) and the distribution of folate coenzymes (Fig 2) were determined in proerythroblasts from control and folate-deficient mice. At the time of their isolation from the spleens at 0 hours in Fig 1A and B), the proerythroblasts from folate-deficient mice had about one-tenth of the total folate content as those proerythroblasts isolated from control mice. During culture in folate-deficient medium, the proerythroblasts from folate-deficient mice had persistently low total folate content (Fig 1B) Culturing the folate-deficient proerythroblasts in folate-deficient medium repleted the total folate content (Fig 1B) such that at 32 and 44 hours of culture (Fig 1B) the total folate content was similar to erythroblasts from control mice cultured in control medium (Fig 1A). When proerythroblasts from control mice were cultured in folate-deficient medium, they had decreased total folate content as compared with when they were cultured in medium containing folic acid.
Fig 1. Total folate content of erythroblasts in culture. Control (A) and folate-deficient (B) erythroblasts were cultured in either folate-deficient (□) or control medium (■) and harvested at the times indicated. The total folate concentration was determined by Lactobacillus casei bioassay. Data are the means ± SEM of three experiments. *P < .02 (A) or P < .002 (B) when folate levels are compared between folate-deficient medium and control medium at the respective hours of culture.

(Fig 1A). The decline over time in the total folate per cell in cultures of control cells in Fig 1A is due to the marked decrease in cell size that occurs during erythroid differentiation.33,34 However, throughout the 44-hour culture period in folate-deficient medium, the control erythroblasts maintained a total folate content that was greater than their counterparts from folate-deficient mice cultured in folate-deficient medium (Fig 1). Also, these control erythroblasts completed their differentiation into reticulocytes in folate-deficient medium without undergoing apoptosis.

Because folate-deficient erythroblasts begin to undergo apoptosis at 20 hours of culture in folate-deficient medium,8 the distribution of folate coenzymes was examined at the time of their isolation (0 hours) and after 20 hours of culture. When compared with control erythroblasts, the folate-deficient cells at 0 hours had similar percentages of tetrahydrofolate and 5-formyltetrahydrofolate, an increased percentage of 5-methyltetrahydrofolate (15.3 ± 1.0 v 7.3 ± 0.7 [mean ± 1 SEM]) and a decreased percentage of 10-formyltetrahydrofolate (21.4 ± 2.4 v 34.3 ± 1.9). This pattern of coenzyme distribution in folate-deficient cells persisted after 20 hours of culture in folate-deficient medium. However, after 20 hours of culture in control medium, the folate-deficient erythroblasts had similar distribution of coenzymes as did control erythroblasts in either folate-deficient or control medium. Although no single folate coenzyme form became totally depleted in folate-deficient erythroblasts that were cultured in folate-deficient medium, the persistence of decreased absolute levels of all folate coenzymes in these cells leads to critical intracellular changes that can induce apoptosis.

Although all of the folate coenzymes were decreased in the folate-deficient erythroblasts, the inhibition of their apoptosis by the addition of thymidine to the folate-deficient medium indicated that decreased 5,10-methylenetetrahydrofolate played an important role in the fate of folate-deficient erythroblasts.8 In the conversion of dUMP to dTMP, 5,10-methylenetetrahydrofolate provides a methylene group and reducing equivalents. Decreased levels of 5,10-methylenetetrahydrofolate results in an increased ratio of dUMP/dTMP and ultimately to increased rates of uracil misincorporation into DNA. The steady-state level of misincorporated uracil in DNA was measured in both control and folate-deficient erythroblasts during culture in either control or folate-deficient medium (Fig 2). The freshly isolated proerythroblasts from normal and folate-deficient mice had similar levels of uracil misincorporation in DNA. Erythroblasts from control mice had similar levels of uracil misincorporation throughout their culture in either control medium or folate-deficient medium (Fig 2A). When folate-deficient erythroblasts were cultured in folate-deficient medium, they had significantly increased uracil misincorporation in DNA as compared with when they were cultured in the control me-

Fig 2. Uracil misincorporation into DNA in cultured erythroblasts. Control (A) or folate-deficient (B) erythroblasts were cultured in folate-deficient (□) or control medium (■). DNA was extracted from cells collected at various times. The uracils were removed from the DNA with uracil-DNA glycosylase, derivatized with 3,5-bis(trifluoromethyl)benzyl bromide, analyzed by gas chromatography-mass spectrometry, and expressed per 10^6 thymines in the DNA sample. Results are the means ± 1 SEM of 7 to 17 separate samples. *P < .05 in steady-state levels of uracil in DNA in folate-deficient erythroblasts cultured in folate-deficient as compared with control medium.
of intracellular p53 and p21 protein accumulation and, blasts isolated from control mice. Despite this 10-fold differ-

ence in folate levels between folate-deficient and control erythroblasts. When erythroblasts were cultured in folate-deficient medium as opposed to control medium (Fig 3). Thus, the minority of folate-deficient erythroblasts cultured in control medium or from control proerythroblasts cultured in either control or folate-deficient medium (Fig 4B). Thus, the minority of folate-deficient erythroblasts that were cultured in folate-deficient medium and that were able to survive the in vitro apoptosis produced significantly larger reticulocytes than did their counterparts cultured in control medium or control erythroblasts cultured in either control or folate-deficient medium.

The one characteristic of megaloblastic anemia not noted in our previous in vivo murine study using the folate-deficient diet was erythrocyte macrocytosis. In those studies, weaning mice were fed the folate-free diet for only 6 weeks before being refed folate. The weaning mice have a greater body growth than adults and they have a greater folate requirement for survival. Many weaning mice cannot survive 8 weeks on the folate-free diet. However, if the adult mice are fed the folate-free diet, they all survive for more than 10 weeks and their anemia is accompanied by macrocytosis. In Fig 5, adult mice fed the folate-deficient diet for 9 weeks had an increased MCV that accompanied their development of anemia. After 9 weeks, mice fed the folate-free diet had a mean ± 1 SEM of 7.7 ± 0.4 nmol folate/g of liver, whereas mice fed the control diet had 36.6 ± 2.2 nmol folate/g of liver (P < .001).

**DISCUSSION**

Proerythroblasts isolated from folate-deficient mice had about one-tenth the total folate content as did proerythroblasts isolated from control mice. Despite this 10-fold difference in folate levels between folate-deficient and control proerythroblasts, the uracil in DNA, the p53 and p21 protein content, and the percentage of apoptotic cells were the same in both populations of proerythroblasts. These results indicate that irreversible changes in the folate-deficient erythroblasts had not yet occurred and that correction of the intracellular folate deficiency could prevent the death of the erythroblasts in culture. Indeed, if folic acid is provided in the medium during the first 8 hours of culture, the folate-
deficient cells can be rescued and complete normal differentiation into reticulocytes. If the provision of folic acid is delayed until 20 hours of culture, only two-thirds of the cells that would undergo apoptosis can be rescued. Equal amounts of uracil in DNA are found in folate-deficient and control proerythroblasts when they are first isolated (Fig 2), but when the folate-deficient proerythroblasts are cultured in folate-deficient medium, uracil misincorporation in DNA increased significantly when compared with culture in control medium. At 32 through 44 hours of culture in folate-deficient medium, p53 protein accumulation persisted at high levels in folate-deficient cells and p21 protein progressively accumulated (Fig 3). The most prominent apoptosis in these cells occurs at 32 hours and later times of culture. The 32-hour period of culture corresponds to the basophilic and polychromatophilic stages of erythroblast differentiation in the bone marrow. Thus, these late stages of erythroid differentiation appear to be especially susceptible to apoptosis due to folate deficiency. At the late basophilic and early polychromatophilic stages of their development, the erythroblasts begin to accumulate hemoglobin and they undergo a dramatic reduction in size. They have completed one division in vitro and have one remaining division before enucleating. Our previous in vivo results with mice fed the folate-free diet

![Image](https://via.placeholder.com/150)

**Fig 4.** The number of reticulocytes (A) and reticulocyte profile sizes (B) at various times during the in vitro differentiation of control and folate-deficient proerythroblasts. Cytocentrifuge preparations were made of control proerythroblasts cultured in control medium ( ), control proerythroblasts cultured in folate-deficient medium ( ), folate-deficient proerythroblasts cultured in control medium ( ), or folate-deficient cells in folate-deficient medium ( ). Reticulocytes were counted in a blinded manner at 100 magnification. Reticulocyte sizes were determined by a computerized calculation of light microscopic images. For each culture, determination of reticulocyte numbers was made by counting consecutive microscopic fields until 300 nuclei (free or intracellular) were counted. For each culture, the determination of reticulocyte size was made from 30 consecutive reticulocytes. All data are the means ± 1 SEM of three separate cultures. *P < .05 when data from folate-deficient cells cultured in folate-deficient medium are compared with all other treatments.

![Image](https://via.placeholder.com/150)

**Fig 5.** The hematocrit and MCV changes induced by folate deficiency in adult mice. Mice were fed either control or folate-free diet for 9 weeks. Blood cell counts were determined using an automated counter that was programmed for murine blood cells. All data are the means ± 1 SEM of 10 mice. *P < .001 when comparing folate-deficient mice with control mice at the weeks shown.
until they developed megaloblastic anemia are consistent with the major proportion of apoptosis occurring at the basophilic-polychromatophilic stages of development. When these folate-deficient mice became pancytopenic after 6 weeks of the folate-free diet, they had (1) increased numbers of colony forming units-erythroid (CFU-E), (2) decreased numbers of reticulocytes, and (3) many immature cells but very few late stage erythroblasts in their bone marrow. Ferrokinetic studies were also consistent with the loss of cells at the late stages of erythroid development when the erythroblasts had begun to synthesize hemoglobin.

Our results show that nutritional folate deficiency can lead to changes in DNA, increased levels of p53 and p21 proteins, and apoptosis in Friend virus-infected erythroblasts. Partial differentiation of erythroleukemia cells in response to chemical inducers has been associated with p53-independent increases in p21 protein and suggests a role for p21 in erythroid differentiation. Our results in Fig 3 are consistent with this differentiation-related function of p21. The late-stage accumulation of p21 protein in folate-deficient erythroblasts, which is most prominent at 44 hours, may be due to altered nucleotide pools, or relative persistence of undifferentiated cells at this time. Alternatively, the increased p21 protein may be due to a p53-dependent mechanism. However, p53-null mice can develop megaloblastic anemia (Koury et al, unpublished data). The p53 accumulation in folate-deficient erythroblasts occurs temporally with the p21 accumulation, which, in turn, may inhibit cell cycle progression. This arrest of cell cycle in folate-deficient erythroblasts may prevent their apoptosis and would be consistent with the previous studies showing an antiapoptotic effect of p21 protein.

Our in vitro data in Fig 4 indicates that macrocytosis related to folate-deficient erythroblasts is determined during the late stages of erythroblast development. Folate deficiency may lead to increased reticulocyte size by several mechanisms. One is that folate deficiency may select for the erythroblasts that normally produce the largest reticulocytes. If the erythroblast apoptosis of folate deficiency specifically spares the few percent of erythroblasts that normally will produce the largest reticulocytes, then the mean reticulocyte size will be significantly increased. A second mechanism is that folate deficiency inhibits the normal decrease in erythroblast size that occurs during the terminal two divisions of erythropoiesis. A third mechanism is that, with folate deficiency, the few erythroblasts that do not undergo apoptosis have successfully repaired DNA damage during our results show that nutritional folate deficiency can lead to changes in DNA, increased levels of p53 and p21 proteins, and apoptosis in Friend virus-infected erythroblasts. Partial differentiation of erythroleukemia cells in response to chemical inducers has been associated with p53-independent increases in p21 protein and suggests a role for p21 in erythroid differentiation. Our results in Fig 3 are consistent with this differentiation-related function of p21. The late-stage accumulation of p21 protein in folate-deficient erythroblasts, which is most prominent at 44 hours, may be due to altered nucleotide pools, or relative persistence of undifferentiated cells at this time. Alternatively, the increased p21 protein may be due to a p53-dependent mechanism. However, p53-null mice can develop megaloblastic anemia (Koury et al, unpublished data). The p53 accumulation in folate-deficient erythroblasts occurs temporally with the p21 accumulation, which, in turn, may inhibit cell cycle progression. This arrest of cell cycle in folate-deficient erythroblasts may prevent their apoptosis and would be consistent with the previous studies showing an antiapoptotic effect of p21 protein.

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CFU-E, and proerythroblasts and thereby further shifts the erythropoietic population toward these earlier stages. This shift in the population toward immature erythropoietic progenitor cells is characteristic of megaloblastic hematopoietic tissue. Although the erythroid lineage is shown in this model, the clinical findings of pancytopenia and our murine studies showing expansion of myeloid and megakaryocytic progenitor cells in folate-deficient mice indicate that nonerythroid hematopoietic lineages are similarly affected during megaloblastic anemia.

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