Rescue of Embryonic Lethality in Reduced Folate Carrier-deficient Mice by Maternal Folic Acid Supplementation Reveals Early Neonatal Failure of Hematopoietic Organs*

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The reduced folate carrier (RFC1) is an important route by which the major blood folate, 5-methyltetrahydrofolate, is transported into mammalian cells. In this study we determined the consequences of inactivation of RFC1 in mice by homologous recombination. While RFC1-null embryos died in utero before embryonic day 9.5 (E9.5), near-normal development could be sustained in RFC1+/− embryos examined at E18.5 by supplementation of pregnant RFC1+/− dams with 1-mg daily subcutaneous doses of folic acid. About 10% of these animals went on to live birth but died within 12 days. These RFC1+/− mice showed a marked absence of erythropoiesis in bone marrow, spleen, and liver along with lymphoid depletion in the splenic white pulp and thymus. In addition, there was some impairment of renal and seminiferous tubule development. These data indicate that in the absence of RFC1 function, neonatal animals die due to failure of hematopoietic organs.

Folate cofactors play a key role in the de novo synthesis of purines and pyrimidines, and folate deficiency has been associated with megaloblastic anemia (1), neural tube defects (2, 3), cardiovascular disease (4, 5), and predisposition to cancer (6). Hence, folate absorption and delivery to folate-requiring peripheral tissues is essential to health. Folate transport in mammalian cells is mediated by a number of different processes. The reduced folate carrier (RFC1) is a facilitative union exchange that mediates folate delivery into a variety of cells of different origin (7, 8). RFC1 has a much higher affinity for reduced folates, including the physiological substrate 5-methyltetrahydrofolate (−2 μM), than folic acid (−200 μM) (9). Folate receptors (α and β), which anchor to cell membranes through a glycosylphosphatidylinositol moiety, transport folates via an endocytic process (10, 11). These receptors have a very high affinity for folic acid (−1 nM) and a lesser, but still high affinity, for 5-methyltetrahydrofolate (12, 13). In addition, there are at least two other folate transport systems in murine leukemia cells, but their molecular basis has not been identified. One mechanism operates optimally at low pH (14, 15) with comparable affinities for MTX, folic acid, and reduced folates and properties similar to the folate transport process in intestine (16). Another poorly characterized system has been proposed as a transporter for folic acid (17).

Recently, two of the folate receptors (α and β) were inactivated in the mouse. Loss of folate receptor-α resulting in early embryonic death associated with a failure of neural tube closure. However, receptor-α null animals could be sustained through day 18 of gestation by oral intubation with administration of 5-formyltetrahydrofolate, but live birth of these animals was not reported (18). Loss of folate receptor-β was not associated with a pathological phenotype (18). In this paper, we examine the consequences of the targeted disruption of the other major folate transporter, RFC1, in particular, the defects that occur during embryonic development and in the early neonatal period. This carrier is of particular importance, since (i) RFC1 is a very efficient transporter; the cycling rate of RFC1 is 2 orders of magnitude faster than that of folate receptor-α (19). (ii) The tissue distribution pattern of RFC1 in normal tissues is different, in part, from that of folate receptor-α. RFC1 is expressed in placenta, liver, lung, and small intestine (20, 21), whereas folate receptor-α has been detected in choroid plexus, lung, thyroid, kidney, breast, placenta, ovary, and testis (22, 23). (iii) RFC1 and folate receptor-α may localize to different regions in polarized cells, i.e. apical versus basolateral membranes, as demonstrated in mammalian retinal pigment epithelium (24). Hence, these spatial relationships may play a role in the vectorial transport of folates. The results of the current study shed further light on the critical role that RFC1 plays in embryonic development and in meeting folate demands in hematopoietic tissues.

MATERIALS AND METHODS

Construction of RFC1 Targeting Vector—A mouse genomic Charon 35, 129/ola phage library was screened with a PatI/NarI fragment (662 base pairs) of exon 4 (25). Three positive clones were identified; one contained the exons 3–6 (25). HindIII fragments of this clone were subcloned into plasmid Bluescript (Strategene). The targeting construct was generated by a two-step insertion of HindIII fragments into plasmid PKGneo. A 1.3-kb fragment containing only a small part of exon 3 was treated with Klenow polymerase and cloned into an EcoRI site, whereas a 7-kb fragment containing exons 4–6 was inserted into a HindIII site of PKGneo.

Electroporation of Embryonic Stem Cells—The targeting vector was linearized at the single XhoI site and electroporated into 2.0 × 10⁷ WW 6 embryonic stem cells (26). Neomycin (150 μg/ml)-resistant clones were screened by PCR using a forward primer (p1) specific for the upstream RFC1 gene, 5′-CAGGACAGAACATGTGAGACAGAC-3′; a reverse primer (p2) specific for the downstream RFC1 gene, 5′-CTGCTCCTTGGTTGAAATTAC-3′; and a reverse primer (p3) specific for the neomycin cassette in PKGneo, 5′-GCCAGAAGCGAAGGACAGAAC-3′.
Positive clones were identified by a 1.6-kb PCR fragment specific for the disrupted RFC1 and a 1.3-kb fragment specific for the wild-type RFC1 gene. Two positive clones, RFC1–99 and RFC1–113, were identified from a total number of 49 clones. The correct targeting event was also shown by SacI digestion of high molecular weight DNA and Southern blot analysis using the PstI/Nor1 fragment directed at exon 4 of RFC1.

**Generation of Mice with the RFC1 Mutation—**Chimeric mice were generated by injecting C57BL/6 blastocysts with 8–12 embryonic stem cells derived from RFC1–99 and RFC1–113 clones. RFC1–99 gave rise to a male chimeric animal, while RFC1–113 produced a female chimera. Both mice transmitted the RFC1 mutation through the germ line. Heterozygous mice were maintained, and mating was initiated to generate homozygous mutants.

**Western Blot Analysis and Measurement of MTX Influx—**Primary embryonic fibroblasts were established from 15.5-day fetuses and cultured in Dulbecco’s modified Eagle’s medium using a standard protocol (27). The fibroblast cells were trypsinized, washed twice with cold PBS, and sonicated in cold PBS supplemented with a proteinase inhibitor mixture (P8340, Sigma, 10% (v/v)). Total lysates (10 μg) were treated with dithiothreitol-free loading buffer at room temperature and resolved on a 12% SDS-polyacrylamide gel. Subsequent blotting and detection were performed according to the ECL Plus Western blotting detection systems (Amersham Pharmacia Biotech) with an RFC1 antibody directed to the C terminus of the carrier (28). MTX was used as a model transport substrate for RFC1 (29). MTX influx was assayed in fibroblasts growing on, and adherent to, the bottom of 20-ml glass scintillation vials with 0.5 μM [3H]MTX as described previously (30).

**Maternal Folate Supplementation—**Folate supplementation was administered by subcutaneous injections of RFC1−/− dams. Initially, folate supplementation was begun 1 week before matings; later, supplementation (most of the animals that received a folic acid dose of 1 mg/day) was started at the time of appearance of the vaginal plug. The latter supplementation schedule appeared to improve the mating efficiency. Folate supplementation continued until pregnant dams were sacrificed or RFC1-null pups died or were sacrificed. Folic acid and 5-formyltetrahydrofolate were dissolved in water at appropriate concentrations, neutralized to pH 7.0, sterile-filtered, and injected with a 26-gauge needle in a volume of 0.1 ml.

**Histopathological Examination—**The neonatal mice were sacrificed before death, and their organs were examined and then immersed in 10% neutral buffered formalin for fixation. Sections from all organs were cut at 4–5 μm and stained with hematoxylin and eosin (HE). The E18.5 embryos were fixed, then serial coronal sections of the entire embryos were processed and embedded to obtain HE-stained cross-sections. Serial sectioning of the paraffin-embedded blocks of tissues from neonates and sectioning of embryos were undertaken, as warranted, to ensure complete evaluation of representative sections of all organs.

**RESULTS**

**Generation of RFC1−/− Mice—**A targeted disruption of an RFC1 allele was generated in embryonic stem cells by homologous recombination. A large part of exon 3, which harbors the initiation codon ATG, was replaced with a neomycin cassette as illustrated in Fig. 1a. Heterozygous progeny of chimeric animals were identified by genomic PCR and Southern blot analysis of SacI-digested tail DNA (Fig. 1, b and c, respectively). Two breeding colonies derived from two independently targeted embryonic stem cell clones were studied and gave the same results.

**Effect of RFC1 Inactivation on Embryonic Development—**Mice heterozygous for RFC1 were mated to generate F2 offspring. From these matings 284 pups from 37 litters were obtained and genotyped. One-hundred and eighty-four pups were heterozygous for RFC1, and 100 were wild-type (Table I). No homozygous RFC1-null pups were obtained, indicating that the mutation was embryonic lethal. RFC1−/− mice were physically indistinguishable from their wild-type littermates.

Studies were conducted to determine at which stage embryos ceased to develop. At day 11.5 no RFC1−/− embryos were identified from a total number of 49 (Table I). However, embryonic tissues could be isolated from two small implants and were homozygous for RFC1 disruption. At day 9.5, 11 smaller and markedly deformed nonviable RFC1−/− embryos were identified among 95 fetuses; examples are illustrated in Fig. 2. Additionally, there were 10 (10%) completely reabsorbed implants (Table II). Hence, the RFC1-null embryos died before day 9.5.

**Rescue of Embryonic Lethality by Maternal Folate Supplementation—**In an attempt to circumvent the folate transport defect in RFC1-null embryos, RFC1−/− dams were injected subcutaneously with folic acid at doses of 10, 25, 100, 250, 500, or 1000 μg/day beginning 1 week before conception and continuing during gestation and for 2 weeks after the pups were born. Folic acid is a very poor substrate for RFC1 (8, 9), and at the blood levels generated with the high doses utilized, it is likely that appreciable quantities of folic acid may be delivered into cells by passive diffusion as well as other secondary transport routes (14, 15, 17). There were no live births of RFC1−/− pups until a dose of 1 mg of folic acid/day was administered to RFC1−/− dams. Eighteen (9.3%) RFC1−/− mice were obtained from 194 live births in 39 litters (Table I). No RFC1−/− neonates were identified when RFC1−/− dams were injected with 1 mg/day 5-formyltetrahydrofolate, a reduced folate with a high affinity for RFC1, but a much lower affinity for folate receptors than folic acid (9, 12, 15).

**Characterization of RFC1-null Neonatal Mice Rescued by Folic Acid Supplementation—**RFC1−/− mice born from RFC1−/− dams supplemented with folic acid, aside from being pale, appeared normal during the first few days of life but then showed gross retardation, irritability, and increasing weakness, dying at or before day 12. Four RFC1−/− mice were euthanized for pathologic studies at postpartum days 9, 10, 11, and 12, along with four RFC1−/− and four RFC1−/+ littermates. The body weights of these RFC1−/− animals (3.1 ± 0.2 g) were significantly lower (paired t test, p < 0.0025) than the weight of the RFC1−/+(6.9 ± 0.4 g) or RFC1−/− (6.7 ± 0.4 g) neonates (Fig. 3a). There were no significant pathologic differences between wild-type and heterozygous mice. However, the spleen and...
Characterization of E18.5 Embryos Rescued by Folic Acid Supplementation—E18.5 embryos were examined histologically to determine the extent to which development was sustained prior to birth by folic acid supplementation of dams at a dose of 1 mg/day of folic acid. The percentage of RFC1-null fetuses (20%) was close to the predicted 25% for the homozygous genotype (Table II). Histologic examination on RFC1-null (n = 6), heterozygous (n = 4), and wild-type (n = 4) embryos indicated almost complete protection from adverse effects of RFC1 inactivation by folic acid supplementation. Erythropoiesis was active in liver (Fig. 4, a versus b) and bone marrow (Fig. 4, c versus d), with comparable numbers of nucleated red cells as compared with wild-type and heterozygous littermates. Splenic erythropoiesis was mildly to markedly impaired in only two of the six RFC1−/− embryos (Fig. 4, e versus f). The thymus (Fig. 4, g versus h) and kidneys in RFC1-null embryos were not different from RFC1−/+ and RFC1+/+ embryos.

**RFC1 Expression and MTX Influx in Embryonic Fibroblast Cells**—Three independent embryonic fibroblast cell lines were established for each genotype from three 15.5-day fetuses from dams that received folic acid supplementation to confirm that RFC1 had been completely inactivated in mice (Table II). An antibody against the C terminus of RFC1 detected a 58-kDa protein in wild-type fibroblast cells, the same size as found in murine leukemia L1210 cells (Fig. 1d) (28). The level of expression of RFC1 in heterozygous fibroblasts was decreased as compared with that of wild-type cells. No RFC1 protein was detected in RFC1-null fibroblasts. As indicated in Fig. 1e, MTX influx was decreased by 28 and 82% in RFC1−/− and RFC1−/+ fibroblasts, respectively, as compared with wild-type cells. The small residual transport activity in RFC1-null cells was apparently due to alternative folate transport route(s) in the fibroblasts.

**DISCUSSION**

Rapidly proliferating tissues require an adequate supply of folate substrate to meet cellular demands for one-carbon moieties for a variety of cellular biosynthetic processes. RFC1 is a well characterized folate transporter with a high affinity for the physiological folate substrate 5-methyltetrahydrofolate and has been the subject of intensive studies because of its critical role in antifolate transport and resistance (7, 8). However, it was unclear as to its role in the delivery of folates to a diverse spectrum of tissues due to the presence of multiple folate transport routes in mammalian cells. To address the role of the RFC1 gene in mouse embryonic development and neonatal life,

**TABLE I**

| Folate supplementation (subcutaneous) | Total pups | Litters | Average litter size | RFC1−/+ | RFC1−/− | RFC1+/− |
|--------------------------------------|------------|---------|---------------------|---------|---------|---------|
| None                                 | 0          | 284     | 37                  | 7.7     | 100 (35) | 184 (65) | 0       |
| Folic acid                           | 10         | 13      | 2                   | 6.5     | 2 (15)   | 11 (89)  | 0       |
| 25                                   | 7          | 16      | 3                   | 3.5     | 5 (71)   | 2 (29)   | 0       |
| 100                                  | 16         | 3       | 2                   | 5.3     | 9 (56)   | 7 (44)   | 0       |
| 250                                  | 18         | 2       | 9                   | 2 (11)  | 16 (89)  | 0        | 0       |
| 500                                  | 22         | 4       | 5.5                 | 6 (27)  | 16 (73)  | 0        | 0       |
| 1000                                 | 194        | 39      | 5.0                 | 60 (31) | 116 (60) | 18 (9.3) | 0       |
| 5-Formyltetrahydrofolate             | 1000       | 39      | 6                   | 6.5     | 10 (26)  | 28 (76)  | 0       |

**TABLE II**

| Embryonic stage | No. litters | No. implants | No. resorbed | No. embryos | Embryonic genotype |
|-----------------|-------------|--------------|--------------|-------------|--------------------|
|                 |             |              |              | RFC1−/+     | RFC1−/−           | RFC1+/− |
| No supplementation with folic acid |              |              |              |             |                    |        |
| E11.5           | 6           | 53           | 6 (11)*      | 49          | 16 (32)           | 31 (63) | 0       |
| E9.5            | 14          | 105          | 10 (10)      | 95          | 30 (31)           | 54 (57) | 11 (12) |
| Supplementation with folic acid at a dose of 1 mg/day |              |              |              |             |                    |        |
| E15.5           | 3           | 20           | 0            | 20          | 4 (20)            | 11 (55) | 5 (25)  |
| E18.5           | 7           | 54           | 0            | 54          | 18 (33)           | 24 (44) | 11 (20) |

*Some tissues could be isolated from two partially reabsorbed embryos and were genotyped and found to be homozygous for RFC1 disruption.

**FIG. 2. Comparison of RFC1−/− and RFC1+/+ embryos harvested at E9.5.** The RFC1-null embryos are smaller and morphologically deformed as compared with the well developed wild-type embryo (upper left).

The thymus of RFC1-null mice were extremely small, and these organs and the liver were very pale (Fig. 3b). Histologic examination indicated a marked absence of hematopoiesis in the bone marrow (Fig. 3, c versus d) of RFC1−/− mice, and extramedullary hematopoiesis was also absent in spleen (Fig. 3, e versus f) and liver. The lymphocyte population in the white pulp of the spleen (Fig. 3, e versus f) and in the cortex of the thymus was markedly diminished (Fig. 3, g versus h) in the RFC1−/− mice. Postnatal development of renal medullary tubules and the seminiferous tubules in the testis was also impaired. There were no abnormalities in other organs, including stomach, small intestine, proximal and distal colon, cecum, heart, lungs, uterus, urinary bladder, eye, brain, pancreas, or salivary gland.

**Characterization of E18.5 Embryos Rescued by Folic Acid Supplementation—**E18.5 embryos were examined histologically to determine the extent to which development was sustained prior to birth by folic acid supplementation of dams at a dose of 1 mg/day of folic acid. The percentage of RFC1-null embryos with double aortic arches (20%) was close to the predicted 25% for the homozygous genotype (Table II). Histologic examination on RFC1-null (n = 6), heterozygous (n = 4), and wild-type (n = 4) embryos indicated almost complete protection from adverse effects of RFC1 inactivation by folic acid supplementation. Erythropoiesis was active in liver (Fig. 4, a versus b) and bone marrow (Fig. 4, c versus d), with comparable numbers of nucleated red cells as compared with wild-type and heterozygous littermates. Splenic erythropoiesis was mildly to markedly impaired in only two of the six RFC1−/− embryos (Fig. 4, e versus f). The thymus (Fig. 4, g versus h) and kidneys in RFC1-null embryos were not different from RFC1−/+ and RFC1+/+ embryos.

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**DISCUSSION**

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It is of interest that folic acid, but not 5-methyltetrahydrofolate, prolonged the life of RFC1-null mice up to 12 days postpartum, while the latter extended the viability of folate receptor-α-null embryos to day 18. At the high blood levels of

the gene was disrupted by homologous recombination. The complete absence of carrier in RFC1−/− mice was verified by the lack of RFC1 protein and a marked decrease in MTX influx in embryonic fibroblast cells.

RFC1−/− embryos died before E9.5. Embryos lacking folate receptor-α die at a similar development stage (18). Thus, RFC1 and folate receptor-α simultaneously play distinct roles in embryonic folate homeostasis, and the presence of one does not compensate for the loss of the other. This is likely related to their different tissue expression patterns (20–23) or different localization if expressed in the same cell type (24). These findings are consistent with the critical requirement for folates in normal embryogenesis (31). In both cases high doses of folate administered to dams rescued embryos. However, the amounts of folate used were 3 orders of magnitude higher than that recommended for pregnant women (0.4 mg/day) to prevent neural tube abnormalities (2, 3).

It is of interest that folic acid, but not 5-methyltetrahydrofolate, prolonged the life of RFC1-null mice up to 12 days postpartum, while the latter extended the viability of folate receptor-α-null embryos to day 18. At the high blood levels of folic acid generated in these animals, ~100 μM, adequate folate delivery could have been achieved via a transport route that has a higher affinity for folic acid than 5-formyltetrahydrofolate (17). Also, because 5-formyltetrahydrofolate is a racemic mixture, and the D isomer does not have biologic activity, it could compete with the L isomer and inhibit its utilization. Hence, transport is not fully stereospecific (32), and limited data suggest mammalian folylpolyglutamate synthetase can utilize both 6R and 6S diastereomers of folates (33, 34).

While folic acid supplementation can maintain near-normal embryonic development up to at least E18.5 in RFC1−/− embryos and some animals go on to live birth, the life of animals beyond postpartum day 12 cannot be sustained. Prior to birth, high folate concentrations achieved by folic acid injection, with transport or diffusion across the placenta barrier where high levels of folate receptor are expressed (23), provides adequate folates for the developing embryo. However, possibly due to low concentrations of folic acid in the mother’s milk and inactivation of intestinal RFC1 that is involved in folate absorption across the pup’s small intestine (21, 35), where folate receptors are not expressed (36), there is apparently insufficient folate available to sustain the life of pups long after birth.

The major pathological changes occurred in tissues of hematopoietic origin in RFC1-null neonatal mice, suggesting that cells of other origins can receive adequate folate via transport route(s) other than RFC1. The lack of any pathological changes
in intestine of neonatal animals, which normally express high levels of RFC1, raises the possibility that this carrier may not be the only folate transporter in this tissue although folate receptor is not expressed in intestine. It is possible that the small intestine of the neonatal mouse can absorb sufficient folate to support proliferation and maturation of crypt cells when only one allele is affected and the wild-type allele is homozygous state would not have physiological consequences although the level of RFC1 protein in RFC−/− embryonic fibroblasts was decreased as compared with that of wild-type cells, and transport was reduced by ∼30%. Hence, a mild decrease in RFC1 activity is well tolerated at least in mice on the usual folate-rich diet. It is therefore likely that mutations or polymorphisms in RFC1 that markedly impair function in the homozygous state would not have physiological consequences when only one allele is affected and the wild-type allele is expressed. Recently, a polymorphism (G80A) in RFC1 has been identified, but its role in folate transport or folate homeostasis remains to be established (38).

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