Mucosal Iron Transport by Rat Intestine

By Michael A. Savin and James D. Cook

Using highly sensitive 2-site immunoradiometric assays, we examined the relationship between iron absorption from closed intestinal loops and transferrin and ferritin concentrations in isolated duodenal mucosal cells. As in prior studies, mucosal ferritin correlates inversely with iron absorption and directly with body iron stores as measured by the concentration of nonheme iron in liver. Mucosal transferrin, on the other hand, varies directly with both the total mucosal uptake of radioiron and the proportion of this radioiron transferred from the mucosa to the carcass. The highest correlation with iron absorption was observed with the transferrin:ferritin ratio in isolated mucosal cells. These results suggest that there are two functionally distinct iron-binding compartments in the duodenal mucosa. One is a strong compartment, ferritin, and the other is a transport compartment, transferrin. Control of iron absorption by the intestinal mucosa is closely tied to the balance between these two intracellular iron compartments.

IRON ABSORPTION is a physiologically-regulated process that serves as the primary mechanism for maintaining body iron balance. Absorption is increased during periods of iron deficiency and decreased when body iron stores are in excess. There is considerable evidence that the regulation of iron absorption occurs in pathways that operate within the duodenal mucosal cell.1-3 However, despite intensive investigation, the precise nature of the mechanism for mucosal iron transport has yet to be elucidated.

Two iron-binding proteins have been identified in the homogenized intestinal mucosa in several species. One of these proteins is an isoferritin, the principle iron storage protein in tissue such as liver and spleen.1,2,4-15 The second protein has properties similar, if not identical, to transferrin.7,8,12-17 Moreover, the pattern of iron-binding by this protein during iron absorption is reported to reflect behavior of the cell vis-à-vis iron absorption and may therefore mediate iron transport by the mucosal cell.7,12,15,18

To investigate the possible role of mucosal transferrin and ferritin in the regulation of iron absorption, we developed 2-site immunoradiometric assays (IRMA) capable of detecting nanogram quantities of both rat transferrin and rat ferritin. These assays were used to examine the relationship between iron absorption and intracellular concentrations of the two proteins in isolated mucosal cells.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250–350 g were used in all studies. Three groups of animals with varying iron status were used. A control group of normal animals was maintained on a diet of laboratory rat chow containing 415 μg iron/g pellet. A second group of rats was made iron deficient by removing 3–5 ml blood at weekly intervals while maintaining the animals on a low diet containing 8 μg iron/g food (Nutritional Biochemical Corp., Cleveland, Ohio) until the hemoglobin concentration was less than 6 g/dl and the transferrin saturation less than 15%. Several weeks were required for the development of iron deficiency anemia. A third group of iron-loaded rats was given an intraperitoneal injection of 25 mg iron as iron dextran 2 wk prior to study.

Iron Absorption Measurements

Absorption of a test dose of ferrous sulfate tagged with 59Fe was measured in vivo from closed duodenal loops. Individual test doses were prepared as follows. Stock solutions were prepared fresh each day, and individual test doses were prepared immediately prior to administration to the rats. Iron stock solutions contained 5000 μg elemental iron as ferrous sulfate per ml in 0.1 N HCl. Radioiron stock solutions were prepared by dilution with 0.1 N HCl of a commercial 59Fe solution (approximately 2000 μCi/ml in 0.5 N HCl) to a concentration of 100 μCi per ml. Immediately prior to administration, test doses were prepared by dilution of 1 part iron stock solution and 1 part radioiron stock solution with 18 parts iron-free water. Each individual dose contained 50 μg elemental iron as ferrous sulfate tagged with 1 μCi of 59Fe in a volume of 0.2 ml with a pH of approximately 2.1.

After an overnight fast, animals were anesthetized with 50 mg/kg sodium nembutal, and a 4 cm segment of duodenum beginning just beyond the pulorus was ligated at both ends while care was taken to preserve the blood supply. The 0.2 ml iron test dose was introduced into the isolated segment through a 25 gauge needle. Absorption was terminated 30 min later by removing the gut segment from the carcass and the animal killed with an overdose of sodium nembutal. The gut segment was opened immediately, flushed with normal saline to remove unabsorbed radioactivity, and counted in a NaI(Tl) scintillation counter. Carcass radioactivity was measured in a stationary field viewed by four 6-inch diameter NaI(Tl) detectors used for whole-body counting in man.19 Standards containing the same quantity of radioactivity as that originally introduced into the closed loop were diluted to the same volume as the gut and carcass specimens, respectively, and counted simultaneously. Absorption was calculated as a percentage of the radioiron originally introduced into the gut segment. Radioiron remaining with the washed gut segment and the carcass will be referred to as gut and carcass absorption, respectively, and the sum of these as total absorption.

Preparation of Mucosal Cell Homogenates

Immediately after counting the gut segment, mucosal cells were isolated by mechanical vibration as previously described.20 The

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isolated cells were washed three times in calcium- and magnesium-free Hank’s balanced salt solution (GIBCO, Grand Island, N.Y.), and suspended in 1 ml 0.005 M tris-HCl buffer, pH 7.4. The cells were disrupted by ultrasonication, and particulate cellular debris was removed by centrifuging for 10 min in the cold at 10,000 g. The supernatant was stored a −20°C until used for measurements of transferrin and ferritin by IRMA.

The mean percent recovery of total gut segment 59Fe in the mucosal suspensions was 70%, with a range of 52%-92%. In normal rats, the mean recovery was 73% of total gut segment 59Fe, in iron-deficient animals 72%, and in iron-loaded animals 65%.

**Mucosal Transferrin and Ferritin**

The concentrations of both proteins were measured by a 2-site IRMA previously described for serum ferritin measurements in man.\(^{21}\) Briefly summarized, a diluted sample of the mucosal supernatant was added to a polystyrene tube that had been coated with rabbit antisera raised against either purified rat ferritin or transferrin. After 48-hr incubation, the tube was rinsed and an excess of 125I-labeled antibody was added which complexed with the insolubilized antigen. After a further 24-hr incubation, the tube was aspirated and washed to remove unbound labeled antibody, and the residual radioactivity measured in a well-type scintillation counter. A standard curve was derived from measurements on a blank and 7 standards containing 0.25-100 ng protein/ml. Linearization of the standard curve was achieved by a logit/log transformation and a computer searching technique.\(^{21}\) The concentrations of mucosal ferritin and transferrin were expressed as ng/mg total mucosal protein as measured by the Lowry method.\(^{22}\)

In establishing the IRMA for rat ferritin, recrystallized rat liver ferritin was purified by the technic of Yu and Fineburg\(^{23}\) from animals previously injected with iron dextran. The sensitivity and reproducibility of the assay were virtually identical to serum ferritin measurements in man.\(^{23}\) As previously reported,\(^{24}\) the rat assay was unsuitable for serum measurements because of large unexplained variations when serial measurements were performed in the same animal. However, the assay was highly reproducible when used for measurements of tissue ferritin. With assays of rat liver an excellent correlation (r = 0.88, p < 0.001) was observed between IRMA ferritin and total nonheme iron as measured by the method of Torrance and Bothwell\(^{25}\) (Fig. 1). When recrystallized ferritin was added to mucosal homogenates, recovery ranged from 80%-100%.

The methods for purification of rat plasma transferrin and the development of the IRMA for the protein are described in detail elsewhere.\(^{26}\) During initial efforts to purify the protein using standard preparative techniques, significant contamination with serum immunoglobulins was detected by immunoelectrophoresis. The following procedure was devised to provide a transferrin preparation free of contaminating proteins. Transferrin was initially purified from iron saturated rat plasma by the following steps: (A) precipitation of other proteins with 6,9-diamino-2-ethoxyacridine lactate; (B) sequential precipitation of transferrin with acid ethanol; (C) ion exchange chromatography of DEAE-agarose; and (D) gel filtration on Sephadex G-200. Contaminating immunoglobulins were removed from this preparation by absorption against rat immunoglobulin immobilized on cyanogen bromide activated Sepharose. The adsorbed transferrin was used to prepare anti-transferrin antisera in rabbits. Transferrin antibodies were then purified from the antisera and immobilized on cyanogen bromide activated Sepharose. The resulting anti-transferrin immunoadsorbent was used as a reagent to purify transferrin from rat plasma without intervening steps and the anti-transferrin antisera was used in the transferrin IRMA as described above.

The IRMA for rat transferrin was initially validated in terms of the circulating protein. Assay sensitivity ranged from 0.25 to 5 ng transferrin/ml, which necessitated an initial serum dilution of 1:2 × 10\(^6\). When purified rat transferrin was added to duodenal mucosal homogenates to increase transferrin concentration by 100 ng/ml, recoveries of 85%-100% were obtained with a reproducibility of ±10%. Moreover, when rat plasma was serially diluted to obtain iron-binding capacities ranging from 50-500 μg/dl, an excellent correlation was observed with IRMA.\(^{26}\)

Because serum contains a high concentration of transferrin compared to that found in mucosal homogenates, studies were performed to determine whether mucosal transferrin could be due to contamination with serum. In the first study, mucosal and serum transferrin were measured by IRMA in 22 iron-deficient rats; mucosal transferrin ranged from 226-472 ng/mg mucosal protein while serum transferrin varied from 3.1-8.9 mg/dl serum. Because no significant correlation was observed between these two measurements (r = 16, p > 0.10), it is unlikely that mucosal transferrin was due simply to serum contamination. The possibility of serum contamination was further tested by injecting 125I-labeled rat transferrin intravenously into normal rats. The labeled transferrin, iodinated by the method of Bolton and Hunter,\(^{27}\) had a specific activity of 15 μCi/μg transferrin. When 500,000 cpm was injected into normal rats, plasma radioactivity at 5 min, 15 min, 30 min, and 24 hr ranged from 14,000 to 2200 cpm/ml. It was not possible in any instance to detect radioactivity in homogenates from isolated duodenal mucosal cell suspensions in these animals. Based on a specific activity for plasma transferrin of 4600 cpm/mg and an average mucosal protein concentration of 10 mg/ml, contamination by 100 ng/ml protein or more would have been detected with the counting method employed. This level is about one half the mucosal transferrin concentration in iron-loaded rats and is substantially less than that observed in either normal or iron-deficient animals. Thus, even in iron-loaded rats, more transferrin was detected than could be accounted for by contamination. Moreover, the fact that mucosal transferrin in iron-loaded rats was as low as 16 ng/ml makes significant serum contamination unlikely.
Tissue Iron Measurements

Body iron status was determined from measurements of total nonheme iron in liver homogenates as described by Torrance and Bothwell. The results were expressed as µg iron/g wet weight liver. These measurements were not performed in iron-loaded animals because of potential interference from residual iron dextran.

RESULTS

Measurements of iron absorption and mucosal iron-binding proteins in normal, iron-deficient, and iron-loaded rats are summarized in Table 1. Total absorption in normal and iron-loaded rats was similar, 24.8 and 20% of the 50 µg radioiron dose, respectively. Carcass and gut absorption values also were similar in normal and iron-loaded rats. Indeed, none of the differences in gut, carcass, or total absorption between normal and iron-loaded rats were statistically significant.

Iron absorption in iron-deficient rats was markedly higher than in normal and iron-loaded animals. A total of 53.7% of the dose was absorbed and most of this increase was due to higher carcass absorption. Carcass and total absorption were both significantly higher in iron-deficient than in either normal or iron-loaded rats (p < 0.0001 for all comparisons). Gut radioactivity, on the other hand, was very similar in all three groups. Mean gut absorption was 13.8, 11.0, and 16.6% of 50 µg in normal, iron-loaded, and iron-deficient rats respectively (p > 0.10 for all comparisons). Thus, the major effect of iron deficiency was on the fraction of absorbed radioactivity transferred to the carcass rather than residual gut absorption. Additional evidence of this is presented in Table 2. In the combined group of normal and iron-deficient rats, storage iron as measured by liver nonheme iron was highly correlated with carcass absorption (r = −0.84) and with total absorption (r = −0.86), whereas the correlation with liver nonheme iron and gut absorption was not significant (r = −0.28).

These absorption values from closed intestinal loops are very similar to results reported by Wheby et al. They observed that 47% of the absorbed dose had been transferred to the carcass at 35 min in normal rats as compared to 45% in the present study. Similarly, they observed that 75% of the absorbed dose was localized in the carcass in iron-deficient rats given 50 µg iron as compared with 69% in the present study.

Ferritin was detected by IRMA in mucosal cell homogenates from all three animal groups, ranging from 10–321 ng/ml protein in individual rats. Values were closely related to body iron status with the lowest mean in iron-deficient animals and the highest in iron-loaded animals (20 and 124 ng ferritin/mg cell protein, respectively). As with iron absorption, mucosal ferritin in normal animals (mean, 98 ng/ml) was closer to that in iron-loaded animals (mean, 124

| Table 1. Relationship Between Iron Absorption, Iron Stores, and Mucosal Transferrin and Ferritin in Normal, Iron-Deficient, and Iron-Loaded Rats |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| No. | Liver Nonheme Iron (µg/g) | Iron Absorption Carcass (% of dose) | Gut (% of dose) | Total | Mucosal Trsf(F)† (ng/mg protein) | Mucosal Ferr(F)† (ng/mg protein) | T/F Ratio |
| No. | 12 | 74 ± 4* | 11.1 ± 1.8 | 13.8 ± 0.9 | 24.8 ± 2.1 | 306 ± 24 | 98 ± 18 | 5.9 ± 1.9 |
| | (57-93) | (5-28) | (9-19) | (15-45) | (193-466) | (16-218) | (1.4-23.3) |
| Iron- | 17 | 15 ± 2 | 7.1 ± 2.5 | 16.6 ± 1.0 | 53.7 ± 2.6 | 478 ± 40 | 20 ± 3 | 23.4 ± 2.6 |
| deficient | | (10-31) | (13-77) | (10-23) | (35-77) | (325-645) | (10-36) | (14-41) |
| Iron- | 9 | — | 9.0 ± 1.6 | 11.0 ± 1.0 | 20.0 ± 2.3 | 178 ± 45 | 124 ± 32 | 4.5 ± 1.9 |
| loaded | | (4-16) | (8-16) | (11-29) | (16-337) | (19-321) | (0.1-12.5) |

*Mean ± 1 SE (range).
†Trsf, transferrin; Ferr, ferritin.

Table 2. Correlation Coefficients for Liver Nonheme Iron, Iron Absorption Parameters, and Mucosal Cell Concentrations of Transferrin and Ferritin

<table>
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<th>No.</th>
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<th>Carcass Absorption</th>
<th>Total Absorption</th>
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<th>Transferin</th>
<th>Traf:Ferr Ratio*</th>
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<td>-.86</td>
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<td>-.81</td>
<td>-.84</td>
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*Traf, transferrin; Ferr, ferritin.
†All correlation coefficients with the exception of this value are significant at the 1% level.
Correlations were observed when these mucosal 
significant correlation between mucosal ferritin and 
absorbed from the gastrointestinal lumen, mucosal 
parameters (Fig. 2). Correlations with total absorption 
showed the highest correlation with iron absorption 
status. Body iron stores as measured by liver nonheme 
proteins were related to other parameters of iron 
transport: mucosal ferritin and transferrin where no difference was observed between 
and iron-loaded animals, a significant difference in mucosal transferrin was observed with normal 
and iron-loaded rats ($t = 2.82, p < 0.025$).

Correlation coefficients for all parameters in this 
study are presented in Table 2. With the exception of 
gut absorption versus liver nonheme iron, all correlations were significant at the 1% level. There was 
significant correlation between mucosal ferritin and 
transferrin ($r = -0.59, p < 0.001$), but even higher 
correlations were observed when these mucosal proteins were related to other parameters of iron 
status. Body iron stores as measured by liver nonheme 
iron in normal and iron-deficient rats correlated significantly with both mucosal ferritin and transferrin ($r = -0.60$ and $0.79$, respectively), but the latter showed the highest correlation with iron absorption 
parameters (Fig. 2). Correlations with total absorption 
were similar for mucosal ferritin and transferrin ($r = 0.70$ and $0.73$, respectively), but the highest correlation with total absorption was observed with the ratio of transferrin:ferritin ($r = 0.95$). It is also apparent from Table 2 that the correlation between mucosal iron protein and carcass absorption was nearly as high 
as with total absorption, indicating that carcass absorption, or mucosal transfer, is a more important 
index of mucosal response than is residual gut radioactivity.

DISCUSSION

The mechanisms by which the intestinal mucosa 
regulates iron absorption from the gastrointestinal tract remain unknown despite several decades of 
intensive study. Since Granick first proposed that 
mucosal ferritin constitutes a block to unwanted iron 
absorbed from the gastrointestinal lumen, mucosal 
ferritin has been the focal point of many theories on 
the regulation of iron assimilation. Granick's theory 
fell into disfavor when it was demonstrated that no 
absolute block existed, his theory was later revised by 
Crosby et al. who suggested that the "ferritin apparatus" of the mucosa is conditioned during its development 
in the crypts of Lieberkühn by the level of body iron stores. According to their hypothesis, mucosal 
synthesis of ferritin is normal in states of iron repletion 
and, as a result, iron entering from the lumen is 
trapped in mucosal ferritin. In iron deficiency this 
apparatus is defective in that little ferritin is produced 
and, as a result, iron entering from the lumen is 
impeded from movement into the cell into circulation. Support for this concept came from other 
laboratories that confirmed the apparent conditioning 
of mucosal cells during development and showed that 
these cells could be induced to synthesize ferritin in 
response to parenteral or oral iron. This observation identified a possible mechanism whereby the 
ferritin content of the cell could be conditioned by the 
quantity of iron in the cell's environment.

The importance of ferritin as a regulator of iron absorption was not statistically significant ($t = 0.79, p > 0.10$). In iron-deficient rats, however, ferritin was significantly lower than in either normal or iron-loaded rats.

The concentration of mucosal transferrin ranged 
widely, from 16–645 ng/ng cell protein, in the composite group of 38 animals. In contrast to ferritin, the lowest concentration of mucosal transferrin was observed in iron-loaded rats (mean, 178 ng/mg) and the highest level in iron-deficient rats (478 ng/mg). The mean transferrin in iron-deficient rats was significantly different than that in either normal or iron-loaded animals ($t = 4.82$ and $6.58$, respectively, $p < 0.001$). In contrast with iron absorption and mucosal ferritin where no difference was observed between 
normal and iron-loaded animals, a significant difference in mucosal transferrin was observed with normal 
and iron-loaded rats ($t = 2.82, p < 0.025$).
absorption has been challenged by other investigators. Britt and Raval found that iron-deficient animals retained the capacity to synthesize mucosal ferritin in response to oral iron and Schade found ferritin synthesis in mucosal cells to be normal in animals with depressed iron absorption due to inflammation. Worwood and Jacobs reported that incorporation of oral iron into mucosal ferritin was greater in iron-loaded than in normal rats, further challenging the role of ferritin as the sole mechanism for regulating iron assimilation by the intestinal mucosa.

It should be noted that most studies of mucosal ferritin in animals have measured either the proportion of radioiron uptake by the mucosa that enters the ferritin pool or the rate of ferritin synthesis in response to oral or parenteral iron. These tracer techniques have provided valuable information about the kinetics of mucosal iron transport but have not defined the relationship between iron absorption and the basal concentration of mucosal ferritin. Other mucosal ferritin studies used either immunologic techniques or nonheme iron measurements, but the limited sensitivity of the assay required the use of large segments of the entire intestinal wall. The sensitive 2-site IRMA used in the present study obviated these technical difficulties and allowed determinations to be performed on isolated mucosal cells that had been freed from contamination by submucosal tissue. This study leaves no question about the intimate relationship between iron status of the mucosal cell as measured by ferritin and its uptake and transfer of luminal radioiron.

There is considerable indirect evidence that proteins other than ferritin may be involved in iron transport by the mucosa. Studies examining the distribution of radioiron in mucosal homogenates after in vivo exposure to Fe in the lumen have shown that iron is bound to at least two different mucosal proteins. One of these is ferritin and another appears to be similar, if not identical, to transferrin based on electrophoretic mobility and chromatographic behavior. The time course of labeling mucosal fractions with radioiron combined with chaser experiments suggests that this protein has a direct role in iron transport by the mucosa because the turnover of iron through this compartment is high during absorption. On the other hand, the ferritin pool is rapidly labeled during absorption but retains its iron rather than releasing it to the carcass. Anand et al. and Mason and Taylor have used an immunohistochemical technique to study the distribution of iron-binding proteins among several human tissues. Using peroxidase-labeled specific antisera to stain fixed surgical speci-
with increasing iron deficiency, transferrin increases and serum ferritin falls, whereas in iron overload, the converse is true.

An intriguing finding in the present study was that iron absorption correlated better with the ratio of mucosal transferrin:ferritin than with either of these parameters separately. One possible explanation is that one parameter is more sensitive to changes in iron assimilation when iron stores are increased, while the other is more sensitive when iron stores are diminished. Support for this is shown in Fig. 2. When absorption exceeds 40%, mucosal ferritin falls to a low and relatively constant level while mucosal transferrin shows a continual increase. If these two proteins monitor different ends of the spectrum in iron status, the ratio would be a better correlate of iron absorption across a wide range in iron status.

These findings suggest that mucosal transport of iron from lumen to carcass is a function of the balance between the size of the iron transport and storage compartments. The intracellular distribution of luminal iron would be determined by the relative abundance of storage protein and transport protein. In iron-replete animals, the storage compartment is large and the transport compartment small, while in iron-deficient animals the converse is true with the result that iron is transported through the cell rather than diverted into ferritin. Thus, the relative abundance of transferrin in relation to ferritin would channel iron into one of the two intracellular compartments. This mechanism would place the burden of mucosal transport regulation within the intracellular milieu of the mucosa; this conclusion is consistent with previous data indicating that the brush border membrane of the cell does not participate in the regulation of iron absorption. 20

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