Ferroportin is expressed on the mucous granule membrane of a subpopulation of goblet cells in the duodenum of the rat

P.S. Oates and C. Thomas
Physiology, School of Biomedical and Chemical Sciences, the University of Western Australia, Crawley, Western Australia

Summary. Ferroportin is a basolateral transporter involved in the release of iron from cells. In addition to expression on the basolateral membrane of enterocytes, ferroportin is also seen on the microvillus membrane. This led us to consider that ferroportin might be expressed by other cells of the intestine where it contributes to iron metabolism. Ferroportin gene and protein expression in rat duodenum was studied by in situ hybridisation and immunohistochemistry, respectively in rats with different efficiencies of iron absorption. Ferroportin mRNA localised to enterocytes of the villus only. Ferroportin was demonstrated in enterocytes and in 30% of goblet cells. In goblet cells it localised to the mucous granule membrane. In iron-loaded intestine some goblet cells contained iron suggesting that ferroportin may transport iron into the mucous granule where it would be lost during discharge of mucous. The finding of ferroportin in iron deficient goblet cells also suggests an additional role to iron excretion.

Key words: Intestine, Goblet cells, Differentiated, Progenitor, Enterocytes, Iron, Absorption, Mucopolysaccharides

Introduction

Iron absorption takes place by duodenal enterocytes and involves at least two transport proteins, one that takes up iron from the intestinal lumen; the other releases the iron to the body. Uptake of Fe(II) takes place by divalent metal transporter (Oates and Morgan, 1996a; Fleming et al., 1997; Gunshin et al., 1997), while ferroportin/IREG-1/metal transporter protein 1 (MTP1)/SLC40A1 (Abboud and Haile 2000; Donovan et al., 2000; McKie et al., 2000; Thomas and Oates, 2002). These cells have in common the ability to export iron suggesting that ferroportin performs this function. Furthermore, mutations to ferroportin result in an iron overload phenotype, suggesting that a loss of function by ferroportin to transport iron from the cell leads to cellular iron overload (Montosi et al., 2001; Njajou et al., 2001). In addition, it was shown in vitro that incubation of HEK293 cells with the 25 amino acid peptide, hepcidin caused ferroportin to be internalised. Loss of membrane ferroportin was associated with increased cellular iron levels and ferritin, but decreased iron responsive protein II expression (Nemeth et al., 2004), indicating that ferroportin is required for iron efflux (Nemeth et al., 2004; Yamaji et al., 2004; Yeh et al., 2004).

In addition to the export of iron from the enterocyte we recently showed that in freshly isolated enterocytes, the enterocyte cell lines IEC-6 and Caco-2 that blocking ferroportin function by a functional antibody inhibited iron uptake (Thomas and Oates, 2004a,b). This suggests that ferroportin also functions at the apical membrane. Supporting this we recently detected ferroportin expression along the microvillus membrane of enterocytes and in polarised Caco-2 cells (Thomas and Oates, 2004a). This suggests that in addition to iron export, ferroportin has another function. Understanding the function of ferroportin will assist in elucidating the mechanism and regulation of iron absorption. Therefore, identifying the sites of ferroportin synthesis and expression in cells along the crypt-villus axis will be crucial in defining these processes.

In view of this we studied ferroportin gene and protein expression by in situ hybridisation and immunohistochemistry, respectively. The expression of mucopolysaccharides and the presence of iron in goblet cells along the villus axis in the duodenum of rats with
Ferroportin in the rat duodenum

variations in the efficiency of iron absorption were also investigated by histochemistry.

Materials and methods

Production and validation of a ferroportin antibody

A synthetic peptide corresponding to amino acids 247-264 of rat ferroportin (i.d. number AAK77858) was conjugated to keyhole limpet hemocyanin and then used to immunize a rabbit for the production of an anti-ferroportin antiserum as described previously (Thomas and Oates, 2002). Validation of this antibody has previously been reported (Thomas and Oates, 2002, 2004b).

Animals

The Animal Welfare Committee of the University of Western Australia has approved the animals used in this study. Nine outbred male, Wistar rats were obtained from the Animal Resource Centre (Murdoch, Western Australia). Three groups of 3 rats were fed a semipurified diet low, normal (70 mg/kg) or high (2% carbonyl iron) in iron for 2 weeks. This regimen has been shown to alter iron absorption (Oates and Morgan, 1996a). The composition and the form of the iron used in these diets have been reported previously (Oates and Morgan, 1996a).

At the time of study the animals were injected with 0.5 ml of 60 g/L Nembutal given intraperitoneal. After deep anaesthesia the abdominal cavity was opened and a 2 cm length of the duodenum distal to the pylorus was removed and fixed in buffered formal saline for 24 hrs. The tissue was processed for wax embedding as a composite block containing 9 duodenums from the 3 conditions studied.

Ferroportin cDNA production and riboprobe synthesis

A partial ferroportin cDNA was produced using a full-length template from a ferroportin clone (Accession number: AF394785.3) that we previously produced (Thomas and Oates, 2002). Primers were designed to generate a PCR fragment and subcloned into T-Easy vector (Promega). Sequencing confirmed the composition of the cDNA as rat ferroportin and its position within the vector. In vitro transcription of antisense and sense ferroportin transcripts were performed using T7 and SP6 polymerases, respectively, a riboprobe system (Promega) and α35S-uridine 5'-triphosphate (10 μCi/μl) as label (Jeffrey et al., 1994).

Ferroportin expression by in situ hybridisation

In situ hybridisation was performed using 0.5 million disintegrations per minutes of 35S labelled antisense and sense riboprobes and wax sections according to our previous description. Serial sections were exposed for 2, 4, 6, 13, 20, 27 and 35 days.

Ferroportin expression by immunohistochemistry

Wax embedded tissue was serially cut at 5 μm dewaxed and rehydrated by phosphate buffered saline (PBS). Endogenous peroxidase and aldehydes were blocked as described previously (Oates and Morgan, 1996b). Immunodetection of ferroportin involved using an anti-rat ferroportin antibody that had either been incubated overnight in PBS or PBS plus 50 ng/ml of purified immunizing peptide. The antibody was diluted with MEM at 1:2000 and incubated at 4°C overnight. The secondary antibody used was a goat anti-rabbit biotin (Santa Cruz) and subsequent amplification by the Vectastain ABC kit (Vector Laboratories).

The incidence of goblet cells expressing acid or neutral mucopolysaccharides

To determine the total number of goblet cells per crypt-villus unit, sections of the composite block was subjected to alcian blue staining to detect acid mucopolysaccharides and in a serial section stained with AB + periodic acid-Schiff’s (PAS) reagent in order to reveal all goblet cells (Spicer et al., 1965; Clark, 1981). The number of goblet cells expressing acid or total mucopolysaccharides was then counted in over 50 well-oriented villus axes.

The incidence of goblet cells expressing ferroportin

Because of the dominant and uniform expression of ferroportin in enterocytes that represent over 90% of cells lining the villus, the likelihood of identifying ferroportin positive goblet cells at low power magnification was considered poor and equivocal. We approached this problem by looking for obvious ferroportin negative goblet cells and compared this number to the total number of goblet cells present using AB+PAS staining. It became apparent that we could not account for all goblet cells. Therefore to determine the extent of ferroportin expression by goblet cells, 10 well-defined villi axes per rat were video imaged at x40 while immersed in an aqueous mounting medium. The coverslip was then removed and the section subjected to AB+PAS staining to detect total goblet cell number and mounted in DEPX mounting medium. The villi previously imaged were again identified and imaged again to determine total goblet cell numbers. The individual images of cells along the villus were then montaged-using Adobe Photoshop v8.0. The two images were then placed side by side and assessed independently by two investigators. Firstly, ferroportin negative goblet cells were counted then the total number of goblet cells in the same villus using AB+PAS stained sections. Goblet cells identified by AB+PAS and being ferroportin positive were therefore identified. To corroborate this we repeated the procedure on another set of sections this time looking at villi at x100 objective magnification.
Distribution of goblet cells along the length of the villus

To determine the distribution of goblet cells along the villus axis, the same AB+PAS stained digital images described above were used. The length of the villus was first measured and then divided into upper-, middle- and lower-thirds. The number of goblet cells in each segment was then counted. The data is expressed as a percentage of total goblet cells in each segment.

Determining co-localisation of ferroportin and iron in goblet cells

The tissue was first prepared for detection of ferroportin, then reacted with Perl’s Prussian blue stain (Clark, 1981) to identify iron, and finally by AB+PAS to reveal goblet cells. After each reaction specific villi were identified and imaged.

Statistics

Data between conditions was analysed by the analysis of variance with partitioning according to the Tukey test. Significance was considered at p<0.05.

Results

Goblet cell number in the villus of the duodenum of rats with varying iron stores

The number of goblet cells expressing acid mucopolysaccharides increased with the iron content of the diet (Table 1). That is, goblet cell expression of acid mucopolysaccharides was low in rats fed the iron deficient diet and highest in those fed the iron-loaded diet. Interestingly, there was no difference in the total number of goblet cells in the three conditions studied as assessed by combined AB + PAS staining (Table 1). Therefore, as expected in the iron deficient state there was goblet cells that expressed neutral mucopolysaccharides only.

Table 1. Goblet cell numbers in the duodenum of iron deficient, control and iron loaded rats along well-defined villus units. The number of goblet cells positive for acid, total (acid + neutral) mucopolysaccharides, the percentage of total goblet cells found in the upper-, middle- and lower-thirds of the villus and the percentage of goblets cells expressing ferroportin compared with total goblet cell numbers. Results are expressed as the mean ± SD.

<table>
<thead>
<tr>
<th>MUCOPOLYSACCHARIDE TYPE/FERROPORTIN</th>
<th>IRON DEFICIENT</th>
<th>IRON CONTROL</th>
<th>IRON LOADED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>18±1 c,l</td>
<td>26±2 l</td>
<td>32±3</td>
</tr>
<tr>
<td>Total</td>
<td>24±2 l</td>
<td>25±2</td>
<td>28±3</td>
</tr>
<tr>
<td>% Upper-third</td>
<td>24±10</td>
<td>26±9</td>
<td>22±4</td>
</tr>
<tr>
<td>% Middle-third</td>
<td>37±6</td>
<td>38±9</td>
<td>33±6</td>
</tr>
<tr>
<td>% Lower-third</td>
<td>39±7</td>
<td>36±9</td>
<td>45±9</td>
</tr>
<tr>
<td>% Ferroportin positive (% total)</td>
<td>31±6</td>
<td>30±11</td>
<td>31±8</td>
</tr>
</tbody>
</table>

Acid mucopolysaccharides identified by Alcian blue (AB) staining and total goblet cell numbers by combined AB + periodic acid, Schiff's (AB-PAS) staining of at least 50 complete crypt-villi units from 4 different sections. Significant different expression of acid mucopolysaccharides in goblet cells of iron deficient compared with control (c) or iron loaded animals (l). c,l: significant difference from acid mucopolysaccharide within iron deficient group. nd: not detectable.

Ferroportin expression along villi of the duodenum

In enterocytes, ferroportin was found throughout the enterocyte but was strongest along the basal membrane and cytoplasm (Fig. 1B). It was also seen weakly along the lateral membrane (Fig. 1D). Some expression was seen immediately above the nucleus (Fig. 1D). The extent of expression in enterocytes was strongest in the iron deficient state and least with iron loading.

We also showed that 30% of the total villus goblet...
cell population is positive for ferroportin expression on the mucous granule membrane (Fig. 1). This was the case irrespective of the iron content of the diet (Table 1).

Control studies

Importantly, these distributions of ferroportin were lost when the tissue was incubated with antibody and purified immunizing peptide (Data not shown).

Ferroportin mRNA expression

Only villus epithelium express ferroportin mRNA, crypt epithelium and the cells within the lamina propria did not express this gene (Fig. 2A).

Although some goblet cells contained grains in the theca the pattern of these grains did not always follow the contours of the goblet cell (Fig. 2A,C). Therefore it was not possible to determine whether goblets cells express ferroportin by in situ hybridisation.

Goblet cells contain iron and the mucous membrane express ferroportin

Iron loading increased the amount of iron in enterocytes. In addition, towards the villus tip some goblet cells stained positive for Perl’s Prussian blue in the mucous granules (Fig. 3A,B) and the membrane of

![Fig. 2. Ferroportin mRNA expression in the duodenum. Tissue was hybridised with either antisense (A, C) or sense (B) riboprobes and expression determined by in situ hybridisation. The tissue was exposed to the emulsion for up to 35 days. No discernible grain distribution was seen in the crypt region (below arrows in A and B) between antisense and sense labelled probes indicating that ferroportin mRNA was not produced by cells of the crypt. Ferroportin mRNA was only detected in enterocytes lining the villus (A and C). The lamina propria and goblet cells were negative (A and C). Higher power magnifications of goblet cells (arrows) showed no apparent expression over goblet cells (C). Bars: 10 µm.](image1)

![Fig. 3. Represents the same tissue firstly immunoreacted to detect ferroportin (A), then with Perl’s Prussian blue to identify iron deposits (B) and finally with AB + PAS to identify goblet cells (C). Two goblet cells (C) are shown to express ferroportin in their mucous granule membranes (A), and that these granules are positive for iron (B). Ferroportin positive mucous membrane granules are outlined by arrows. Bar: 10 µm.](image2)
these granules were ferroportin positive (Fig. 3B,C).

Discussion

In this study we investigated sites of ferroportin expression along duodenal villi of rats under conditions in which the efficiency of iron absorption was altered (Oates and Morgan, 1996a). As previously shown iron deficiency increased ferroportin expression in enterocytes along the basal and lateral membranes (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). In addition to membrane expression, strongest intracellular expression was seen in the basal cytoplasm and directly above the nucleus. This expression was markedly reduced when the rats were fed an iron-loaded diet. Thus the expression of ferroportin in enterocytes was inversely related to iron loading (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000).

During this investigation it became apparent that some goblet cells also express ferroportin, but these cells were difficult to quantitate by counting at low power because the villus structure is dominated by enterocytes that exhibit strong ferroportin expression. To overcome this technical problem we video imaged the villus at high power and then stained the sections with AB+PAS to identify all goblet cells. The same villi were identified again and total goblet cell numbers quantitated. From the two images we showed that 30% of goblet cells expressed ferroportin in the mucous granule membrane, irrespective of the efficiency of iron absorption. From these studies it was not possible to determine whether all goblet cells express ferroportin at some point in their migration along the villus and that the protein is lost during a cycle of mucous discharge. However, since the goblet cells expressing ferroportin were evenly distributed, as were goblet cells along the length of the villus axis, it suggests that only a subpopulation of these cells express ferroportin. This method may be useful in determining the expression of a protein in a minor population of cells.

The significance of ferroportin in the mucous granule membrane of some goblet cells requires comment. It is possible that ferroportin acts to transport iron into the mucous granule, causing it to be concentrated and permitting the iron to be lost during mucous granule discharge. It has been suggested that goblet cells excrete iron (Refsum and Schreiner, 1980, 1984), although since iron excretion by the body is relatively small, the physiological relevance of this route appears limited (Dubach et al., 1955; Green et al., 1968, LeSage et al., 1986, Britton et al., 1994). Nonetheless, it has previously been suggested that goblet cells may account for a 50% loss of body iron when animals are taken from a diet containing carbonyl iron to one deficient in iron (Oates et al., 2000a). To test the hypothesis that ferroportin positive mucous granule membranes contain iron we performed Perl’s staining of iron-loaded duodenum in order to reveal the presence of iron deposits. Supporting this we found that some goblet cells was positive for iron and ferroportin.

The hypothesis that ferroportin operates intracellularly to transport iron into mucous granules is new and may also be relevant to the enterocyte since abundant ferroportin is seen in a supranuclear position. Thus it is possible that ferroportin operates at this site to move internalised iron into an organelle/vesicles during the absorption of iron. If this is the case then this may in part account for the inhibition in uptake seen in our previous studies when a blocking antibody to ferroportin was used. Further studies are required to clarify this issue (Thomas and Oates, 2004a,b).

Although we attempted to find ferroportin mRNA expression in goblet cells by in situ hybridisation we were unsuccessful. A similar finding of ferroportin protein expression without detection of the mRNA encoding ferroportin has been reported in neurons, astrocytes and epithelium of the choroid plexus of the brain (Wu et al., 2004). This suggests that the technique of in situ hybridisation may not be sensitive enough to pick up the small amounts of mRNA in goblet cells. Alternatively, it is possible that ferroportin may be acquired from adjacent enterocytes. It is known that enterocytes release membrane vesicles called exosomes (Van Niel et al., 2001, 2003). If exosomes express ferroportin and these are acquired by goblet cells then this could account for the presence of ferroportin in cells that do not appear to express the gene. Certainly exosomes are taken up by other cell types (de Gassart et al., 2004).

In addition to iron excretion there are several studies that show mucous from goblet cells may play a role in iron absorption (Wein and Van Campen, 1991; Conrad et al., 1999; Simovich et al., 2003). Absorption of iron is made difficult because iron precipitates at the neutral pH of the intestine making it unavailable for absorption (Conrad et al., 1999). It has been suggested that mucous derived from goblet cell traps and binds iron, circumstances that would make the iron available to the enterocyte for absorption (Conrad et al., 1999). Supporting this hypothesis, Conrad and co-workers (1999) showed that mucous could bind iron-Fe(III). The ability of mucous to chelate iron would make it able to donate iron to the enterocyte. Although these properties of mucous would favour iron absorption others have shown that in iron deficiency when iron absorption is highest mucous production is reduced (Wein and Van Campen, 1991). It is therefore likely that the amount of mucous produced under iron deficient conditions can bind iron in excess of that required for absorption. This suggests that if mucous plays a role in iron absorption it must be in other ways that possibly involve ferroportin.

In the process of determining goblet cell numbers we looked at AB and AB+PAS positive goblet cells in rats with variations in iron loading. We found that total goblet cell numbers as determined by AB+PAS staining were not different among the three groups, however AB positive goblet cell numbers were reduced by iron
deficiency. Since AB stains acid mucopolysaccharides and PAS identifies the presence of neutral ones it appears that goblet cells have differentially reduced their expression of acid mucopolysaccharides while maintaining neutral mucopolysaccharide expression in iron deficiency. The finding of variations in acid mucopolysaccharide expression in a subpopulation of goblet cells is consistent with our finding of expression of ferroportin in some goblet cells. Others have also noted heterogeneous expression of mucin isoforms along the crypt-villus axis and in goblet cell lines (Etzler and Braunstrator 1974; Stanley and Phillips, 1999).

Acknowledgements. We acknowledge the National Health and Medical Research Council of Australia for funding this work (grant #211940) and Alan Light for technical assistance.

References


Accepted January 21, 2005