JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Moving Toward a More Physiological Model: Application of Mucin To Refine the in Vitro Digestion/Caco-2 Cell Culture System

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The objective of this study was to determine if a combination of commercially available mucin and an 8 μm microporous membrane insert can be used to replace the 15 kDa molecular weight cutoff (MWCO) dialysis membrane used in an established in vitro digestion/Caco-2 cell culture system. Although the current model with the 15 kDa membrane correlates well with human studies, use of mucin may improve the system as the mucus layer is suspected to play a physiological role in Fe absorption. Use of mucin may also enable more complete assessment of iron bioavailability from large molecular weight forms of Fe such as heme and ferritin Fe. A range of foods or Fe (i.e., $FeCl_3$ $\pm$ ascorbic acid, cooked beef, red bean, white bean, soybean, horse spleen ferritin and plant-type ferritin) were subjected to in vitro digestion. In the presence of mucin, significantly more Fe was taken up from the heme Fe (86%) and ferritin (91%) samples and significantly less Fe was taken up from the white bean samples ($\sim$70%) relative to the 15 kDa membrane. The results indicated that the forms of iron interact with mucin. The mucus layer has a significant effect on Fe uptake. Further refinement and characterization of the mucin method is needed before it can be deemed to be a suitable replacement for the dialysis membrane.

KEYWORDS: Mucin; iron; iron bioavailability; in vitro digestion; Caco-2

INTRODUCTION

Iron deficiency is the most common nutritional disorder in the world, affecting billions of people in both developed and developing nations (*1*). The direct primary cause of Fe deficiency is poor dietary Fe bioavailability, due to diets high in inhibitors (i.e., phytate, polyphenolics) and low in promoters (i.e., meat, ascorbic acid) of Fe uptake (*2*). Moreover, increased consumption of staple food crops such as wheat, maize, and rice that tend to be low in Fe concentration and less consumption of legumes and other pulse crops high in Fe are contributory factors to Fe deficiency anemia (*3*, *4*).

Dietary strategies to combat Fe deficiency include fortification, consumption of a diverse diet that includes meat, and biofortification, a process that involves improving Fe concentration and bioavailability in staple food crops such as rice, beans, maize, and wheat. All of these strategies require the ability to assess a broad array of factors and potential food interactions. Such interactions are too numerous to be addressed via in vivo studies; thus, nutritionists and food scientists have pursued in vitro methods (*5*).

The current state of the art with in vitro measurement of Fe bioavailability is an in vitro digestion/Caco-2 cell culture system (*6*, *7*). It has been documented to be a sensitive in vitro tool for Fe availability measurement with characteristics such as low cost, ease of use, and widespread acceptance. It has been applied for the evaluation of iron availability from commercial iron preparations (*8*), infant formula (*6*), fruit juices (*9*), rice of different genotypes (*10*), and maize of different varieties (*11*). Although the current model with the 15 kDa membrane is well validated in human studies, some further refinement and modification may make the system more physiological and enable more accurate assessment of iron bioavailability from large molecular weight forms of Fe such as heme and ferritin Fe.

Iron absorption occurs and is regulated in the duodenum and jejunum (*12*). The small intestine excretes numerous endogenous ligands that bind metals to help them remain soluble even at neutral pH (*13*). In 1943, Hahn postulated that a mucosal receptor was responsible for the regulation of iron absorption (*14*). In the early 1990s, Conrad and co-workers identified a series of iron-binding proteins in duodenal mucosa of rats and proposed a mucin−integrin mobilferrin pathway for iron absorption (*15*). In their experiments, mucin was isolated and shown to be iron binding at an acidic pH when iron is soluble. Once an iron−mucin complex was formed, it kept the iron soluble even at elevated pH values close to a neutral value. At neutral pH, mucin can also accept iron from its chelates of ascorbate, fructose, and histidine (*16*). These findings pointed to the fact that intestinal mucin delivers inorganic iron to

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10.1021/jf061684i CCC: $33.50
Published on Web 10/25/2006

intestinal absorptive cells in an acceptable form for absorption. In more recent work, both divalent metal transporter (DMT-1) and mobilferrin were shown to be concentrated in the intestinal mucosa due to increased binding of the proteins to mucin (*17*).

The present study represents the initial efforts to refine the established in vitro digestion/Caco-2 cell model. In this work, dialysis membranes of different kilodalton MWCOs were compared to a mucin solution placed on the cell monolayer prior to the start of the intestinal uptake period. A series of sample digests were placed on the kilodalton MWCO dialysis membranes and mucin solution/8 μm membrane insert for the comparison of Caco-2 cell iron uptake through evaluation of ferritin formation.

MATERIALS AND METHODS

Chemicals, Enzymes, and Hormones. Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental Design. Two sets of experiments were conducted. Dialysis membranes of 3.5, 15, and 50 kDa MWCO were compared to a mucin solution placed on the Caco-2 cell monolayer with an 8 μm membrane insert placed above the mucin solution to allow for the removal of some of the digests to help keep the cells attached. Iron bioavailability from a large variety of foods and chemical forms was assessed on the basis of Caco-2 cell ferritin formation.

Experiment 1. The objective of this experiment was to compare Fe bioavailability from a broad range of foods containing several forms of Fe [i.e., $FeCl_3$, $FeCl_3$ + ascorbic acid (AA), cooked beef, cooked red bean, cooked white bean, uncooked whole soybean, horse spleen ferritin (HSF)] when either dialysis membranes of 3.5, 15, and 50 kDa MWCO or a mucin solution was placed on the Caco-2 cell monolayer prior to intestinal digestion. Most dietary heme iron is in meat, and ~80% of the total iron in beef is heme iron. Therefore, the iron bioavailability of beef can be seen as an indication of heme iron bioavailability when MWCO inserts are compared with mucin/8 μm membrane.

Experiment 2. The objective of this experiment was to compare a 15 kDa MWCO dialysis membrane/Caco-2 cell culture system with a mucin/8 μm insert/Caco-2 cell culture system on Fe bioavailability from a suite of samples including $FeSO_4$, $FeSO_4$ + AA, reconstituted HSF, reconstituted HSF + AA, reconstituted HSF + fish, reconstituted plant-type ferritin (P-HSF), reconstituted P-HSF + AA, reconstituted P-HSF + fish, cooked reconstituted plant-type ferritin (CP-HSF), CP-HSF + AA, and CP-HSF + fish.

Sample Preparations. *Experiment 1.* Stock solutions of $FeCl_3$ of 1040 μg of Fe/mL (18.6 M) in 0.1 M HCl (Sigma 1-9011) was used in experiment 1 to provide a Fe concentration of 50 μmol/L per 30 mL of sample digest. To prepare for $FeCl_3$ sample digest, 80.5 μL of $FeCl_3$ stock solution was added to 20 mL of 140 mM NaCl, 5 mM KCl, pH 2, solution to initiate the gastric phase digestion and made up to a final volume of 30 mL of solution at the start of intestinal digestion. The $FeCl_3$ + AA sample digest was prepared in a similar manner except that 100 μL of the freshly prepared AA stock solution (52.8 mg/mL) was added to get a molar ratio of Fe/AA of ~1:20. Both white bean (Ituri Matata variety from Kenya) and red bean (JCSCA variety from Kenya) samples were cooked and stored at −20 °C until ready to use. Soybean sample (from a local grocery store) was used without any cooking procedure. To initiate the gastric phase digestion of the bean samples, 1.0 g of the white bean, red bean, or soybean sample was added to 20 mL of 140 mM NaCl, 5 mM KCl, pH 2, solution and made up to a final volume of 30 mL of sample digest at the start of intestinal digestion. Beef digest was prepared by starting with 0.5 g of the cooked beef and made up to a final volume of 30 mL of sample digest at the start of intestinal digestion. Stock solution of horse spleen ferritin (Sigma F4503, 91 mg of ferritin/mL) was used to prepare the horse spleen ferritin sample digest, and the Fe concentration in the final 30 mL of digest was 50 μmol/L.

Experiment 2. A 3.3 mmol/L $FeSO_4$ stock solution was made fresh from ferrous sulfate salt, and the final Fe concentration in the sample digest was 50 μmol/L. To prepare for $FeSO_4$ sample digest, 113 μL of $FeSO_4$ stock solution was added to 5 mL of 140 mM NaCl, 5 mM KCl, pH 2, solution to initiate the gastric phase digestion and made up to a final volume of 7.5 mL of solution at the start of intestinal digestion. $FeSO_4$ + AA sample digest was prepared in a similar manner except that 100 μL of the freshly prepared AA stock solution (13.2 mg/mL) was added to get a molar ratio of Fe:AA of ~1:20.

Reconstituted HSF or P-HSF was prepared from apoferritin (Calzyme, San Luis Obispo, CA) according to the method given in references *18* and *19*. The reconstituted ferritins are essentially indistinguishable from natural forms (*19*, *20*). To prepare for CP-HSF, P-HSF was cooked at 160 °F (i.e., 72 °C) for 1 h according to USDA meat-cooking regulations (*21*). One hundred and thirteen microliters of the reconstituted HSF, P-HSF, or CP-HSF stock solution (Fe concentration = 3.3 mM) was added to 5 mL of 140 mM NaCl, 5 mM KCl, pH 2, solution to initiate the gastric phase digestion and made up to a final volume of 7.5 mL of sample digest at the start of intestinal digestion. Reconstituted HSF + AA, P-HSF + AA, or CP-HSF + AA sample digest was prepared in a manner similar to that of reconstituted HSF, P-HSF, or CP-HSF except that 100 μL of the freshly prepared AA stock solution (13.2 mg/mL) was added to get a molar ratio of Fe/AA of ~1:20. Cooked, lyophilized fish muscle tissue was used as a source of "meat factor" in this experiment. The preparation procedure for the fish is the same as in previous work (*22*). Fish acid extract was dissolved in 0.01 N HCl to achieve a concentration of 100 mg/mL. Ten milliliters of the above solution was processed through a stirred ultrafiltration cell (Millipore, U.S. model 8200) fitted with a 5000 molecular weight cutoff membrane, and the filtrate was collected. Reconstituted HSF + fish, P-HSF + fish sample, or CP-HSF + fish sample digest was prepared in a manner similar to that of reconstituted HSF + AA except that 100 μL of the fish acid extract solution filtrate was added to a final volume of 7.5 mL of sample digest.

Cell Cultures. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 29−32. Cells were seeded at a density of 50000 cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM^3, GIBCO, Grand Island, NY) with 10% v/v fetal bovine serum (FBS, GIBCO) and 25 mmol/L HEPES (Sigma, St. Louis, MO). Cells were maintained at 37 °C in an incubator with a 5% CO_2, 95% air atmosphere with constant humidity, and the medium was replaced every 2 days. Cells for each study were used 14 days postseeding. Under these conditions, the amount of cell protein measured in each well (2.8 mg of protein/well) was found to be highly consistent from well to well within each culture plate.

In Vitro Digestion/Caco-2 Cell Culture Method. In vitro digestion and enzyme preparation methods have been described in detail elsewhere (*6*). Briefly, uptake samples were combined in 50 mL tubes as described above. To initiate the gastric phase of digestion, a 140 mM NaCl, 5 mM KCl, pH 2, solution was added to the sample tube. After pH adjustment to 2 with 1 N HCl, 0.5 mL of pepsin solution was added to each tube, and the mixtures were incubated for 1 h at 37 °C on a rocking platform (model RP-50, Laboratory Instruments, Rockville, MD). After incubation, the pH was raised to 5.5−6.0 with 1 M $NaHCO_3$, and 2.5 mL of pancreatin/bile solution was added to each mixture. The pH was then adjusted to approximately 7.0, and the final volume contained within each tube was adjusted by weight using a 140 mM NaCl, 5 mM KCl, pH 6.7, solution. At this point, the mixtures were referred to as "digests".

To initiate the intestinal digestion period, a 1.5 mL aliquot of the digest was placed into the upper chamber of a two-chambered system formed by placing well inserts fitted with MWCO dialysis membranes into plate wells containing Caco-2 cell monolayers. Plates were covered and incubated at 37 °C in an incubator with a 5% CO_2, 95% air atmosphere with constant humidity for 2 h on a rocking platform (approximately 12 oscillations/min). Following 2 h of incubation, the inserts were carefully removed and an additional 1 mL of DMEM was added to each well. The cell plates were then re-placed into the incubator to allow time for ferritin formation. Cells were harvested after 22 h (24 h from the start of the intestinal digestion).

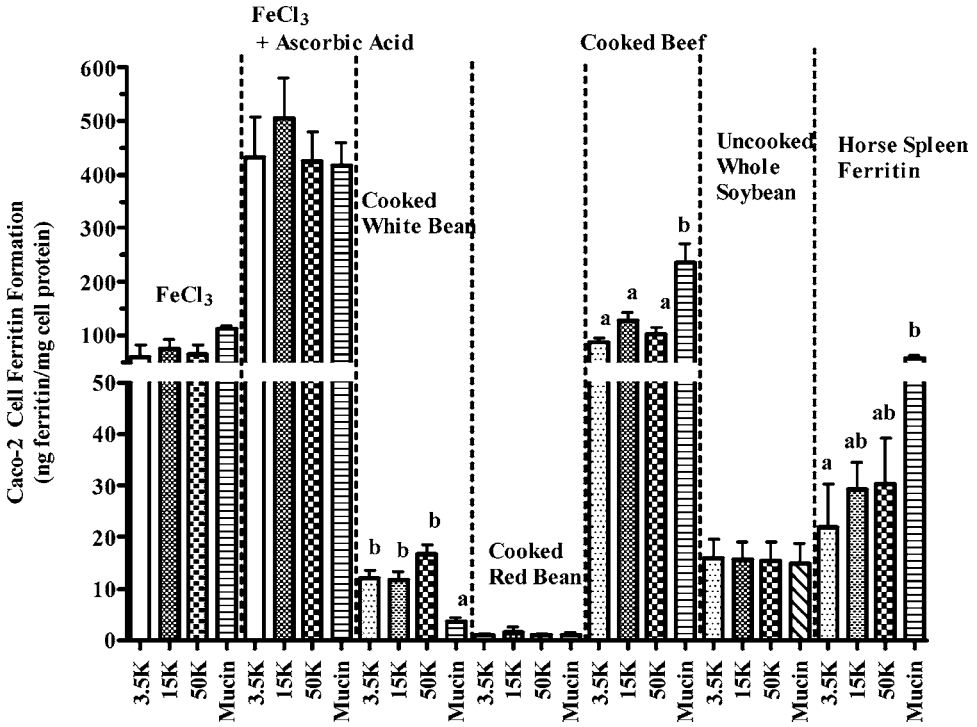

Figure 1. Caco-2 cell ferritin formation in response to digests, MWCO membranes, and mucin application. Digests contained a constant level of iron concentration of 50 μmol/L and a molar ratio of Fe/AA of 1:20 when AA was present. Values are mean ± SEM. Each data set represents two separate experiments done on three different days. Bars with no letters in common are significantly different ($n = 6$, $p < 0.05$).

Preparation and Application of Mucin on Caco-2 Cell Monolayer. Mucin stock solution of 25 mg/mL was made from porcine stomach mucin (M 1778, Sigma) with a pH 2.0 salt solution of 140 mmol/L NaCl and 5 mmol/L KCl. The mucin supplied by Sigma originally contained 274 ppm of Fe, a relatively high Fe concentration indicating that it readily bound Fe in the gastrointestinal tract. For this mucin to be useful in our system and not produce an artificial inflation of Caco-2 cell ferritin formation, the mucin was first treated with a resin to reduce the iron concentration to approximately 16 ppm of Fe. Chelex-100 (Bio-Rad, Hercules, CA) chelating resin was mixed with mucin stock solution, and the mixture was poured into a glass column (i.d. = 1.5 cm, length = 45 cm) to allow for the draining through of mucin solution with iron being removed. Mucin stock solution was stored at −20 °C when not in use. The mucus gel covering the gastroduodenenal mucosa constituted ∼3−5% (∼30−50 mg/mL) of mucin plycoproteins and 95% of water, lipids, nucleic acids, and other proteins (*23*). Personal communications with Dr. Adrian Allen (Department of Physiological Sciences, University of Newcastle upon Tyne, U.K.) suggested a range of 5−10 mg of mucin/mL would be appropriate to apply on the Caco-2 cell monolayer. An evaluation of a range of mucin concentration (1.5−12.5 mg/mL) applied on the Caco-2 cell monolayer was done to find that a mucin concentration of 5 mg/mL provided sufficient protection to the cell monolayer while keeping iron contamination at a minimum. After the application of mucin and sample uptake, the Caco-2 cell monolayer was seen to firmly attach to the plate, which proved the existence of functional tight junctions. The cell protein content of each well was a good indicator of the total number of cells present, and these values did not differ from the wells that were not exposed to mucin and the in vitro digest. Thus, a 5 mg/mL mucin solution was used for all of the experiments in the present work. Prior to each experiment, the iron-removed mucin stock solution was thawed and diluted with DMEM to give a final concentration of 5 mg of mucin/mL. Eight micrometer transwell tissue culture membrane inserts (Corning Inc.) were placed on the mucin solution before sample digests were added.

Protein and Ferritin Assay. All glassware used in sample preparation and analyses was rinsed with 10% HCl and 18 MΩ deionized water before use. Caco-2 cell ferritin formation served as a marker of cell Fe uptake. Caco-2 cell protein content of samples was measured, after solubilization in 0.5 mol/L NaOH, using a Bio-Rad DC protein assay kit, which is a commercial semimicro adaptation of the Lowry assay (Bio-Rad Laboratories). An immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Serum Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 μL sample of the sonicated Caco-2 cell monolayer in 18 MΩ deionized water, harvested in 2 mL, was used for each ferritin measurement.

Statistical Analyses. Data were analyzed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). ANOVA with Tukey's post-test was used to compare differences among means. When appropriate, data were transformed to achieve equal sample variances. Differences among means were considered to be significant at $P \leq 0.05$.

RESULTS

In general, the MWCO of the dialysis membrane did not influence Fe availability from the whole range of samples. In the presence of a mucin solution and an 8 μm membrane insert, significantly more Fe was taken up from the heme Fe and ferritin sample (∼86−173 and ∼85−150%, respectively) and significantly less Fe was taken up from the white bean samples (∼70%) (**Figure 1**). There was no difference in Fe absorption from $FeCl_3$, $FeCl_3$ + AA, red bean, and soybean when either dialysis membranes of 3.5, 15, and 50 kDa MWCO or a mucin solution and 8 μm pore size membrane insert were applied to the Caco-2 cell culture system (**Figure 1**).

Fe bioavailability from reconstituted HSF, reconstituted HSF + AA, reconstituted HSF + fish, reconstituted P-HSF, CP-HSF, and CP-HSF + AA was significantly higher when mucin/8 μm membrane insert was used in place of a dialysis membrane of 15 kDa MWCO (**Figure 2**). Although there was no significant difference, more cell ferritin was seen formed from uptake of $FeSO_4$, $FeSO_4$ + AA, P-HSF + AA, P-HSF + fish, and CP-HSF + fish when mucin/8 μm membrane insert was applied instead of the 15 kDa MWCO dialysis membrane (**Figure 2**).

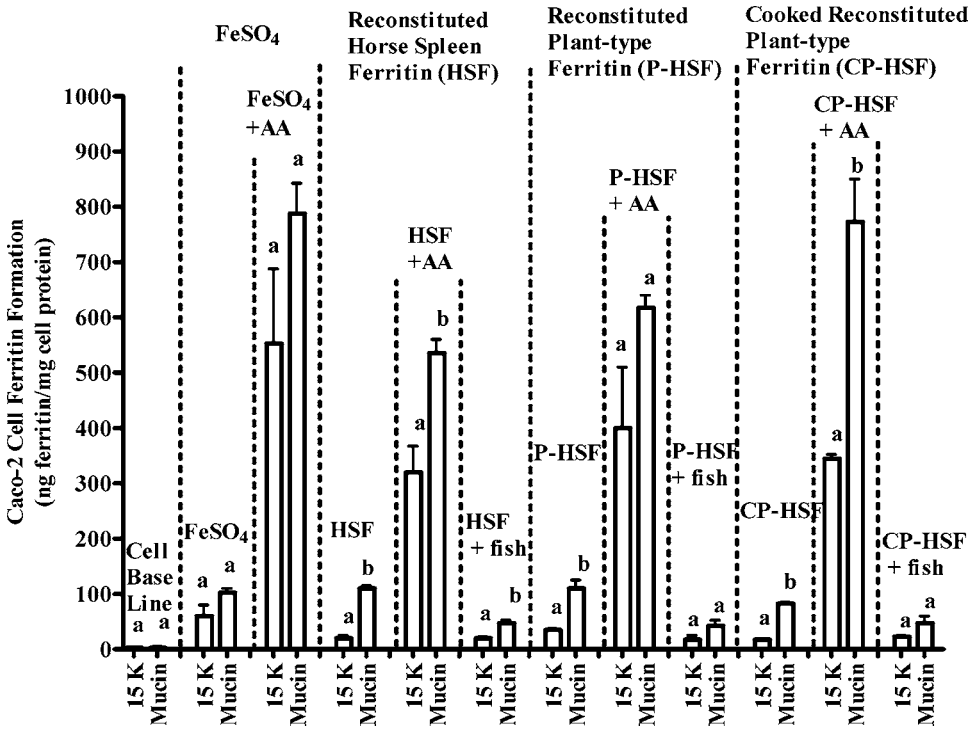

Figure 2. Caco-2 cell ferritin formation in response to digests with both 15 kDa MWCO membranes and mucin treatment. Ascorbic acid was present at a molar ratio of Fe/AA at 1:20 when AA was present. Fish was added in the form of diluted fish acid extract solution. Digest iron concentration was 50 μmol/L. Ferritin was chemically reconstituted in the same manner as reported by Lonnerdal et al. (*19*). Values are mean ± SEM, $n = 3$. Data within each sample treatment between 15K and mucin were analyzed by unpaired *t* test after logarithmic transform. Each data set represents three separate experiments done on the same day. Bars with no letters in common are significantly different ($p < 0.05$).

There are some key issues that may negate the use of mucin as a replacement in the dialysis membrane method. One of the problems relates to cell survival. Follow-up experiments to the ones published in this paper observed occasional cell loss when mucin was used in place of the MWCO membranes. Obviously, when cell loss happens, the whole experiment is compromised and has to be repeated to avoid misinterpretation of results, which raises the cost in terms of sample and reagent usage, human labor, and experiment time. It indicates that despite the best efforts of trained technicians, the mucin conditions are not easily reproduced. An attempt to reduce cell loss by reducing the regular 24 h cell uptake time to ~4.5 h was unsuccessful due to the low level of cell ferritin formation under the shortened period of time.

DISCUSSION

Gastroduodenal mucus is secreted as a water-insoluble gel adherent to the mucosal surface and is also present as a viscous, soluble form in the lumen. In 1835, Nicolas Theodore de Saussure introduced the term "mucin" to describe substances isolated from mucus (*24*). In 1865, the pioneering work by Eichwald led to the knowledge of mucin as a combination of protein and carbohydrate (*25*). The current knowledge on mucin generalizes it as "a group of glycoproteins that is the main component of the mucus layer that covers the epithelium of the gastroduodenal intestinal tract (GIT) as well as all epithelia of mammals" (*26*). The main function of the mucus is to protect the epithelium from chemical, enzymatic, physical, and bacterial aggressors that may be present in the intestinal lumen (*27*). The breakdown of mucus gel by either proteolysis or physical and chemical damages can result in the release of mucin into the lumen and thereby in the chyme, where further proteolysis of mucin is hindered by the oligosaccharide coat, which covers up to 80% (by weight) of the protein backbone (*27*).

In the gut, mucin is produced by goblet cells in the intestinal mucosa and secreted into the intestinal lumen, where it forms an insoluble coating of the epithelial surface. The mucus layer not only lubricates the gastrointestinal epithelium but also helps with the digestive processes by creating a digestion zone in which enzymes are immobilized near the epithelium surface for longer periods of nutrient hydrolysis and absorption (*26*). The mucus layer also functions as a protective permeability barrier to allow the diffusion of suitable nutrients to be absorbed while blocking large compounds from filtering onto the epithelium (*26*). Ions and small molecules (molecular weight < 1000) can pass through the mucus layer, but large molecules such as proteins (molecular weight > 17000) are reported to be impermeable (*28*).

Due to the important function of mucus in animal gastroduodenal digestive system, it would be ideal to grow goblet cells together with Caco-2 cells to have a coculture in vitro system that is more physiological to evaluate nutrient uptake such as iron absorption by the human body. Coculture systems of Caco-2 cells and mucus-secreting goblet cell clone HT29-MTX were developed to study the permeability of a range of different drugs to compare with either human or rat data (*29*, *30*).

The currently identified iron absorption pathways include uptake of heme iron, ferric iron, and ferrous iron. Heme iron absorption starts when heme is taken into enterocytes as a metalloporphyrin through an endosomal process, whereas nonheme ferric iron was thought to be taken up through a β3 integrin and mobilferrin pathway (IMP) and ferrous iron is absorbed via a DMT-1 pathway (*12*). However, recent evidence

suggests that this might also be an endosomal pathway (*31*). The fact that large quantities of both mobilferrin and DMT-1 are found in goblet cells and intraluminal mucins in the gut of iron-deficient rats indicates that these iron uptake proteins are secreted with mucin into the intestinal lumen to bind iron to facilitate uptake by cells (*17*, *32*). The mucin serves to chelate and render the otherwise insoluble ferric iron available for transport into the intestinal mucosal cell.

The results in the present study indicate that the different forms of iron interact with mucin and that the mucus layer has a significant effect on Fe uptake. The observation that iron from beef (i.e., a combination of heme and nonheme Fe) and ferritin iron from horse spleen ferritin had significantly higher cell uptake when mucin was applied instead of the MWCO membranes supports this hypothesis. Moreover, iron from white beans had significantly lower bioavailability when mucin was applied on the surface of Caco-2 cells in place of the MWCO membranes, which also indicates interaction between the iron form and mucin in an in vitro cell culture system. Iron forms such as $FeCl_3$, $FeCl_3$ + AA, and iron from red bean and soybean had the same levels of bioavailability whether MWCO membranes or mucin was applied. In summary, the mucin treatment alters the Fe bioavailability relative to the dialysis membrane conditions, thus lending evidence to support the possibility that a mucin layer has a significant effect on food iron bioavailability when the Fe form is heme or from large molecules such as ferritins. Whether this observation is a real effect, or simply an artifact of the in vitro model conditions, needs to be determined in future experiments.

Ferritin iron bioavailability has long been debated. Recently reconstituted ferritins showed high bioavailability for both humans and rats (*18*, *19*). Our previous work indicated that reconstituted ferritin iron responds to promoters and inhibitors in a similar manner as ferrous sulfate. The similar cell uptake and iron release results from ferritins and ferrous sulfate suggested that ferritin iron exchanges easily with other compounds in a meal (*33*).

The observation that reconstituted ferritins and ferrous sulfate responded in a similar manner with the presence of promoters when either 15 kDa MWCO membrane or mucin was applied is consistent with our previous findings. Caco-2 cell ferritin formation for both ferritins and ferrous sulfate was higher when mucin was applied in place of the 15 kDa MWCO membrane. The cooking process of ferritin did not alter the bioavailability of its iron, and the cooked ferritin had basically the same response as noncooked ferritin in the presence of promoters AA and fish whether 15 kDa MWCO membrane or mucin was applied, although iron bioavailability was also higher in the case of mucin.

Another issue remaining is the fact that removal of Fe from the mucin is necessary before it can be used in an experiment. As mentioned under Materials and Methods, the mucin product supplied by Sigma is relatively high in Fe (274 ppm). This observation alone indicates the potential for mucin to complex Fe. Obviously contamination in mucin compromises the precision and accuracy of the results if the present iron in mucin is not removed.

Finally, the mucin applied in the present study was from a product produced by a pepsin digestion of the mucus from the gastric surface of the porcine stomach. With this processing treatment it is not of the same polymeric structure as that produced by the goblet cells in the intestine. However, the availability and relatively low cost of this product and the lack of an available source of undigested mucus made it attractive to test as an alternative to the dialysis membrane approach.

In summary, the different forms of iron interact with mucin and have a significant effect on Fe uptake. Although these results are interesting, we do not recommend the use of this commercial mucin product for the reasons mentioned above. A more effective and physiological approach may be coculture of goblet cells and Caco-2 cells (*29*, *30*). This coculture approach may ultimately refine this in vitro method and eliminate the need for the dialysis membrane. Experiments in the coculture method are ongoing and will be published in the future.

ACKNOWLEDGMENT

We thank Pei Pei Cheng, Mike Rutzke, Mary Bodis, and Zhiqiang Cheng for assistance in this research.

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Received for review June 15, 2006. Revised manuscript received September 15, 2006. Accepted September 25, 2006.

JF061684I