Transport of Iron Bound to Recombinant Human Lactoferrin from Rice and Iron Citrate Across Caco-2 Cell Monolayers

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The possibility of using recombinant human lactoferrin from rice (rhLF) makes it necessary to study its differences from the protein of milk. In this work, the binding of different iron-saturated forms of rhLF to Caco-2 cells was studied. Iron-saturated rhLF bound in higher proportion than the apo-form, but, the data obtained for specific binding were not compatible with receptor-mediated binding. Competition assays showed the same binding capacity for human milk lactoferrin as for rhLF to Caco-2 cells. Another basic protein of milk, lactoperoxidase, was found to compete with rhLF for binding to Caco-2 cell membranes, suggesting an electrostatic interaction. The transport of iron (¹⁵⁹Fe) bound to rhLF and to citrate and the transport of rhLF (¹²⁵I-labeled) were studied on Caco-2 monolayers. Transport of iron was found to be significantly greater when bound to citrate than to rhLF. The amount of intact lactoferrin that traversed the Caco-2 monolayers was very low, suggesting degradation of it across these cells.

Key words: Caco-2 monolayers; cell binding; iron transport; rice recombinant human lactoferrin

Lactoferrin is an iron-binding glycoprotein of the transferrin family that was originally discovered in milk. Afterwards, it was detected in other external fluids including tears, saliva, and mucosal secretions, and also in the secondary granules of polymorphonuclear leukocytes. It has a molecular mass of 80 kDa and a bilobal structure, and each lobe can reversibly bind one ferric iron with the participation of bicarbonate. Lactoferrin is involved in many different functions, among them, the regulation of iron absorption in breast-fed infants. This possible function is supported by the fact that human lactoferrin has been found to bind to enterocytes. However, the role of lactoferrin in iron absorption in the gut is still not clear. In fact, some studies in rats have shown that a diet with lactoferrin as a supplement improves iron status, while others have not found any positive effect of lactoferrin on iron levels, or have shown a negative effect.

It is known that lactoferrin levels are higher in human milk than in bovine milk throughout lactation. Therefore, in some countries, manufacturers add bovine lactoferrin to infant formulas in order to increase the low content of this protein and make them more similar to human milk, but it is not known whether the activity of lactoferrin of bovine origin is the same as that of human origin when administered to infants. Production of recombinant human lactoferrin (rhLF) in various transgenic systems has been achieved, and this allows considering the use of this protein for different applications. The production of human lactoferrin in rice is especially interesting, as it is an easy and safe way to obtain this protein. Rice is a recommended food for infants and is used in allergen exclusion diets. Hence, lactoferrin from rice might be a useful ingredient in functional products, like formula milks or special products designed to alleviate diarrhea symptoms. Since human lactoferrin is a natural component of breast milk and the human body is also exposed to lactoferrin present in tears and saliva, it is not anticipated that the addition of recombinant human lactoferrin from rice to the diet is unsafe, and this has been corroborated in some studies. The main physico-chemical and biochemical characteristics of recombinant human lactoferrin from rice have been found to be practically the same to those of the protein isolated from milk. In this respect, the behavior of recombinant lactoferrin from rice is very similar to that of lactoferrin from human milk when studied by differential scanning calorimetry, indicating a comparable structure. However, differences in the glycosylation of lactoferrin of different origins have been reported. Lactoferrin from human milk has typical glycans of mammals, such as 2-6-linked neuraminic acid, β1-4-linked galactose, and α1-6-linked fucose, while lactoferrin from rice has typical glycans of vegetables, such as α1-3-linked fucose and β1-2-linked xilose. These differences might be important in some biological functions of the protein. Hence, it is important to study the biological activity of rhLF by different approaches if this protein is intended to be used as a supplement in special products. In this work, we studied the ability of rhLF to bind to Caco-2 cells and also the transport of iron bound to the protein through monolayers of these cells grown in bicameral chambers, an established model of the intestinal barrier, in order to make comparisons with previous studies done using human lactoferrin of other origins. The aim of this work was to acquire more data on the characteristics of rhLF from rice to determine whether its functionality in certain models is comparable to that of milk origin, supporting its use in functional products.

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Abbreviations: hLF, human milk lactoferrin; rhLF, recombinant human lactoferrin from rice
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Materials and Methods

Source of lactoferrin. Recombinant human lactoferrin from rice (rhLF) was kindly provided by Ventria Bioscience (Sacramento, CA). It was supplied in three forms, apo (0.05 mg of Fe/g LF), holo (1.3 mg of Fe/g LF), and as isolated from rice (0.98 mg of Fe/g LF). Lactoferrin purity was checked by SDS–PAGE, which showed a band corresponding to a protein with a molecular mass of about 80 kDa and purity higher than 90%. Hence, it was used without further purification.

Cell culture. Human colon carcinoma Caco-2 cells TC7 clone were kindly provided by the Department of Physiology of the Veterinary Faculty of Zaragoza University (Zaragoza, Spain). Cells were routinely grown in DMEM supplemented with 20% v/v fetal bovine serum, 2 mm 1-glutamine, 1% non-essential amino-acids, 100 U/ml penicillin, 100 μg/ml streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel), 25 mM glucose, and 2.5% HEPES (Sigma, Poole, UK). Cells were grown in 25 cm² tissue culture flasks to confluence and, were maintained at 37°C in 5% CO₂.

For the binding assays, cells were seeded into plastic tissue culture dishes 35 mm diameter at a density of 10⁵ cells/cm² and incubated for 15 d to obtain differentiated Caco-2 cells; the medium was replaced by fresh medium every other d.

For the transport studies, Caco-2 cells were seeded into Transwell bicameral chambers (Costar, Hygh Wycombe, UK) 3.0 to obtain differentiated Caco-2 cells; the medium was replaced by 500 μM type I (Boehringer, Mannheim, Germany), as follows, a volume of 0.2 mg/ml solution of collagen in 0.1 M acetic acid was added to each chamber and after removing the excess, and left to dry under sterile conditions before seeding the cells. The well contained 200 μl of culture medium in the upper chamber and 800 μl in the lower chamber which was replaced with fresh medium every d. Confluent cultures of differentiated cells were obtained after 21 d. Monolayer integrity was checked by measuring transepithelial electrical resistance (TEER) with an epithelial voltmeter (World Precision Instruments, New Haven, CT), as described by Alvarez-Hernandez et al., and by phenol red exclusion as described by Halleux and Schneider.

Caco-2 cell membrane binding assays. Biotinylination of lactoferrin. Biotinylination of the iron-saturated form of rhLF was performed as described by Rejjman et al. Fifty molar excess of N-hydroxy-succinimide-biotin (NHS-biotin; Sigma, Poole, UK) dissolved in DMSO was added to a solution of lactoferrin at a concentration of 2 mg/ml in phosphate buffered saline (PBS) (pH 7.4). The polycarbonate membrane of the inserts had been coated with rat tail collagen type I (Boehringer, Mannheim, Germany), as follows, a volume of 50 μl of a 2 mg/ml solution of collagen in 0.1 M acetic acid was added to each chamber and after removing the excess, and left to dry under sterile conditions before seeding the cells. The well contained 200 μl of medium in the upper chamber and 800 μl in the lower chamber which was replaced with fresh medium every d. Confluent cultures of differentiated cells were obtained after 21 d. Monolayer integrity was checked by measuring transepithelial electrical resistance (TEER) (≥800 Ω cm²) and by phenol red exclusion (<1.5% per h). After washing of both upper and lower chambers with serum-free medium, solutions of recombinant human lactoferrin from rice (rhLF) previously radiolabeled were added to the upper chamber.

In the experiments on iron transport, rhLF was previously iron saturated to 60% with ⁵⁹Fe-citrate (specific activity, 4.5 mCi/mg, Amersham Biosciences, Little Chalfont, UK) for 8 h and then unlabeled ferric nitroacetate (FeNTA) was added to saturate the protein completely during overnight incubation. To eliminate free iron, the protein was applied to a desalting column, PD-10 Sephadex G-25 (Amersham). To carry out the transport studies, the labeled protein was added to the upper chamber at a concentration of 50 μg/ml in serum-free medium. The transport of iron from Fe-citrate was studied by adding an equivalent amount of ⁵⁹Fe-citrate without any carrier protein. The lower chamber contained culture medium with apo-human transferrin (apo-hTF) (1 mg/ml) as iron acceptor in all wells. The medium was removed from the lower chamber for analysis at 1, 5, and 24 h of incubation, and was replaced by fresh medium also containing apo-hTF. At 24 h of incubation, the medium was taken from the upper chamber and the monolayer was washed with Hank’s balanced salt solution (Sigma), mixing the washings with the upper medium. After the addition of the original volumes of fresh culture medium to the upper and lower chambers, TEER was measured in order to check monolayer integrity after the assay. The radioactivity associated with the medium from the lower chambers at different durations of incubation, and also to that of the upper chambers and to the inserts containing the Caco-2 cell monolayers at 24 h was measured in a LKB Wallac 1428 Compugamma Universal Gammascanner (Turku, Finland).

The experimental conditions used to study the transport of rhLF were the same as those used in the iron transport studies. RhLF was iodinated by the chloramine T method, and the free ¹²⁵I was removed by chromatography in PD-10 Sephadex G-25 columns. The integrity of the ¹²⁵I-rhLF that passed across the Caco-2 monolayers was measured by precipitation of 300 μl of the lower chamber medium with 10% w/v trichloroacetic acid (TCA) and by determination of radioactivity associated with the pellet and to the supernatant after centrifugation.

Statistical analysis. The experiments were performed at least 3 times using freshly prepared samples. Mean and standard deviations were calculated from all the data obtained in the assays. The data were statistically evaluated by t test or ANOVA using the SPSS 14.0 package for Windows.
Results

Binding of rhLF to Caco-2 cell membranes

The results of the binding studies on recombinant human lactoferrin from rice (rhLF) in the forms of iron saturation (holo, as-isolated, and apo) to Caco-2 membranes are shown in Fig. 1. Although the forms of rhLF bound to these cells in a saturable manner, the non-specific binding obtained was very high for the three forms of the protein. Significant differences ($p < 0.05$) between holo- and apo-rhLF binding were found at the highest concentrations assayed ($1.5$ and $2.2\, \mu\text{mol/l}$). The data obtained for the specific binding of recombinant lactoferrin were subjected to Scatchard plot analysis, but the values did not adjust with good linear correlation for any of the assays.

In the present work, competition assays for evaluation of the binding of biotinylated recombinant human lactoferrin (B-rhLF) in the forms of iron saturation (holo, as-isolated, and apo) to Caco-2 membranes with increasing concentrations of unlabeled rhLF, human milk lactoferrin (hLF), human transferrin (hTF), lysozyme, and lactoperoxidase were done. The results obtained for the holo form are shown in Fig. 2; these were fairly similar to those obtained for the other forms of rhLF. At a constant concentration of B-rhLF ($0.4\, \mu\text{M}$), increasing concentrations of unlabeled rhLF, hLF, and lactoperoxidase competed with B-rhLF for binding to Caco-2 cell membranes but, increasing concentrations of unlabeled hTF and of lysozyme did not decrease the binding of B-rhLF.

Iron and rhLF transport across Caco-2 monolayers

The passage of iron ($^{59}\text{Fe}$) across the Caco-2 monolayers was significantly greater from iron citrate than from iron-saturated rhLF ($p < 0.05$) at all durations of extraction ($1$, $5$, and $24$ h) (Fig. 3). Great variability was observed in the percentage of iron transported by citrate, as compared to that from rhLF. The values of the $^{59}\text{Fe}$ radioactivity percentage associated with the upper chamber and cell monolayers and transported to the lower chamber at 24 h of incubation (accumulating the values of radioactivity associated with the medium of the lower chambers obtained from the three extractions) is shown in Table 1. The amount of iron transported across the monolayer was very low, even for citrate, whose value for accumulated radioactivity was about $2\%$. The proportion of iron associated with the cell monolayers was similar for the two iron carriers.

The passage of rhLF across the Caco-2 monolayers was determined by measuring $^{125}\text{I}$-activity in the lower chamber: the results are shown in Figs. 4 and 5. The percentage of accumulated $^{125}\text{I}$-activity at 24 h associated with rhLF in the lower chamber was $22.8\%$ and that
associated with the cells, 3.4%. The integrity of the $^{125}$I-rhLF that passed across the Caco-2 monolayers was estimated by measuring the radioactivity associated with the TCA-precipitable fraction, what was 4.4%. This indicates that most of the protein was degraded in the passage across the cell monolayer. It was confirmed that no degradation of the protein occurred in the upper compartment over the incubation period, since it was found intact at 1, 5, and 24 h of incubation when checked by TCA precipitation. Therefore, only 1% of the total protein added to the upper chamber traversed the membrane intact.

**Discussion**

In this work, we studied the ability of recombinant human lactoferrin from rice (rhLF) to bind to Caco-2 cell membranes and to transport iron through a monolayer of these cells. It was found that the three forms of rhLF bound to the Caco-2 cell membranes in a saturable manner. However, higher binding of the holo-rhLF than of the apo-rhLF was found, with significant differences at the highest concentrations, of 1.5 and 2.2 μmol/L. This was also observed by Davidson and Lönnerdal, who suggested that the affinity of the apo-human lactoferrin for the brush-border membranes of the infant Rhesus monkey small intestine was lower than that of lactoferrin saturated with iron. The analysis by the Scatchard method of the data obtained for the binding of rhLF to Caco-2 cell membranes under the experimental conditions used in the present work did not permit us to obtain the binding parameters of a putative receptor. In previous work by Sánchez et al., binding of human milk lactoferrin to Caco-2 cells was also reported, though the data obtained did not support the existence of a specific well-defined receptor, even though both the lactoferrin and the cells used were of human origin. Recently, we also studied the interaction of recombinant human lactoferrin from *Aspergillus awamori* with Caco-2 cell membranes and of lactoferrin from human milk (hLF). It was found that the binding of both types of lactoferrin to Caco-2 cell membranes was saturable, but the dissociation constant could be calculated only for the binding of apo-rhLF from *A. awamori*.

The results obtained in the competition experiments indicated that lactoferrin from human milk competed with rhLF for the binding to Caco-2 cell membranes. This confirms the similarity in the behaviors of the two proteins. The absence of competition by human transferrin demonstrated that rhLF does not bind to the transferrin receptor present in Caco-2 cells. This has also been demonstrated in various other cell types, mouse peritoneal cells, MAC-T bovine mammary epithelial cells, and monocytes.

Although some researchers have identified a so-called receptor for lactoferrin in cells, including intestinal cells, immune system cells, and cells of the lung, liver, kidney, and heart, the existence of a unique receptor for lactoferrin in all cells is still unproven. Furthermore, it has been observed that the binding of lactoferrin to membranes of cells, such as liver cells and a prononycotic cell line, is reduced in the presence of other basic proteins, such as lysozyme or lactoperoxidase, suggesting a form of non-specific binding to Caco-2 cell membranes. We have studied the effect of these basic proteins on the binding of rhLF from rice to Caco-2 cell membranes. Lactoperoxidase, with a molecular weight similar to that of lactoferrin, markedly inhibited the binding of rhLF. However, lysozyme, with a molecular weight lower than that of lactoferrin, did not inhibit lactoferrin binding, despite its basic nature. In fact, it has been reported that only the dimerized form of lysozyme inhibits the binding of lactoferrin, and in the present work lysozyme was used in the monomer form. These results are in agreement with those previously reported for hLF and rhLF from *Aspergillus awamori*. Moreover, the importance of the basic cluster present in the N-terminus of human lactoferrin in its binding to the human colon carcinoma cells HT29-18-C1 has been reported based on the use of variants of the protein without this part of the molecule. In work by Suzuki et al., who studied the interaction of lactoferrin chimeras with Caco-2 cells, the importance of a subdomain of the N-terminus was also determined. These findings confirm the importance of electrostatic interactions in the binding of lactoferrin with intestinal...
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cells, even if a receptor for this protein is expressed under certain conditions.

The characteristics of lactoferrin receptors reported in studies are not homogeneous in terms of molecular mass and number and affinity of binding sites. This suggests that lactoferrin binding to some cells is not mediated by a receptor as a well-defined protein. Some regions with high affinity for lactoferrin may exist in the cellular membrane, and interaction with them might be similar to receptor-mediated binding. Expression of a Ca^{2+}-dependent lectin, called intelectin, in the small intestine, has been reported as structurally identical to the intestinal lactoferrin receptor of the human enterocyte brush border. Intelectin can bind lactoferrin and other ligands such as the carbohydrate chains of the bacterial cell wall that contain galactofuranose, but does not bind other proteins such as bovine serum albumin or transferrin. However, as is shown by the work of Suzuki et al., lactoferrin-receptor/intelectin is expressed in many different human fetal and adult tissues, the expression of this protein in Caco-2 cells is very low. This might explain the results that we obtained in our study of low specific binding of lactoferrin to Caco-2 membranes.

The great bioavailability of iron and the higher concentration of lactoferrin in human milk compared with that of bovine milk lead us to suspect a role of lactoferrin in intestinal iron absorption in breast-fed infants. Here, we studied the passage of iron bound to rhLF from rice as compared to iron citrate through a monolayer of differentiated Caco-2 cells grown in bicameral chambers. This system extensively used to study intestinal iron uptake, as well as the effects of different carrier molecules on this uptake.

Data obtained for iron (59Fe) transport across Caco-2 cell monolayers showed significant differences in the transport of iron bound to rhLF and citrate. From the latter it was greater than from rhLF. This result is in agreement with previous studies in which iron transport higher from iron citrate than from human milk lactoferrin or from recombinant human lactoferrin obtained in Aspergillus awamori was found. However, lactoferrin has been suggested to be a safer iron supplement because the administration of inorganic iron carriers is associated with adverse effects such as constipation, diarrhea and decreased growth. Moreover, considering the amount of iron transported and retained by Caco-2 cells, the values obtained for rhLF from rice were very similar to those reported in previous studies for hLF and for rhLF from Aspergillus awamori, showing close similarities among all these lactoferrins. Sánchez et al. reported that the transport of iron across Caco-2 monolayers was lower from citrate than from hLF, unlike the results found in the present study. However, if we consider the iron transported to the lower chamber together with that retained by the cells, this is practically the same in the two studies, as the proportion of iron bound to citrate transported is 9% and for lactoferrins, around 5%.

In this study, the passage of rhLF across Caco-2 monolayers was also determined. The percentage of accumulated 59Fe-activity associated rhLF in the lower chamber and cells at 24h was 22.8% and 3.4% respectively. If these results are compared with those obtained for human milk lactoferrin and for rhLF from Aspergillus awamori, it is observed that the amount of lactoferrin retained by the cells was similar for the three types of lactoferrin. However, the amount of rhLF from rice transported across the monolayer was higher than that of hLF or rhLF from A. awamori. This might be due to differences in glycan composition among the lactoferrins. It is known that glycans can be involved in molecular recognition events such as cell-protein interaction. Differences in the binding to Caco-2 cells between hLF and rhLF from rice have been reported, and they were attributed to the different glycosylation patterns of the two lactoferrins.

Two mechanisms have been described for the transport of iron from lactoferrin in the intestine. One of them is the binding of lactoferrin to a membrane protein or region of intestinal cells, release of iron at the surface, and subsequent delivery to the cells by a non-vesicular transport pathway, as happens in other cells. The other is that lactoferrin is endocytosed by intestinal epithelial cells and the iron bound to the protein is released by lysosomal degradation, as happens in hepatocytes. The results obtained for rhLF transport in the present indicate that most of the protein that traversed the monolayer was degraded in passage across the Caco-2 cell monolayers, in agreement with endocytosis-mediated transport. Moreover, the rhLF in the medium of the upper compartment was not found to be degraded when analyzed by TCA precipitation at the end of the assay. However, taking into account that the iron transported by rhLF and milk hLF was practically the same and that the passage of rhLF was higher than that for hLF, this indicates that not all the iron bound to rhLF was transported across the Caco-2 cells. This iron might be mainly released at the surface of the intestinal cells, the rhLF transported across the monolayers being in part iron free.

In conclusion, the results obtained in this indicate that rhLF from rice binds to Caco-2 cell membranes in a saturable way, similarly to the interaction shown by human milk lactoferrin with these cells. However, no evidence showing that this binding is mediated by a specific receptor has been found, at least under the experimental conditions used in this study. The transport of rhLF across Caco-2 cell monolayers was higher than that reported previously for hLF, though the total amount of iron transported across the monolayers and internalized by the cells was the same for lactoferrin of both origins. These results suggest that rhLF from rice has behavior similar to that of lactoferrin from human milk, if considered as an iron donor, though the recombinant protein showed some slight differences in the passage through the Caco-2 monolayers.

Taking into account the difficulty of obtaining human lactoferrin on an industrial scale, the availability of recombinant human lactoferrin from rice is very interesting considering its use in special products. The results of our work indicate that the roles of rhLF and hLF in iron absorption are comparable. Furthermore, it has been observed that rhLF is nearly completely degraded in the passage across the Caco-2 cell monolayer, as was previously reported for human milk lactoferrin. This finding indicates a low risk for human lactoferrin from rice of causing allergenicity by crossing the intestinal barrier into the circulation as a whole molecule.
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