Recombinant Human Milk Proteins

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Abstract

Human milk provides proteins that benefit newborn infants. They not only provide amino acids, but also facilitate the absorption of nutrients, stimulate growth and development of the intestine, modulate immune function, and aid in the digestion of other nutrients. Breastfed infants have a lower prevalence of infections than formula-fed infants. Since many women in industrialized countries choose not to breastfeed, and an increasing proportion of women in developing countries are advised not to breastfeed because of the risk of HIV transmission, incorporation of recombinant human milk proteins into infant foods is likely to be beneficial. We are expressing human milk proteins known to have anti-infective activity in rice. Since rice is a normal constituent of the diet of infants and children, limited purification of the proteins is required. Lactoferrin has antimicrobial and iron-binding activities. Lysozyme is an enzyme that is bactericidal and also acts synergistically with lactoferrin. These recombinant proteins have biological activities identical to their native counterparts. They are equally resistant to heat processing, which is necessary for food applications, and to acid and proteolytic enzymes which are needed to maintain their biological activity in the gastrointestinal tract of infants. These recombinant human milk proteins may be incorporated into infant formulas, baby foods and complementary foods, and used with the goal to reduce infectious diseases.

Introduction

It is well recognized that there are several benefits associated with breast-feeding. Breast-fed infants have fewer infections [1], even in higher socioeconomic groups in affluent countries, and when they get sick, the illness is of shorter duration than in formula-fed infants [2]. This, of course, is even more accentuated in developing countries where sanitary conditions are poor and exposure to pathogens is high. The lower prevalence of infections is likely to be due to several factors, but many of these are unique milk proteins...
that have anti-infective properties. Breast-fed infants also have a different growth pattern, different nutritional status and different gut microflora than formula-fed infants. The high bioavailability of nutrients from breast milk is believed to be due in part to proteins in the milk that facilitate nutrient utilization [3]. While many modifications have been introduced to make infant formula more similar to breast milk, e.g. mixing vegetable oils, adding long-chain fatty acids, adding cow’s milk protein fractions, oligosaccharides and minerals/vitamins, the performance of formula-fed infants is still different than that of breast-fed infants [4]. Many biological activities have been associated with various proteins in breast milk, and it has still not been possible to mimic the protein composition of breast milk, as these proteins are species specific [5]. Among the protein are immunoglobulins, lactoferrin, α-lactalbumin, lysozyme, haptocorrin and α1-antitrypsin. Although bovine lactoferrin and α-lactalbumin are commercially available and have been added to infant formula, no biological activities have yet been correlated to these proteins in clinical studies, possibly because they are different in structure from their human milk counterparts. This may be important, as some proteins, like lactoferrin, have specific receptors in the small intestine that only recognize the species-specific protein [6].

By using genetic engineering, human milk proteins can now be produced in microorganisms, plants or milk from dairy animals [7]. Recombinant human milk proteins can thus be produced in large quantities and may be used in products such as infant formula. However, before this can be achieved, the bioactivities of these proteins need to be evaluated, their safety assessed and the attitude of the consumers carefully considered.

**Expression Systems**

*Expression of Human Milk Proteins in Microorganisms*

Microorganisms such as *Saccharomyces* and *Aspergillus* are being used for expression of human milk proteins. Human lactoferrin has been expressed in *Saccharomyces*, but is not commercially available, possibly due to low expression levels [8]. *Aspergillus*, however, is successfully used for the production of recombinant human lactoferrin [9], which is commercially available. Expression levels are very high, making it an attractive system; however, extensive purification is needed and the cost is most likely too high for use as a food additive. Consequently, pharmaceutical applications, being more cost tolerant, are being pursued for recombinant proteins produced in this expression system.

*Expression of Human Milk Proteins in Milk of Dairy Animals*

Some of the first commercial applications for recombinant proteins were obtained following their expression in the milk of transgenic animals.
Pharmaceutical recombinant proteins, such as clotting factors VIII and IX, were early expressed in the milk of sheep and goats. Recombinant human lactoferrin, a major protein component of human milk, is being expressed in transgenic cows [10] and is now commercially available. Purified recombinant human lactoferrin may, however, be too expensive to be used as a food additive or in infant formula. One possible application would be to use cow’s milk containing recombinant human milk proteins, such as lactoferrin, but it has not yet found a market, possibly due to logistics (transport, storage) and/or cost. Human lysozyme has been expressed in the milk of mice [11], and more recently in cows [E. Maga, personal commun.], but, again, there are no commercial applications yet.

Expression of Human Milk Proteins in Plants

Plant biologists have successfully been able to express recombinant proteins in various crops. By using strong promoters, high levels of expression can be achieved. It is also possible to direct the expression so that specific parts of the plant can be utilized, depending on the species. Fruit (bananas), seeds (rice, barley), leaves (tobacco) and tubers (potatoes) are all used for expression of various proteins.

Human lactoferrin has been produced in tobacco [12]. Expression levels were relatively low, however, and the protein needs to be purified extensively before it could be considered for any food applications, making it unlikely as a commercially viable product. There have been no tests of activity of this protein, and it is not yet commercially available. Human lactoferrin has also been expressed in potatoes [13], but it appears that expression levels so far are low. This system is attractive in that potatoes are a normal part of the diet of many people; however, it is uncertain if the protein will have any biological activity after the extensive boiling that is used for potatoes.

Rice has been used for expression of soybean ferritin to increase its iron content [14, 15] and is now being used for the expression of several human milk proteins, such as lactoferrin, lysozyme and α1-antitrypsin [16–21]. Very high expression levels can be achieved; e.g. 5 g of human lactoferrin/kg dehusked rice has been expressed in large scale field trials for several generations [17]. Rice has several advantages as an expression system: (1) rice does not contain any toxic compounds (potatoes contain solanin); (2) rice is one of the first ‘non-milk’ foods introduced to infants, which in part is due to its low allergenicity, and (3) expression can be directed so the protein is either expressed as a storage protein (in the seed) driven by the Gt1 promoter, or when driven by the amylase promoter, expressed only during germination, making malting another possibility. In this case, the crop (i.e. rice seeds) does not contain the recombinant proteins; the proteins are only synthesized when the seeds are put in contact with water. Thus, recombinant human milk proteins can be introduced into the diet as rice in itself and be combined with various other food components (e.g. rice cereal), or a protein
extract can be produced, yielding a product with higher protein and lower starch content.

**Expression of Human Milk Proteins in Rice**

We are using rice as an expression system to evaluate the biological activity of select human milk proteins. To date, we have expressed lactoferrin, lysozyme and $\alpha_1$-antitrypsin at very high levels, and large-scale field trials for several generations show that the transgenic rice is stable, expression levels are similar through generations, and the proteins are only expressed in the seeds. The genes were synthesized using codon optimization [22], i.e. the GC (guanine-cytosine) content was increased by nucleotide substitution but without changing any amino acid residue. Sequencing of the recombinant proteins confirmed that the amino acid sequence was identical to that of the native proteins. The signal sequence coding for storage was used. The gene was inserted by the so-called ‘gene gun’ technique and calli were grown in culture. Positive plants, detected by extraction and Western blots, were grown in greenhouses to obtain mature seeds. Seeds positive for the recombinant protein were subsequently grown in fields according to USDA regulations. Purified recombinant human lactoferrin and $\alpha_1$-antitrypsin were found to be glycosylated, but the carbohydrate content was less than that of their native counterparts. In other words, rice does recognize the signals for N-linked glycosylation and the specific sites were glycosylated, but the glycans are smaller than those in the native proteins. The carbohydrates in the rice glycans are similar to those in the human N-linked glycoproteins, i.e. mannose and fucose. The terminal residues, however, are usually mannose or xylose, while the native proteins usually have fucose or sialic acid. This difference in terminal monosaccharides in the recombinant proteins may affect stability (e.g. liver clearance) when used for intravenous applications, but is unlikely to have an effect on food applications or to affect allergenicity. In the case of lactoferrin, which has its own specific receptor in the small intestine [6], we have shown that de-glycosylated human lactoferrin as well as different recombinant forms of human lactoferrin (*Aspergillus*, rice) bind equally well to the receptor, i.e. the glycan moiety of lactoferrin is not involved in the binding to the receptor. Thus, we conclude that the glycosylation of these human milk proteins when synthesized in rice is unlikely to affect their nutritional value.

**Biological Activity of Recombinant Human Milk Proteins**

We have verified that the recombinant human milk proteins have their intended biological activities in vitro (fig. 1). Recombinant human lactoferrin was shown to both bind and release iron at low pH in a manner similar to that of native human lactoferrin [16]. It also bound similarly to the human lactoferrin
A receptor, which is present on the surface of the human intestinal cell line, Caco-2, grown in culture, and with an affinity similar to that of native lactoferrin. In addition, recombinant human lactoferrin was shown to inhibit the growth of enteropathogenic Escherichia coli (EPEC), one of the most common

Recombinant Human Milk Proteins

**Fig. 1.** Comparison of functional and biological activities between nHLf and rHLf. (a) Iron-binding capacity, (b) pH dependent iron-release properties, (c) antimicrobial activity against enteropathogenic *E. coli* (EPEC): 1 μg/ml of either nHLf or rHLf were incubated with 10⁶ cfu/ml of EPEC in the synthetic broth and bacterial growth were measured at various time points by absorbance at 630 nm. Control does not contain any protein but 10⁶ cfu/ml EPEC. (d) binding of nHLf and rHLf to Caco-2 cells. (e) uptake of nHLf and rHLf by Caco-2 cells (from [16]).

receptor, which is present on the surface of the human intestinal cell line, Caco-2, grown in culture, and with an affinity similar to that of native lactoferrin. In addition, recombinant human lactoferrin was shown to inhibit the growth of enteropathogenic *Escherichia coli* (EPEC), one of the most common
causes of diarrhea in infants and children, and at a concentration similar to that of native human lactoferrin. When tested in the conventional assay for lysozyme activity, which is based on the lysis of *Micrococcus lysodeikti*us, the recombinant human lysozyme had an activity similar to that of native human lysozyme. The recombinant human lysozyme also inhibited the growth of EPEC at the same concentration as the native enzyme. Breast milk contains a significant concentration of α₁-antitrypsin [23], and we have hypothesized that this inhibitor of proteolytic activity in the small intestine contributes to the ‘survival’ of some human milk proteins, so that they can exert their physiological activities in the upper small intestine. The recombinant human α₁-antitrypsin inhibited trypsin and elastase to an extent similar to that of native human α₁-antitrypsin [21]. Thus, for these three human milk proteins we have demonstrated that they have activities similar to those of their native counterparts. For two of these proteins, lactoferrin and α₁-antitrypsin, this occurred in spite of some differences in glycosylation.

**Stability of Recombinant Human Milk Proteins**

To be active in the small intestine, the recombinant proteins need to be able to withstand exposure to low pH in the stomach. While the gastric pH of infants rarely is below pH 4–5 for the first 6 months of life, in some cases the pH may be as low as pH 2–3. We therefore exposed the recombinant human milk proteins to low pH, from pH 2 to 5, for 30 min and then adjusted the pH back to neutral. Activities of all three recombinant proteins, assessed as above, were similar to those of the native proteins [16, 18, 21]. If recombinant human milk proteins are to be added to infant formula or baby foods, some degree of processing may be involved. We therefore exposed the recombinant proteins, both in pure form in solution and as added to infant formula, to various heat treatments, ranging from 78 to 100°C for 8 s up to 30 min. Except for the most severe treatment, 100°C for 5 min, which partially inactivated both recombinant and native human milk proteins, these proteins maintained activities similar to those of the native proteins [16, 18, 21].

**Digestive Fate of Recombinant Human Milk Proteins**

Proteins are in general effectively digested by the combined proteolytic activities in the stomach and small intestine. Infants, however, are an exception to this. Infants have low pepsin secretion and the pH of the infant stomach is usually around pH 4–5 [24], a pH too high for significant pepsin activity. In addition, secretion of pancreatic enzymes is immature, limiting the proteolytic activity in the small intestine. Further, some human milk proteins have structures that make them relatively resistant against proteolytic enzymes.
As an illustration of this, we have found significant concentrations of lactoferrin, secretory IgA and α1-antitrypsin in the stool of breast-fed infants [23, 25] and this persists up to at least 4–6 months of age. The proportion of proteins surviving decreases with increasing age, most likely as digestive function matures. It is, of course, also possible that some milk proteins survive passage of the stomach and the duodenum and may exert actions in the upper gut, to become digested further down in the small intestine.

We have developed an in vitro digestive system [26] to evaluate the survival of recombinant human milk proteins. Briefly, the proteins, in pure form or added to infant formula, are exposed to low pH and pepsin for 30 min at 37°C. The pH is then adjusted to pH 7 and pancreatin (a mixture of pancreatic enzymes) is added. The solution is then incubated for 30 or 60 min, and the proteolytic enzymes are inactivated by brief (2–3 min) boiling. The molecular weights of the proteins are assessed by Western blotting, and their activities are assessed as described earlier. For all three proteins we studied, activities remained after treatment, and to an extent similar to that of the native human milk proteins. While some degradation did occur, the major part of the activity remained, and the extent of degradation was similar for recombinant and native proteins.

**Further Testing**

We have shown that recombinant human milk proteins can be produced at very high levels in rice and that they can withstand low pH and heat treatment, as well as proteolytic digestion in vitro. Further efficacy and safety trials in animals and humans will be needed next.

Safety studies in rats will be necessary prior to human trials. For efficacy studies, we have chosen to first use a rat pup model, in which we can assess the resistance against proteolytic activity as well as anti-infective properties (incubate with pathogens). We have also used infant rhesus monkeys. This non-human primate model of human infants has a high degree of validity as their gastrointestinal physiology is very similar to that of human infants, and rhesus milk is reasonably similar to human milk both in nutrient content and with regard to bioactive proteins (e.g. lactoferrin). Another advantage of this model is that they can be fed regular infant formulas, without any modification of nutrient content, exclusively for 4–6 months. The addition of recombinant human milk proteins to infant formula can therefore be evaluated under realistic conditions. This model also allows us to infect the infant monkey with a pathogen, such as EPEC, and evaluate the effect of the added protein on diarrhea prevalence, severity and duration [27]. Such studies, of course, are not possible in human infants.

Once efficacy has been shown in these models, human trials are needed both with regard to efficacy and safety. Recently, we have evaluated the effects of
adding recombinant human lactoferrin and lysozyme to the WHO oral rehydration solution (ORS) used to treat children with acute diarrhea in a prospective, double-blind, randomized controlled trial in Peru. Children receiving ORS with added recombinant human milk proteins had a significant decrease in the duration of diarrhea as compared to children receiving control ORS [Zavaleta and Lönnerdal, in preparation]. These recombinant human milk proteins may be incorporated into infant formulas, baby foods and complementary foods and used in industrialized as well as developing countries with the goal of reducing infectious diseases. However, for infant formula efficacy trials are essential as infant formulas are regulated by federal law, the Infant Formula Act, which does not allow additions of novel components unless benefits have been demonstrated. Thus, addition of components primarily for marketing reasons, which occurs in some countries, will not be allowed.

Conclusions

Expression of recombinant human milk proteins in rice is realistic and a possibility for the addition of bioactive factors to infant formula and baby foods. We have been able to produce several such proteins at very high expression levels and have shown that the transmission is stable through several generations. Large-scale field trials have produced such rice in several tons, making it possible for evaluation of efficacy. In vitro studies have shown that these proteins have activities and stabilities similar to those of the native proteins and that they, to a considerable extent, withstand heat treatment. Further animal work and studies in human subjects are needed to document their efficacy and safety.

References

Recombinant Human Milk Proteins


Discussion

**Dr. Hernell:** I just wanted to add to the discussion that also recombinant human milk bile salt-stimulated lipase produced in transgenic sheep has been subjected to two small clinical trials to treat fat malabsorption in patients with cystic fibrosis. So the recombinant enzyme has been proven safe and has a clinical effect as expected. Then in your list of various expression systems, I missed mammalian cell lines, e.g. Chinese hamster ovary (CHO) cells. If for expression of glycosylated proteins you are concerned about the glycosylation pattern, mammalian cells would probably be a useful alternative because they are likely to yield a glycosylation pattern closer to the native human protein.

**Dr. Lönnerdal:** It is a good point. I am not sure how economically realistic it would be to add CHO-produced proteins to food products. I don’t know what kind of capac-
ity you have to produce them in quantities needed for foods. I am happy that you brought up the glycosylation issue because it is also possible to insert enzymes on the terminal carbohydrates of the human glycan in rice. Thus, you can produce not just the recombinant human milk protein but you can also put in recombinant enzymes that will start mimicking the glycan. That is something that, if needed, can be done, but for lactoferrin we just have not yet seen a need for it. When it comes to \(\kappa\)-casein, where glycan plays a much more fundamental role, something like that may be needed or the use of CHO cells that will provide a mammalian glycosylation pattern.

**Dr. Haschke:** Thank you for introducing the fascinating world of bioactive components. From the industrial stand point as you have mentioned, there are so many variables to overcome to apply this concept. The first one is the safety issue, and safety must finally be demonstrated in infants, even if there is no theoretical reason that there might be a safety question. Phase-1 studies cost a lot of money and are very time-consuming. The other thing is the functional outcome study. You showed one study in which the duration of diarrhea was reduced. This is similar to what we can achieve with lactobacillus GG, which is now added to infant formulas. It is exactly the same outcome; a reduction in the duration of diarrhea from 5.4 to 4 days. So of course probiotics are not 100% safe either. But they are more accepted by the consumer and it is very difficult for the industry to make the consumer believe that there is really an advantage unless we can really show positive effects in favor of the infant receiving it. So we have a long way to go, I don't know whether you would agree with this. In the end, if we are doing something wrong, we will be punished.

**Dr. Lönnerdal:** You are quite right and I have sympathy with you. I am primarily interested in the function of the proteins and not the commercial applications, but I am acutely aware of the fact that the attitudes are very different. In the US the probiotic concept is not an easy-sell at all because these are live bacteria and 'you don't eat that'. In Europe, including Sweden, this is a common part of the diet and there are no negative associations with probiotics, whereas in the US there is a much more adverse reaction. Then you have the opposite: in the US you actually have limited negative publicity and attitudes against recombinant proteins as such, so there is not at all the same pressure on the industry to refrain from this because they are in soy, in corn, and on the market already. So again the attitude will depend on what the market will be.

**Dr. Hernell:** Just a comment to follow that. Today I think no one would even dream of not using recombinant insulin and growth hormone. That shows how fast we are moving. Some 20 years ago these recombinant hormones were new on the market; now it is established routine to use them. No one talks about safety anymore.

**Dr. Klassen:** Can you speculate on what the next step will be to demonstrate the synergistic effect you mentioned? In your opinion what bioactive factors should be combined to achieve further benefit? In Dr. Dewey’s presentation we have also seen that there is a tremendous amount of bioactive components present in milk and hence combinations thereof.

**Dr. Lönnerdal:** I can speculate. I have always believed in Aristotle who said, ‘there is a reason behind everything in Nature’. Why would you have so much \(\alpha_1\)-antitrypsin in breast milk? In a way you can view \(\alpha_1\)-antitrypsin as a ‘break molecule’. We have done in vitro studies, and \(\alpha_1\)-antitrypsin will basically delay digestion by pancreatic enzymes to some extent [1]. The possibility would then exist for some of the human milk proteins to have an effect in the duodenum, and perhaps the upper part of the jejunum, and then, after that, they would start being digested. It is a possibility that we have a synergistic effect by which \(\alpha_1\)-antitrypsin would limit the digestion of some of these bioactive milk components in order to allow them to perform various functions.

**Dr. Telmesani:** There is active research towards animals that can produce organs compatible to humans for transplantation. Is there any possibility and/or are there any studies towards cloning the human mammary gland to produce human milk?
Dr. Lönnerdal: I am not sure how easy it would be. We dreamt about many of these things 20 years ago, and we are moving fast. When it comes to whole organs and tissues like that I am not sure. We have immortalized human cell lines so certainly the possibilities are there to get closer to organs, but we may not reach the stage where we would have ‘human dairy industries’.

Dr. Yun Cao: Does the breast milk of mothers with very premature babies also contain this kind of active protein?

Dr. Lönnerdal: That is a very good question. In fact, for many of these bioactive proteins, the richest source you can find is colostrum and the milk of women who deliver prematurely, because in a way premature milk can be viewed as almost a ‘super colostrum’. Previous research on colostrum and ‘premature milk’ has shown that concentrations of lactoferrin are incredibly high, those of secretory IgA are high, and many of these bioactive proteins are very high in concentration. It is very costly to care for premature infants, and premature milk fortifiers or preterm formula may be applications where you would start to see such proteins added, as it is so important to protect the preterm infant.

Dr. Roggero: Could conservation (human milk bank) or pasteurization alter these biological proteins or substances?

Dr. Lönnerdal: It varies. One of my former students, Dr. Madeleine Sigman, has actually studied banked human milk and found that the extent to which these proteins survive varies with the protein. Lactoferrin is relatively tough, secretory IgA is also relatively tough, whereas lysozyme may be more affected by heat. The problem we have had is that the technical qualities of the milk change with heat treatment, so purifying proteins from breast milk is much more difficult because they tend to aggregate more after pasteurization. But from the infant point of view, it still seems that most of the bioactive proteins are still active after pasteurization.

Dr. Hernell: I just want to add that if you compare fresh and pasteurized human milk with respect to functional effects, the most striking difference is decreased fat absorption from pasteurized milk. The reason being that the milk bile salt-stimulated lipase is rapidly activated at 62.5°C. This temperature, which is typical for the pasteurization of human milk, is critical for both lactoferrin and IgA. A slight increase in temperature will rapidly increase the inactivation of both.

Reference
