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Major Advances Associated with the Biosynthesis of Milk

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ABSTRACT

The mammary gland has an incredible level of organization and a remarkable ability to convert circulating nutrients into milk components. This review highlights four areas of high interest in the biology of milk synthesis where advances over the last quarter-century have resulted in new understanding or revealed new opportunities. First, advances in our understanding of the mechanisms of milk secretion has led to a substantial increase in our knowledge of the intracellular origin of lipid droplets and the identity and potential function of milk fat globule membrane proteins in milk-lipid secretion. Second, recent breakthroughs have advanced our understanding of the nutritional regulation of milk fat and highlighted the interrelations between dietary components, digestive processes in the rumen, and the regulation of mammary synthesis of milk fat. Third, nutritional quality is becoming increasingly important in food choices because of consumer awareness of the links between diet and health. The traditional nutritional value of milk and dairy products is well established, but recent discoveries have identified a number of "bioactive" components in milk with potential to improve human health. Finally, the concept of genetic engineering and the use of animals as "bioreactors" and the "pharming" of proteins not normally found in milk have gained recognition, with the dairy industry ideally suited to take advantage of advances in these areas.
Key words: bioactive fatty acids, human health milk fat secretion, milk fat depression

INTRODUCTION

Milk and dairy products were recognized as important foods as early as 4000 BC as evidenced by rock drawings from the Sahara. Today, the important contributions of milk and dairy products in meeting our dietary requirements for energy, high quality protein, and several key minerals and vitamins are well documented. Over the last quarter-century, milk production by the US dairy industry has increased (77,000 vs. 58,000 million kg), whereas the number of cows has decreased (9 vs. 11 million cows). Advances in lactation biology and our understanding of the biosynthesis of milk have played an important role in these gains in productivity.

Mammary epithelial cells have an incredible level of organization and a remarkable ability to convert circulating nutrients into milk components. Patton recognized the importance of the mammary epithelial cell as a "biological factory" and suggested it would rank second only to the photosynthesis cell as a factor in sustaining mammalian life. The productivity of this biological factory is extensive and in terms of the use of nutrients and energy, the cow should perhaps be viewed as an "appendage to the mammary gland" rather than vice versa.

Most advances in science come in small increments building the road of knowledge. Progress in lactation biology is no different and, during the 20th century, structure–function relationships of mammary epithelial cells and the gland were established, biochemical pathways for the synthesis of milk components identified, and the role of hormones in the development of the mammary gland and the regulation of its function elucidated. Over the last quarter-century, incremental gains in our knowledge of lactational biology have continued and that offers a major challenge in preparing this overview. We have therefore chosen to highlight four areas of high interest in which advances have resulted in new understanding or revealed new opportunities in the biology of milk synthesis.

The first area is advancements in our understanding of the mechanisms of milk secretion, especially the intracellular assembly of milk-lipid droplets and the nature and formation of the milk-fat globule membrane (**MFGM**). van Leeuwenhoek first observed milk-lipid droplets in the light microscope more than 300 yr ago, and Ascherson described the first evidence of membrane material on the surface of the droplets in the mid-19th century. More recently, application of electron microscopy, biochemical and molecular cloning techniques, and gene targeting technologies has led to a

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substantial increase in our knowledge of the intracellular origin of lipid droplets and the identity and potential function of MFGM proteins in milk-lipid secretion. The second area is the nutritional regulation of milk fat. First recognized by Boussingault in 1845, the explanation for why certain diets cause a specific reduction in milk fat production has perplexed producers and scientists for over 150 yr and numerous theories to explain the cause were proposed and subsequently found inadequate. However, recent breakthroughs have advanced our understanding of the interrelations between dietary components, digestive processes in the rumen, and the regulation of mammary synthesis of milk fat. Thirdly, in the last quarter-century, nutritional quality has become increasingly important in food choices because of consumer awareness of the link between diet and health. Foods contain components that may provide benefits to human health beyond those associated with traditional nutrients and a number of these "bioactive" food components have been discovered in milk. Importantly, milk fat has been shown to contain a number of bioactive components, and some of these will be discussed. Lastly, we will consider advances in genetic engineering and how this relates to the dairy industry. The concept of using animals as "bioreactors" and the "pharming" of proteins not normally found in milk has gained recognition, with the dairy industry ideally suited to take advantage of advances in this area.

MECHANISM OF MILK SECRETION

During peak lactation, the mammary gland synthesizes and secretes a prodigious complement of products into milk including proteins, carbohydrates, membrane-coated lipid droplets, water, and ions. How the secretion of such a diverse array of milk components is coordinated at the cellular level remains a major challenge for cell biologists and lactation specialists. Over the past 25 yr, most progress has been made in unraveling potential mechanisms for the assembly and secretion of milk-lipid droplets and the protein composition of the MFGM. Understanding how milk fat is secreted and the role of the MFGM in stabilizing the droplets after secretion is of major importance to the dairy industry because the nature of the cream fraction influences the manufacturing properties and organoleptic qualities of milk and dairy products.

Our understanding of how lipid droplets are assembled within mammary cells has been advanced primarily by Keenan and colleagues, who used a combination of cell fractionation techniques and cell-free assays to identify the initial sites of lipid droplet formation and monitor their subsequent growth and maturation. Triacylglycerols are synthesized in the rough endoplasmic reticulum by membrane-bound fatty acyl transferases. Where in the membrane the newly synthesized lipids aggregate into nascent droplets is still unclear; for example, within the lipid bilayer or on the luminal or the cytoplasmic surfaces. However, at some stage, microlipid droplets (less than 0.5 μm in diameter) are released into the cytoplasm and coated with protein and polar lipids from the reticular membrane. Some of these droplets undergo a series of fusion reactions to form larger cytoplasmic lipid droplets during their transport to the apex of the cell (pathway A in Figure 1). These droplets become relatively large before secretion, but many others are secreted virtually unchanged in size from the time of their initial formation (pathway B in Figure 1). More than 80% of the droplets are under 1 μm in diameter, although the bulk of milk fat is made up of droplets averaging approximately 4 μm. Using an ingenious cell-free assay, Keenan showed that lipid droplets fuse together in vitro upon the addition of calcium, gangliosides, and protein components. Identification of the factors controlling the ultimate size of lipid droplets in milk could have interesting practical applications for the production of milk with different sized droplets and different manufacturing characteristics.

By using proteomics or biochemical approaches, respectively, Howell and Keenan showed that intracellular lipid droplets acquire a number of proteins on their surface either from the rough endoplasmic reticulum during their formation or during transit through the cell. The droplets therefore arrive at the apical surface with a cohort of surface proteins and polar lipids, some of which become bona fide components of the MFGM.

Expulsion of lipid droplets from the cell is completed by a budding process, during which the droplets acquire an outer membrane of proteins and phospholipids from the apical plasma membrane (Figure 1). A major question over the past 25 yr has been which, if any, of the MFGM proteins are required for promoting final formation of the membrane and release of the droplets. Rapid progress in identifying the major MFGM proteins came through the application of molecular cloning techniques, which provided a wealth of information because the complete linear amino acid sequences can be derived from the respective cloned cDNA sequences. Largely through the efforts of Peterson and colleagues in Denmark and Jack and Mather in the United States, sequences of all the major proteins of bovine MFGM were obtained. These proteins include the mucins MUC1 and MUC15, the redox enzyme xanthine dehydrogenase/oxidase, the integral proteins CD36 and butyrophilin, and the lipid-binding protein adipophilin.

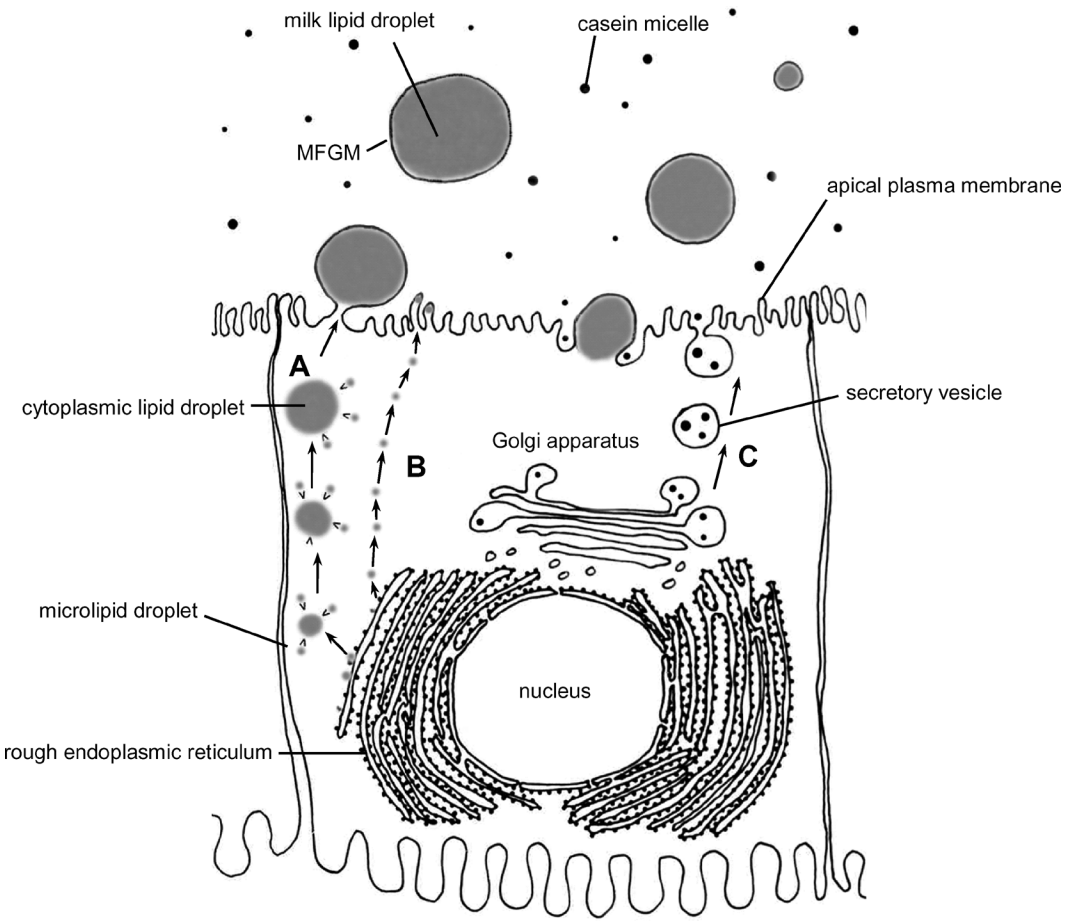

Figure 1. Major secretory pathways in mammary epithelial cells during lactation. A = cytoplasmic lipid droplet pathway; B = microlipid droplet pathway; C = secretory pathway for skim milk components. Adapted from Mather and Keenan (1998).

Evidence for the potential function of 2 of these characterized proteins in lipid secretion was subsequently obtained by the generation of mice in which the respective genes were made defective by replacing key regions with unrelated DNA to block transcription of the targeted gene. Such knockout animals are especially useful for functional studies because they only differ from their wild-type littermate controls in lacking functional copies of the targeted gene. Knockout of the genes encoding either xanthine dehydrogenase/oxidase or butyrophilin in the laboratories of Capecchi and Mather, respectively, generated striking lactation phenotypes. In both cases, the regulated secretion of lipid droplets was blocked and large pools of triacylglycerol accumulated in the cytoplasm. Large droplets escaped from the cell without an intact outer membrane bilayer and fused together in the glandular spaces to form massive aggregates of lipid. These results strongly suggest that both xanthine dehydrogenase/oxidase and butyrophilin function in the secretion of lipid droplets and because the phenotype of both mouse lines was similar, the results imply that the 2 proteins function in the same pathway. It has been proposed that butyrophilin, as an integral protein in the apical plasma membrane, binds to xanthine dehydrogenase/oxidase to promote the envelopment of intracellular lipid droplets with plasma membrane.

From a practical standpoint, these results highlight the importance of the MFGM in stabilizing lipid droplets in milk and the consequences when this membrane is weakened or disrupted. The xanthine dehydrogenase/oxidase and butyrophilin genes are thus obvious candidates to be included in genetic screens of dairy cattle for milk quality and production traits. In contradistinction, the results show that disruption of lipid secretion, per se, is not a promising avenue for the suppression of lipid levels in milk by genetic manipulation, because triacylglycerol synthesis does not shut down as lipid accumulates in the cytoplasm. Reduction in the expression levels or activities of key lipogenic enzymes may

constitute a more promising approach. However, complete inhibition of triacylglycerol synthesis for such purposes is contraindicated by the absolute elimination of lactation in mice that lack a functional diacylglycerol transferase-1 gene.

Progress on our understanding of the regulation and secretion of the other major components of milk has been disappointingly slow over the past 25 yr. Most of the skim milk components, including caseins, many whey proteins, lactose, water, and ions accumulate in Golgi-derived secretory vesicles. Following transport to the apex of the cell, the vesicle membrane fuses with the apical plasma membrane and the contents of the vesicles are discharged into milk, a process known as exocytosis (pathway C; Figure 1). In many secretory systems, exocytosis is regulated and only occurs when the cell is stimulated by specific signals. In other systems, secretion is continuous, or "constitutive." Burgoyne and Wilde showed that milk protein secretion appears to occur by a combination of both mechanisms and that release of at least a fraction of the skim milk proteins is regulated by calcium levels in the cell. This observation deserves further study because methods for manipulating the rate of protein secretion could be used to control the overall levels of protein in milk and aid in the production of recombinant protein in transgenic animals (see later section).

NUTRITIONAL REGULATION OF MILK FAT SYNTHESIS

As discussed above, fat is the major energy component in milk and accounts for many of the physical properties, manufacturing characteristics, and organoleptic qualities of milk and milk products. Fat is also the most variable milk component and the environmental and physiological factors affecting milk fat have been extensively characterized. Nutrition is the predominant factor affecting milk fat and it represents a practical tool to alter the yield and fatty acid composition of milk fat. The most dramatic example of nutritional effects on milk fat is the low-fat milk syndrome, typically referred to as milk fat depression (**MFD**). Through the first half of the twentieth century, when the feeding of dairy cows began to follow "scientific principles," diet-induced reductions in milk fat were observed for a range of common diets including those supplemented with fish or plant oils, diets high in concentrates and low in fiber, and diets low in effective fiber (e.g., from grinding or pelleting of the roughage).

Over the last 50 yr there has been substantial research addressing MFD and practical producer recommendations to minimize the occurrence of MFD have been reviewed by Emery, Erdman, Palmquist, and Sutton. However, an understanding of the biological basis by which certain diets cause a reduction in milk fat has remained elusive. Several general characteristics have been identified that provide insight; changes are specific for milk fat, and fat yield can be reduced by 50% or more with little or no change in the yield of milk or other milk components. Milk fat is composed of fatty acids of varying chain length and degree of saturation; these are derived almost equally from the uptake of circulating preformed fatty acids and the synthesis of new (de novo) fatty acids in the mammary gland. In diet-induced MFD, the yield of all individual fatty acids is reduced, but the decline is greatest for short- and medium-chain fatty acids that are synthesized de novo. Investigations have also established that 2 conditions are needed to observe the reduction in milk fat yield; first, the diet must alter the rumen environment, thereby causing changes in ruminal microbial processes and second, the diet must contain unsaturated fatty acids. Thus, the etiology of diet-induced MFD involves products of rumen bacteria that are produced because of the diet-induced shifts in rumen microbial processes and the presence of unsaturated fatty acids.

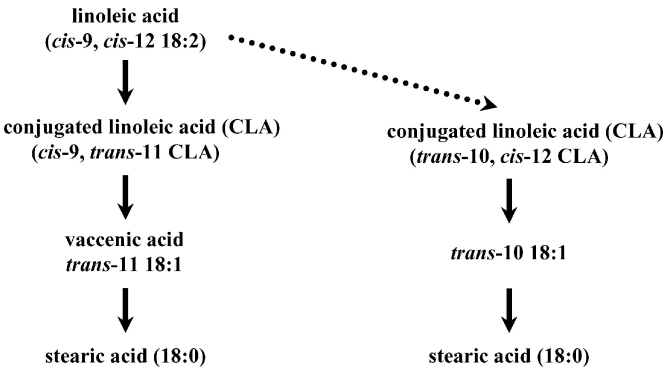

Figure 2. Pathways of the rumen biohydrogenation of linoleic acid under normal conditions (left side) and during diet-induced milk fat depression (dotted line). Adapted from Bauman and Griinari (2003); reprinted with permission from Annu. Rev. Nutr., vol. 23 © by Annual Reviews (www.annual reviews.org).

Davis and Brown were among the first to recognize that increases in the milk fat content of *trans* 18:1 fatty acids (**TFA**) were often associated with MFD. *Trans* fatty acids are formed as intermediates in rumen biohydrogenation, and *trans*-11 18:1 (vaccenic acid; **VA**) is the predominant isomer produced, as illustrated by the pathway for the biohydrogenation of linoleic acid (Figure 2). Schultz, Erdman, and others confirmed the general pattern of increases in milk-fat TFA during diet-induced MFD, although in some studies the increases were poorly correlated to milk fat yield. Thus, the basis by which certain diets cause a reduction in milk fat yield had to be more complex than a simple relationship

to the rumen production of TFA. A key development occurred when Griinari and Bauman used improved analytical techniques and discovered that it was a shift in the pattern of TFA isomers rather than total TFA that was correlated with MFD. Specifically, the milk fat content of *trans*-10 18:1 was increased during diet-induced MFD. Thus, under certain dietary situations, a portion of the linoleic acid undergoes biohydrogenation via a unique pathway that produces *trans*-10 18:1 (Figure 2).

Based on the above results and the characteristics of diet-induced MFD cited earlier, Bauman and Griinari proposed the "biohydrogenation theory." They hypothesized that "under certain dietary conditions the pathways of rumen biohydrogenation are altered to produce unique fatty acid intermediates, which are potent inhibitors of milk fat synthesis." Subsequent studies established that the milk fat content of *trans*-10, *cis*-12 conjugated linoleic acid (**CLA**), another unique biohydrogenation intermediate (Figure 2), was also increased during diet-induced MFD. This proved to be key because investigations with pure CLA isomers by Baumgard and Bauman demonstrated that *trans*-10, *cis*-12 CLA was a potent inhibitor of milk fat synthesis. Thus, *trans*-10, *cis*-12 CLA provides a clear demonstration of the interrelationship between digestive processes in the rumen and metabolism in the mammary gland where a specific fatty acid produced naturally by rumen bacteria affects mammary gene expression, thereby regulating rates of milk fat synthesis.

Effects of *trans*-10, *cis*-12 CLA are specific for milk fat, and there is a curvilinear relationship between the dose of *trans*-10, cis-12 CLA and the reduction in milk fat synthesis; as little as 2.5 g/d leaving the rumen is sufficient to cause a 25% reduction in milk fat production. The mechanism by which *trans*-10, *cis*-12 CLA causes a reduction in milk fat synthesis involves gene regulation in the mammary epithelial cells and the coordinated reduction in key enzymes involved in pathways of milk fat synthesis. To date, *trans*-10, *cis*-12 CLA is the only biohydrogenation intermediate that has been unequivocally identified to inhibit milk fat synthesis, but consistent with the biohydrogenation theory, several lines of evidence suggest that there must be additional fatty acid intermediates that regulate mammary synthesis of milk fat. This is an active area of research and improved analytical techniques indicate that rumen biohydrogenation is complex with numerous intermediates produced, including minor amounts of many different TFA and over 20 different isomers of CLA.

One of the exciting aspects of work in this area has been the broader implication of the research. In addition to applying this knowledge in dairy production to maintain a normal milk fat production when that is desirable, dietary supplements of *trans*-10, *cis*-12 CLA can also be used as a management tool for reducing milk fat in a controlled manner. The ability to selectively reduce milk fat yield could be of economic value in situations in which there is a quota on milk fat yield. It could also benefit cow well-being by reducing energy demands during times when nutrient intake is inadequate, such as during environmental stress or in the transition period at parturition. Overall, an understanding of the role of rumen biohydrogenation intermediates in the regulation of milk fat synthesis offers the exciting opportunity for applications on commercial dairy farms ranging from troubleshooting problems related to milk fat production to developing supplements for use as management tools in the controlled reduction of milk fat.

BIOACTIVE COMPONENTS IN MILK

Historically, the goal of agricultural research has been to increase yield and productive efficiency, with little focus given to improving the nutrient profile and manufacturing properties of food products. More recently, nutritional quality has become an increasingly important consideration in food choices because of greater consumer interest in the link between diet and health. Milk and dairy products are important sources of nutrients in human diets providing energy, high quality protein, and essential vitamins and minerals. However, in some instances, problems may arise when certain individuals drink milk; lactose intolerance and allergies to specific milk proteins are well-known examples. There is also increased recognition that foods contain components that can affect health, and scientists are being asked to clarify the role of specific foods and food components in health maintenance and disease prevention. Consequently, the bioactive properties of a number of components in milk have been examined with regard to a range of health-related variables. Of special interest are the components associated with the prevention of chronic human diseases, and this research has most often involved biomedical studies with cell cultures or animal models. These exciting results have demonstrated that milk contains specific proteins, peptides, and fatty acids that are bioactive components, and the production of fermented milk products has been shown to have the potential to elicit beneficial effects on health-related variables.

A recent National Academy of Sciences Report on "Frontiers in Agriculture Research" identified research on bioactive food components as a key focus area for future research to enhance human health through nutrition. The term "functional foods" has been adopted to describe foods or food components that have beneficial

Table 1. Partial list of bioactive components in milk that have human health implications

Milk protein components	Milk fat components	Other
	Cancer	
Whey proteins	Conjugated linoleic acid	Calcium
Casein	Vaccenic acid	Lactose
Lactoferrin	Sphingolipids	Vitamins A and D
α-Lactalbumin	Butyric acid	Oligosaccharides
Peptides	13-methyltetradecanoic acid	Nucleosides
	Ether lipids	Probiotics
	Cardiovascular Health	
Whey proteins	Conjugated linoleic acid	Calcium
Casein	Stearic acid	Vitamin D
	Omega-3 fatty acids	
	Hypertension	
Whey proteins		Calcium
		Potassium
	Immune Response	
Whey proteins	Conjugated linoleic acid	Probiotics
Milk-fat globule membrane proteins		
	Bone Health	
Peptides	Conjugated linoleic acid	Calcium
		Phosphorus
		Vitamin K

effects on human health beyond that expected based on nutritive value. It is the bioactive components within such foods that give them this functionality. Traditionally, functional food components in fruits and vegetables have been featured for their health-promoting properties, but recent investigations have established that bioactive components are also present in animal-derived foods including milk and dairy products. Table 1 provides a partial listing of the wide range of bioactive components in milk that have been identified as possessing effects of potential benefit to disease prevention and health maintenance. Space constraints limit our discussion of these components but readers are referred to reviews by Parodi, Aimutis, Heller, and Pfeuffer and Schrezenmeir. However, some of the most significant advances have been related to milk fat and fatty acids, and we will provide an overview of these.

Recent advancements in our understanding of the benefits of specific fatty acids in milk fat are of special significance due to the generally negative public perception that a food containing saturated fat is unlikely to be beneficial to human health. However, scientific investigations have clearly established that generalizations about fat and fatty acids are of little value and often lead to public confusion and misunderstanding. Rather, one must consider the biological effects and nutritional value based on individual fatty acids, and this is certainly true for saturated fatty acids. The public often associates saturated fat with risk of coronary heart disease and approximately 60% of the fatty acids in milk fat are saturated. However, the saturated fatty acids in milk fat vary in their structure and many are neutral or have no effect on plasma cholesterol. In addition, a number of fatty acids present in milk fat have been shown to have beneficial effects (Table 1). Supporting this, a number of epidemiological studies have found no association or a slight beneficial association between intake of milk and dairy products with variables related to the risk of atherosclerosis.

One of the most exciting advancements in this area has been the discovery of the potential benefits that CLA isomers may have on human health. Dairy products provide approximately 75% of our dietary intake of CLA. Although a number of different forms of CLA are present in milk fat, including *trans*-10, *cis*-12 CLA discussed earlier, it is *cis*-9, *trans*-11 CLA that is of interest as a functional food component. *Cis*-9, *trans*-11 CLA, also known as rumenic acid (**RA**), represents 75 to 90% of the total CLA in milk fat, and its presence is related to the biohydrogenation of polyunsaturated fatty acids in the rumen. Although RA is an intermediate in the biohydrogenation of linoleic acid (Figure 2), the principal source of RA in milk fat is from endogenous synthesis in the mammary gland. Vaccenic acid, an intermediate in the rumen biohydrogenation of both linoleic and linolenic acid (Figure 2), is the substrate

and its conversion to RA is catalyzed by Δ^9-desaturase, an enzyme present in the mammary gland and other tissues.

The original finding of a biological effect of CLA was by Pariza and colleagues when they identified CLA as an antimutagen present in cooked beef. As research expanded to other biomedical models, a range of additional positive health benefits were identified, but some of these were specific for different CLA isomers. In particular, RA has been shown to have anticarcinogenic activity. In fact, both RA and VA found in milk fat are anticarcinogens, the latter because it can be converted to RA via endogenous synthesis. Cancer is a chronic disease so most research on the cancer-preventive effects of CLA has, by necessity, used cell cultures or animal models. Across a wide range of cancer types and biomedical models, the anticancer effects of CLA have been consistently observed. Of particular importance, Ip and coworkers showed that dietary consumption of VA/RA-enriched butter was effective in reducing the incidence of mammary tumors in a rat model of breast cancer. These critical results are among the first to demonstrate that a naturally produced anticarcinogen, consumed as a component of a natural food, is effective in reducing cancer. Further, these preclinical investigations clearly demonstrate the feasibility of a functional food approach using RA- and VA-enhanced dairy products in the prevention of mammary cancer.

Although less extensively studied, the antiatherogenic effects of RA are also noteworthy. Supplements of a mixture of CLA isomers reduced the development of atherosclerotic lesions and even induced the regression of preexisting lesions in animal models. Reduced total plasma cholesterol and low-density lipoprotein cholesterol concentrations have also been observed in many of these studies. Significantly, recent investigations using pure CLA isomers demonstrated that RA was effective in reducing cholesterol-induced atherogenesis in the classic animal models of atherosclerosis. In addition, VA/RA-enriched butter has been shown to lower total plasma cholesterol and improve the plasma profile of lipoprotein cholesterol compared with a control diet or a diet in which the VA/RA-enriched butter was replaced with TFA from partially hydrogenated vegetable oil.

The typical RA content of milk fat can be increased over 3-fold, and on a metabolic basis, this would allow human dietary intakes to be in the effective range for the beneficial effects observed in the biomedical studies with animal models. The diet of the dairy cow is the most significant factor affecting the milk fat content of CLA, and the RA concentration can be increased several-fold by dietary means; the feeding of fresh pasture or the addition of plant and marine oil supplements to typical dairy rations are the most common methods to enhance RA. Differences in the RA content of milk fat among individual cows are particularly striking, with a 2- to 3-fold variation often observed when cows are fed the same diet. Use of such strategies has allowed for the production of RA-enriched products, including butter that has been used in the studies mentioned above, and 2% milk, the latter being shown to have the same storage and taste characteristics as regular 2% milk.

The range of functional food components in milk is impressive and defining their role in health maintenance and the prevention of chronic diseases such as cancer and atherosclerosis is progressing at a rapid rate. The identification and characterization of bioactive components in milk will not only aid in the development of new products but also help improve the public perception of dairy products in general. As our understanding increases, we have an opportunity to modify milk composition to enhance specific components associated with health benefits and disease prevention and decrease less desirable components. In essence, modification of milk composition could be used as an integral component of an overall strategy to improve the nutrition and health of the consumer.

THE MAMMARY GLAND AS A BIOREACTOR

The lactating mammary gland is a prodigious protein-producing factory. Therefore, it is little wonder that with the advent of genetic engineering of mammals someone would propose making use of the mammary gland to produce large quantities of proteins not normally found in milk. The feasibility of producing a pharmaceutical in the milk of genetically engineered (transgenic) mice was first demonstrated in 1987 as the result of collaboration between US government scientists from the National Institutes of Health and scientists from Integrated Genetics, Inc. (Framingham, MA). Producing pharmaceuticals in a cost-effective manner was the driving force of the newly formed "pharming" industry. A secondary focus envisioned using the mammary gland to produce nutraceuticals or bioactive components. Interestingly, the most obvious potential beneficiary of altering the composition of milk, the dairy industry, has not explored this approach as a method of producing new food products-at least not to date. Currently, dairy animals are bred to produce a uniform commodity that is manufactured into many products postharvest. Transgenic technology offers the opportunity to change that paradigm by generating animals that produce milk ideally suited as the starting material for specific products (e.g., cows that produce milk that is especially well suited for making cheese).

Before the development of animal bioreactors, therapeutic proteins were either harvested from plants and animals or produced in bacterial or mammalian cell-culture facilities. The appeal of the transgenic animal bioreactor system resides in its scalability. Because genetically engineered animals transmit their newly acquired genetic information (transgene) to their offspring in a Mendelian fashion, you need only to accelerate your breeding program to increase manufacturing capacity. That is much more cost effective than building new stainless steel purification or manufacturing facilities. Such a large-scale production facility for the manufacture of proteins by cell culture can take several years to build and cost hundreds of millions of dollars. Another advantage of the animal bioreactor system is its ability to process complex proteins. Many mammalian proteins require posttranslational modifications (e.g., acetylation, amidation, carboxylation, glycosylation, phosphorylation, or signal peptide cleavage) for them to become biologically active. The secretory epithelium in the alveoli of the mammary gland, the site of protein production, has rarely failed to process novel proteins appropriately. That is a distinct advantage over producing protein in bacteria. Prokaryotes lack the posttranslational machinery found in eukaryotes and thus are simply incapable of making the modifications when required. Producing protein in mammalian cells in culture can get around the inadequacies of the bacterial system. However, mammalian cell culture systems normally require the addition of serum to culture media, usually human serum. Using human blood derivatives such as serum, potentially contaminated with unknown or undetected viruses, has become a questionable practice. Finally, producing products in milk is appealing because milk is an easily harvested secretion.

By far the most active area of animal bioreactor research and development has focused on producing pharmaceuticals. The initial focus was on high unit-cost products potentially worth hundreds of thousands to millions of dollars per gram. Most of the sought-after products are biologically active compounds normally found in blood. However, these constituents usually circulate at low concentrations and thus, are expensive to concentrate and purify. Acquiring enough human blood to process is another obvious limitation of the "harvest approach" to obtaining these potentially life-saving compounds. At least 20 such serum proteins, intended as pharmaceuticals, have been produced in mammary glands of animals ranging from rabbits to cows. Anticlotting agents and drugs to treat antithrombin deficiency, angioedema, emphysema, hemophilia, and wound healing have all been successfully produced and harvested from mammary glands of farm animals. Of these products, antithrombin III, produced by GTC Biotherapeutics, Inc., Framingham, MA, and C1-inhibitor, produced by Pharming Group, N.V., Leiden, The Netherlands, appear to be furthest along in the government approval process and are likely to be the first animal bioreactor therapeutics to reach the marketplace.

Production of monoclonal and polyclonal antibodies dominates the list of second-generation products. These antibodies, intended as both diagnostic aids and for use as therapeutics, are being made in the milk of genetically modified goats. The shift from production of high unit-value blockbuster drugs by the industry was caused by economic realities. On the one hand, purification of complex pharmaceuticals from milk turned out to be more costly than had been anticipated. On the other hand, the promise of producing new proteins in milk, cost effectively, has been realized. Thus, antibodies, with their "built-in" strategy for purification, became an obvious potential product even though their value per unit is relatively low.

Proposed changes that reflect a more classical dairy science viewpoint include increasing the casein concentration of milk to increase cheese-making efficiency, which has recently been achieved in transgenic cows in New Zealand. Increasing phosphorylation sites on caseins would improve emulsification properties. Increasing the amount of κ-casein could decrease micelle size thus decreasing gelation and coagulation, and increasing digestibility. Knocking out the α-lactalbumin gene would reduce lactose concentration in milk and consequently decrease water content. The feasibility of doing this has been demonstrated in transgenic mice. Decreasing the water content would have a significant positive financial impact on the cost of shipping milk from the farm to the processing plant. Currently, human lysozyme is being produced in the milk of genetically engineered goats to enhance cheese-making efficiency. The benefits of increasing lysozyme concentration in milk are multifold. It could decrease rennet-clotting time and increase curd strength, leading to faster cheese making and firmer cheese. Because lysozyme can also degrade bacterial cell walls, the aforementioned goats are potentially being protected against mastitis, a bacterial infection of mammary glands. It has already been shown that increasing lysozyme concentration in goat's milk can extend shelf life by causing spoilage bacteria to grow more slowly. A similar study is evaluating the efficacy of expressing a gene encoding lysostaphin in cows' milk to protect against mastitis caused by *Staphylococcus aureus.*

Numerous methods can be used to genetically engineer animals. The three most common ways are pronuclear microinjection of genes, sperm-mediated gene transfer, and somatic cell nuclear transfer (cloning).

Each has advantages and disadvantages. However, all genetic engineering techniques are based on attempting to introduce new genetic information (the transgene) at the earliest stage of embryo development, at or around the time of fertilization. Introduction at an early stage of development is desirable to ensure that the transgene ends up in every cell in the animal's body. It is especially important that the transgene is incorporated into the genes of the gametes, so it can be passed on to subsequent generations. And of course, it is important that the transgene be contained in the epithelial cells of the mammary gland so the transgene product can be secreted into the milk.

The remaining art in the process of creating transgenic animals lies in the design of the transgene. At a minimum, a transgene consists of genetic regulatory DNA and the coding or structural sequence DNA, which encodes the instructions for building the new protein. The regulatory element controls the tissue in which the gene is expressed, stage of development at which the transgene is expressed (turned on), and the amount of gene product that is produced. Embellishments such as genetic enhancers, genetic insulators, recombination signals, and secretion signal sequences are added to improve reliability of transgene expression.

A 20-yr timeline is not unusual for development of a drug. Projections by GTC Biotherapeutics, Inc. (antithrombin III from goats' milk) and Pharming Group, N.V. (C1-inhibitor from milk of rabbits and cows) expect products in the marketplace in 2006. Economic realities, as well as concern by food manufacturers that society is not prepared to accept food products from genetically modified animals, will probably temper the speed of development of such foods. Current acceptance in the United States of soybeans, most of which are harvested from genetically modified plants, does, however, suggest that consumers are willing to eat products produced in genetically engineered organisms. It is likely that acceptance of food products from genetically enhanced animals will come when consumers can recognize a direct and unique benefit of the food. Little consumer resistance is anticipated for life-saving pharmaceuticals produced in genetically modified animals.

CONCLUSIONS

The last quarter-century has witnessed impressive advances in lactation biology and our understanding of the biosynthesis of milk. This review has highlighted four key areas: advances in our understanding of the mechanisms of milk secretion; improved understanding of the nutritional regulation of milk fat synthesis; increased identification and investigation of bioactive components in milk with potential to improve human health; and application of the concept of genetic engineering and the use of the mammary gland as a bioreactor. Further advances in these areas offer the potential to not only improve efficiency and productivity of the dairy industry but also to improve human health and well-being.

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