LACTOFERRIN AND CANCER DISEASE PREVENTION

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ABSTRACT

Lactoferrin (LF) is an iron-binding glycoprotein that composes the transferrin family and is predominantly found in the products of the exocrine glands located in the gateways of the digestive, respiratory and reproductive systems, suggesting a role in the non-specific defence against invading pathogens. Additionally, several physiological roles have been attributed to LF, namely regulation of iron homeostasis, host defence against infection and inflammation, regulation of cellular growth and differentiation and protection against cancer development and metastasis. These findings have suggested LF's great potential therapeutic use in cancer disease prevention and/or treatment, namely as a chemopreventive agent. This review looks at the recent advances in understanding the mechanisms underlying the multifunctional roles of LF and future perspectives on its potential therapeutic applications.

INTRODUCTION

Over the past few decades, clinical and mechanistic studies have indicated many relations between nutrition and health, thus evidence that diet is a key environmental factor affecting the incidence of many chronic diseases is overwhelming. The precise extent of this contribution is difficult to judge, but a reduction of 35% in the age standardized incidence of cancer in the United States has been proposed to be achievable via “practicable dietary means” (Elliot and Ong, 2002; Davis and Milner, 2004; Petricoin and Liotta, 2003). Indeed, several food derived compounds we eat are among the most promising chemopreventive agents being evaluated. Chemoprevention is defined as using chemicals with the goal of preventing, interrupting or reversing the carcinogenic process. Since carcinogenesis is a multistage process, which usually takes many years in humans, there is ample opportunity to intervene and prevent the development of cancer. Chemopreventive agents are ultimately expected to be in widespread use by the general population. Therefore, they need to be non-toxic, inexpensive and available for use by oral via. With the demonstration of Tamoxifen ability to prevent breast cancer in women, the feasibility of chemoprevention in humans has now been well established (Jordan and Morrow, 1999) The full extent of biologically active components in our diet is unknown, and our understanding of their mechanisms of action is even more limited (Petricoin and Liotta, 2003). Much of the available data has been derived from *in vitro* studies with purified compounds in forms and concentrations to which the tissues in our bodies may never be exposed (Davis and Milner, 2004; Petricoin and Liotta, 2003). Furthermore, nutrition research has traditionally concentrated on single issues (such as reducing risk of cardiovascular disease or cancer) in “at risk” individuals, whereas what we need to address is the question of all the possible effects of specific food components in a genetically heterogeneous population. This is especially important for determining unintended risk as well as unintended benefit (Elliot and Ong, 2002; Davis and Milner, 2004).

A promising field of research is clinical studies with cancer preventive proteins existing in milk (McIntosh, 1993; Tsuda et al., 2002), namely lactoferrin (LF). LF known for its inhibitory action on cell proliferation, as well as for its anti-inflammatory and antioxidant abilities, has been described to have anti-carcinogenic properties in several *in vivo* and *in vitro* studies (Baveye et al., 1999; Brock, 2002; Nuijens et al., 1996; Tsuda et al., 2000, 2002; Ward et al., 2002; Ushida et al., 1999; Sekine et al., 1997a; Iigo et al., 1999; Wang et al., 2000; Matsuda et al., 2006; Pan et al., 2007). Nevertheless, it is still poorly understood if orally administrated LF is absorbed from the intestine, and if it exerts its protective effect at the site were it is expected, especially in the case of diseases occurring at sites distant from the gastrointestinal tract.

Many food components in the form of biologically active proteins, peptides, lipids, and other substances survive the digestive processes and can enter the circulation. Thus, it is possible that absorbed intact peptides can exert biological activities during health and disease. Lactoferricin (LFCin) is a peptide fragment produced by acid-pepsin hydrolysis of LF. Although LFCin has attracted considerable interest because of its well established antimicrobial activity (Yamauchi et al., 1993), recent evidence indicates that orally or subcutaneously administrated LFCin also possesses potent *in vivo* activity against cancer cells, namely inhibiting metastasis, angiogenesis and tumour growth (Cho et al., 2004; Yoo et al., 1997a). However, the mechanisms by which LFCin exerts its action are still unknown.

Regardless of LF reported potential, very few studies have covered an integrated and systematic approach to its effects as an anti-cancer agent on breast cancer (Benaissa et al., 2005; Teng et al., 2004; Miyamoto et al., 2005; Giancotti, 2006), although some high-throughput techniques, such as DNA microarrays and proteomics, have already been applied (Forozan et al., 2000; Perou et al., 1999; Shan et al., 2002) without a consistent application of bioinformatics tools for data interpretation. Therefore, it remains a large task to identify the mechanisms by which LF and its digested fragments exert their action and to discover innovative means to supply them to consumers.

This review aims to provide an overview of LF functions and mechanisms of interaction that could be exploited further in developing its potential therapeutic applications, namely in cancer disease prevention and/or treatment.

LACTOFERRIN OCCURRENCE AND STRUCTURE

LF was firstly recognised as a single-chain iron-binding protein (Groves, 1960; Johanson, 1960; Montreuil et al., 1960) and the names given to it, LF or lactotransferrin, firmly assigned it to the family of proteins known as the transferrins. The archetypal members of the transferrin family are LF, serum transferrin and ovotransferrin, with ovotransferrin being equivalent to avian serum transferrin, differing only in its glycosylation (Baker, 1994). These proteins are all glycoproteins that typically exhibit 50-70% pair wise sequence identity (Metz-Boutigue et al., 1984; Pierce et al., 1991; Moore et al., 1997). Between LF and serum transferrins the sequence identity is around 60% and between LF from different species it is around 70% (Baker, 1994; Baker and Baker, 2005; Teng et al., 2002; Bowman et al., 1988), for example bovine LF (bLF) shares 69% amino acid identity with human LF (hLF) (Shah, 2000; Pierce et al., 1991). LF is predominantly found in the products of the exocrine glands located in the gateways of the digestive, respiratory and reproductive systems. Thus, LF can be found in the secreted milk, in tears, synovial fluids, saliva and seminal fluid (Lönnerdal, 2003). Table 1 shows a selection of the LF amounts reported from these various biological fluids. Additionally, LF can be found in blood and plasma LF is derived from neutrophils, which degranulate and synthesize the protein during inflammation (Britigan et al., 1994; Levay and Viljoen, 1995; Iyer and Lönnerdal, 1993; Van Snick et al., 1974). Some physico-chemical properties of LF are summarized in Table 2.

Structurally, LF is folded into two lobes, representing its N- and C-terminal halves (Baker et al., 1994), that show sequence homology with each other and can each reversibly bind one ferric ion along with a synergistic anion, usually bicarbonate (Baker, 1994; Steijns and Hooijdonk, 2000). In these respects it closely resembles transferrin, although its affinity for iron is somewhat higher, allowing iron to be retained at lower pH values (Mazurier and Spik, 1980; Peterson et

al., 2000). This gives LF a more potent iron withholding ability (Baker et al., 2002). The lobes are connected by a peptide of 10-15 residues, which in LF forms a 3-turn α-helix, but in transferrins is irregular and flexible. There are non-covalent interactions, mostly hydrophobic where the two lobes pack together (Haridas et al., 1995).

LACTOFERRIN GENE EXPRESSION AND REGULATION

In situ fluorescence hybridization indicated that the hLF gene is mapped in the region 3p21.3 (Kim et al., 1998; Klintworth et al., 1997; Teng et al., 1987), organized in 17 exons and its total length is about 24.5 kb. A comparison of the structure of the hLF gene with that of other species revealed that it is more closely related to bLF than to murine LF (Furmanski et al., 1989; Geng et al., 1998). The positions of the introns are well matched with those of the bLF gene except only for exons 14 and 15. All the exon-intron boundaries of the gene conform to the GT/AG rule. Although LF is highly conserved among several species, its expression is both ubiquitous and species, tissue, and cell-type specific (Das et al., 1999; Furmanski et al., 1989; Grant et al., 1999; Shigeta et al., 1996; Teng et al., 2002). It is differentially regulated through multiple signalling pathways such as steroid hormone, growth factor, and kinase cascade pathways (Das et al., 1997; Teng et al., 2002; Close et al., 1997).

Expression of the LF gene is both constitutive and inducible. During the differentiation of myeloid cells, the LF gene responds to the developmental signals and is transcribed in the myelocyte and metamyelocyte stages (Berliner et al., 1995; Khanna-Gupta et al., 2000). While LF is constitutively expressed in the wet surface mucosa, LF is estrogen inducible in uterine tissue (Pentecost and Teng, 1987; Teng et al., 1986, 1989). Moreover, the presence of LF in the human reproductive tract has also been localized in the prostate and seminal vesicle of males (Goodman and Young, 1981; Wichmann et al., 1989) and is a major coat protein of human sperm (Hekman and Rumke, 1969).

Since the LF gene promoter consists of multiple response elements for various signalling pathways such as the route for kinase cascade, the estrogenic compounds could act through

response elements other than ERE (estrogen response element) to activate the gene (Teng, 2006; Giancotti, 2006) .

There are few studies on the regulation of LF gene expression other than that by hormones. In a cultured mouse mammary epithelial cell system, LF expression was induced at high cell density in the absence of exogenously added basement membranes or prolactin (Close et al., 1997), although an earlier study suggested that LF expression in a mouse mammary gland explants is stimulated by prolactin (Green and Pastewka, 1978). Close and co-workers (1997) demonstrated that LF expression in mammary epithelial cells is mediated by changes in cell shape and actin cytoskeleton. This observation offers an explanation for the suppression of LF expression in monolayer culture and in malignant tissues in which inappropriate cell shapes and cytoskeletal structures are manifested under these conditions. Multiple signalling pathways and diverse regulatory proteins regulate LF gene expression in either a positive or negative manner (Teng, 2006). Although the mechanism of regulation is still unknown, the factors involved in LF gene expression could provide clues as to where LF is needed and under what conditions.

LACTOFERRIN METABOLISM AND RECEPTORS

LF is produced in neutrophils and stored in specific granules and possibly in tertiary granules (Iyer and Lönnerdal, 1993; Lönnerdal, 2003; Van Snick et al., 1974). The steroid-thyroid receptor super family works in concert to modulate LF gene expression supporting the hypothesis that LF levels are hormone dependent (Levay and Viljoen, 1995; Giancotti 2006; Teng, 2006). LF, unlike myeloperoxidase and some other granular products, is not synthesized as a larger precursor and was found to be unphosphorylated (Olsson et al., 1988). The neutrophil LF within the granules can either be secreted into the surrounding tissues or blood (Van Snick et al., 1974), or the granules can fuse with phagosomes (Maher et al., 1993). Secretion from polymorphonuclear cells into the circulation is dependent on degranulation factors, which in turn appear to be dependent on the activation of guanylate cyclase, cGMP and proteinkinase C (calcium dependent). This occurs in both aerobic and anaerobic conditions, is

unaffected by the presence of hydrogen sulphide and is stimulated by IL-8 and surface bound IgG (Maher et al., 1993; Kahler et al., 1988). Plasma LF levels generally increase in iron overload, inflammation, infectious diseases, and during tumour development, demonstrating a multifactor stimulatory mechanism for LF release from neutrophils (Levay and Viljoen, 1995, Bullen et al., 2006).

LF removal from circulation appears to occur in one of two ways. First, LF can be removed from the circulation, as well as from the interstitial spaces, by what would appear to be receptor-mediated endocytosis into phagocytic cells such as macrophages, monocytes and other cells of the RES (reticuloendothelial system), with subsequent transfer of the iron to ferritin (Olofsson et al., 1977; Van Snick et al., 1974, 1976). However, some controversy with regard to the cells involved in this way of LF removal still exists (Ismail and Brock, 1993). The alternative mode of LF removal would be its direct uptake by the liver by liver endothelial cells and hepatocytes (Hu et al., 1993). Bennet and Kokocinski (1979) showed that labelled LF was rapidly cleared from the circulation by the liver and spleen. Nevertheless, it is still not established whether LF, like transferrin, is recycled (Birgens et al., 1988). Further research is needed to fully understand LF metabolism in the human adult. The kidneys appear to play a role in LF clearance from the circulation since both LF and LF fragments were found in the urine of infants (Hutchens et al., 1991). Both faecal and urinary elimination of LF, however, need further investigation because significant controversy still exists (Desai et al., 2007; Hirata et al., 2007; Yamauchi et al., 2006).

LF receptors have been identified in the gastrointestinal tract, on leukocytes and macrophages, platelets, and on bacteria. The surface of the LF molecule has several regions with high concentrations of positive charge responsible for one of the features that distinguishes LF from other transferrins and for some unique properties (Lampreave et al., 1990; Zakharova et al., 2000; Baker, 1994; Van Berkel et al., 1997). The most striking region of positive charge comprises the N-terminus of the polypeptide chain (with the sequence GRRRRS in hLF) which projects from the protein surface and the adjacent C-terminus of helix 1 where residues 27-30 have the sequence RKVR (Baker et al., 2002; Rochard et al., 1989). This region provides a site

for binding heparin (Van Berkel et al., 1997) and glycosaminoglycans (Mann et al., 1994) and is very likely the site that binds DNA. It may also be important for the ability of LF to bind to many cell types, possibly through binding to the negatively charged phospholipids groups of the cell membranes (Yu and Schryvers, 1993). The similarity of the N-terminal sequence (GRRRRS) to a common nuclear localisation signal sequence motif in eukaryotes could further explain the observation that LF can enter the nucleus (He and Furnmanski, 1995). Contiguous with the N-terminal patch is helix 1, which forms the main part of the bactericidal "lactoferricin" domain (Bellamy et al., 1992), which is characterised by its display of surface arginine residues. Despite their virtually identical fold, other transferrins do not share this bactericidal activity, presumably because they lack the necessary surface features. The other main concentration of positive charge is in the inter-lobe region, associated with the helix that joins the two lobes. This also appeals as a likely DNA binding region, both because of the charge and because of the cleft that is formed between the lobes, which could create a docking site.

LACTOFERRIN FUNCTIONS

Because of its close resemblance to transferrin, initial research on LF function was directed towards establishing functions related to its iron-binding properties, namely iron absorption, antimicrobial activity, and modulation of iron metabolism during inflammation. However, despite their structural similarities, LF differs from its serum counterpart in several important aspects including location and functional activity. LF has been proposed to play a role in intestinal iron absorption, regulation of cellular proliferation and differentiation, protection against microbial infection, anti-inflammatory responses, regulation of myelopoiesis, immunomodulation and cancer prevention (Pan et al., 2007; Ward et al., 2005; Naidu, 2002; Min and Krochta, 2005; Steijns and Van Hooijdonk, 2000; Baveye et al., 1999; Nuijens et al., 1996; Levay and Viljoen, 1995; Iyer and Lönnerdal, 1993; Sanchez et al., 1992). Table 3 summarizes some of the established physiological roles for LF and its mechanisms.

The reported antimicrobial activities (Santagati et al., 2005; Valenti et al., 1998; Levay and Viljoen, 1995) of LF highlight the many possible modes by which it can contribute to host protection against microbial infections at the mucosal surfaces, namely by growth inhibition as a result of iron scavenging (Nemet and Simonovits, 1985; Brock, 1980), disruption of the bacterial cell membranes (Yamauchi et al., 1993; Ellison and Giehl, 1991; Al-Nabulsi and Holley, 2006) or blocking of cell-virus interactions (Andersen et al., 2001; Ikeda et al., 2000; Siciliano et al., 1999; Giansanti et al., 2002).

Regarding LF anti-inflammatory activity several mechanisms of action by blocking or inhibiting key mediators of the inflammatory response have also been proposed, such as binding to LPS (Miyazawa et al., 1991); inhibition of several cytokines (TNF-α and IL-1β) (Machnicki et al., 1993; Crouch et al., 1992; Slater and Fletcher, 1987; Baveye et al., 1999) or binding to bacterial CpG motifs (Britigan et al., 2001). Moreover, LF was found to elevate the number and increase the activity of T and B lymphocytes and NK cells (Dhennin-Duthille et al., 2000; Goretzki and Mueller, 1998), stimulate the release of a number of cytokines (Hangoc et al., 1991), increase phagocytic activity and cytotoxicity of monocytes/macrophages (Birgens et al., 1984; Van Snick and Masson, 1976), accelerate the maturation of T and B cells, and elevate the expression of several types of cellular receptors (Adamik et al., 1998; Bennett and Davis, 1981; Frydecka et al., 2002; Zimecki et al., 1991, 1995). Many immunological mechanisms are critically dependent upon cell–cell interactions; the number and affinity of interactions between two cells can often affect the nature of downstream events. The ability of LF to bind to cell surfaces is likely to affect these parameters, and could thus give rise to altered immune responses (Legrand et al., 2005).

Lactoferrin and iron metabolism

Despite much research into the interactions of LF with cells and tissues, there is still no good evidence that it plays any role as an iron-transport molecule or indeed is involved in "mainstream" iron metabolism (Brock, 2002). Nevertheless, it is consensual that LF influences the iron availability (Levay and Viljoen, 1995).

Iron is an element necessary for many metabolic functions of cells including cell proliferation. Therefore, cells and organisms have developed elaborate mechanisms of uptake and storage of iron to facilitate the utilization of this element in metabolic processes and in cell proliferation and at the same time to keep concentrations of free iron at a minimum. In normal physiological processes, iron uptake, storage and utilization are carefully regulated. Cellular damage arising from free radicals, generated by available iron, is kept at a low level by cellular defences and DNA repair systems (Liehr and Jones, 2001).

While iron supplementation and iron enrichment of foods is widely accepted, there have been persistent concerns about potential deleterious effects, because iron enhances colon and mammary carcinogenesis in rodents and because elevated body iron stores increase the risk of several cancers in humans (Weinberg, 1984, 1992; Selby and Friedman, 1988; Stevens et al., 1988). There is also indirect, but substantial evidence supporting a role of iron metabolism in breast cancer. For instance, a six-fold higher tissue ferritin concentration has been measured in malignant carcinoma of the breast compared to normal or benign tissue (Weinstein et al., 1982; Elliott et al., 1993). Furthermore, transferrin and tumour cell transferrin receptor proteins were elevated in breast carcinoma compared to normal or benign cells (Faulk et al., 1980; Rossiello et al., 1984).

All these data taken together indicate that iron enhances tumour development in humans. Certainly, proliferating tumour cells require iron and therefore tumour tissue may have increased transferrin receptor and ferritin levels.

Current evidence suggests that while LF plays no major role in normal iron homeostasis (Ward et al., 2005), it may contribute to alterations in iron metabolism during infection or inflammation. In addition, the iron-binding function of LF may contribute to other physiological functions.

Lactoferrin and human cancer

Many functions have been described for LF over the years, but concerning its anti-tumour role and mechanisms involved there is still a big controversy on whether LF's iron-binding activity

is implicated or not. Lonnerdal and Iyer (1995) state that it is likely to be the iron-binding properties of LF that contribute to its anti-tumour properties, since free iron may act as a mutagenic promoter by inducing oxidative damage to nucleic acid structure (Weinberg, 1984, 1992,1996). It is thought that LF may bind iron locally in tissues, therefore reducing the risk of oxidant-induced tumourigenesis (Stevens et al., 1988). Moreover, estrogenic hormones appear to regulate the uptake of iron and its utilization in proliferative processes (Liehr and Jones, 2001; Teng, 2006). Nevertheless, much research needs to be carried out in future to outline in more detail the regulation of iron metabolism by estrogen. Tumours may thus arise in cells, which are damaged by such processes and which at the same time are stimulated to proliferate by hormone receptor-mediated mechanisms. The possible contribution of iron to genetic lesions and to cell proliferation indicates that this element plays a crucial role in the development of estrogen-dependent neoplasms such as breast or uterine cancers (Weinstein et al., 1982; Elliot et al., 1993; Faulk et al., 1980; Rossiello et al., 1984). Therefore, such hormone responsive tumour development may be prevented by iron chelators (Ghio et al., 1992; Lund and Aust, 1990; Giancotti, 2006). Another possibility is the restriction of dietary iron intake and dietary iron supplements, or the diet supplementation with LF, as part of a strategy to reduce tumour development. Such studies need to be carried out in the future.

On the other hand, several other mechanisms have been described for the LF anti-tumour role, such as regulation of NK cell activity (Damiens et al., 1998; Matsuda et al., 2006), modulation of expression of G1 proteins (Damiens et al., 1999; Xiao et al., 2004), inhibition of VEGF(165)-mediated angiogenesis (Norrby et al., 2001), and enhancement of apoptosis (Yoo et al., 1997b; Sakai et al., 2006).

Damiens and co-workers (1998) investigated LF involvement, at inflammatory concentrations, in cancer progression. It was reported that LF has a significant effect on NK cell cytotoxicity against haematopoietic and breast epithelial cell lines. By pre-treatment of either NK cells or target cells with LF, it was demonstrated that the LF effect is due both to a modulation of NK cell cytotoxicity and the target cell sensitivity to lysis. It was also shown that LF inhibits epithelial cell proliferation by blocking the cell cycle progression. Furthermore, Damiens and

co-workers (1999) found that treatment of breast carcinoma cells MDA-MB-231 with hLF induced growth arrest at the G1 to S transition of the cell cycle by modulating the expression and the activity of key regulatory proteins.

The effect of orally administered iron-unsaturated bLF on angiogenesis induced by VEGF165 and IL-1-α in adult rats was assessed by Norrby and co-workers (2001). LF treatment was found to significantly inhibit the VEGF165-mediated response in terms of microvessel spatial extension, overall vascularity and incidence of crossover. Thus, the oral administration of LF thus appears to be of potential interest as an anti-angiogenesis treatment modality in the clinical setting.

Additionally, the activity of bovine LFCin to induce apoptosis in THP-1 human monocytic leukemic cells was considered (Yoo et al., 1997b). The results achieved suggested that LFCin, but not LF itself, is able to induce apoptosis in THP-1 human monocytic tumour cells, and that its apoptosis-inducing activity is related to the pathway mediated by production of the intracellular reactive oxygen species (ROS) and activation of Ca^{2+}/Mg^{2+}-dependent endonucleases.

Finally, there have also been a number of animal studies showing that LF can inhibit development of experimental tumours (Ushida et al., 1998; Tsuda et al., 2002).

Although the results achieved by several researchers point to a clear anti-tumour role of LF (Benaissa et al., 2005), the mechanisms by which it is exerted are not fully understood, thus further work on this subject is warranted.

LACTOFERRIN AS A CHEMOPREVENTIVE AGENT

Several studies have been done to evaluate the effect of orally administrated LF in healthy or diseased human beings and animals (Tomita et al., 2002; Teraguchi et al., 2004). To the date, it has become evident that the oral administration of LF exerts various beneficial effects against diseases (Table 4), namely as a chemopreventive agent. For the practical use of chemopreventive agents, it is important to know whether their action is limited to only

inhibition. Therefore, use of chemopreventive agents should follow appropriate indications based on reliable information regarding beneficial preventive and adverse promoting or toxic effects (Tsuda et al., 2000). A thorough follow-up of patients or populations during and after the administration of chemopreventive agents is obviously necessary for effective assessment and conclusions to be drawn. For general public use, the agents require safety approval based on a long-term toxicity or carcinogenicity testing. Further development of appropriate *in vivo* animal assay systems to provide reliable information regarding organotropism and adverse effects is also critical for this purpose. To this regard, use of naturally occurring compounds is advantageous because most of them are ingested routinely as food components. Fiber, unsaturated fatty acid, carotenoids, flavonoids, phenolic compounds, especially polyphenols, and now bLF could be promising compounds in this respect. bLF as a food ingredient is thought to be safe, because there is a long dietary history of its use. People who live on dairy products must have ingested bLF for a long time, as raw milk and natural cheese contain high amounts of this protein. A human clinical study in chronic hepatitis C patients showed that high bLF oral doses, up to 7.2 g/body/day were well tolerated (Okada et al., 2002).

Troost and co-workers (2001) demonstrated that over than 60% of administrated bLF survives the passage through adult human stomach and enters the small intestine in an intact form. On the other hand, analysis of the gastric contents revealed that LFCin B was formed at a molar concentration corresponding to 4.5% of ingested bLF (Kuwata et al., 1998a). When the animals were given free access to milk containing bLF at 40 mg/ml (482 μmol/l), the levels of bLF fragments containing the LFCin B region in the contents of the stomach, upper small intestine, and lower small intestine were approximately 200, 20 and 1 μmol/l, respectively (Kuwata et al., 1998b; Kuwata et al., 2001). Some parts of ingested LF are likely to be not fully digested and to be present in the lower gastrointestinal tract. These intact bLF and partially digested bLF peptides, which retain biological activities, may exert various physiological effects in the digestive tract.

Orally administrated LF exhibits several beneficial effects at sites other than the digestive tract. In the case of infants or adults with injury in the intestine, it is possible that ingested LF enters

the blood circulation and acts systemically. However, neither bLF nor functional bLF fragments (LFCin B-containing peptides and anti-bLF antibody-binding peptides) were at a level higher than the detection limit (1 mg/ml) detected in the portal blood of normal adult rats orally given bLF at a maximal dose of 5 g/kg (Wakabayashi et al., 2004). Therefore, LF-related molecules are not likely to be transported from the intestine into the circulation. Hence, it is rational to consider that oral administered bLF and its digested products act initially on the intestinal immune system and augment the protective immunity systemically (Teraguchi et al., 2004).

The protective effect of LF against chemically induced carcinogenesis, tumour growth and/or metastasis have been demonstrated in an increasing number of animal model experiments, namely directed to specific organs, such as esophagus, tongue, lung, liver, colon and bladder (Bezault et al., 1994; Wang et al., 2000; Shimamura et al., 2004; Yoo et al., 1997a; Iigo et al., 1999; Tsuda et al., 2002; Ushida et al., 1999; Tanaka et al., 2000; Fujita et al., 2002; Masuda et al., 2000; Sekine et al., 1997a; Varadhachary et al., 2004; Kuhara et al., 2000). Table 5 summarizes the effectiveness of orally administrated LF–related compounds on cancer. Despite the evidence that LF possesses chemopreventive activity, little is known about (i) its anti-cancer activity against established tumours; (ii) its ability to potentiate chemotherapy, as described with other immunotherapeutics; or (iii) the immune mechanisms by which its anti-tumour activity is mediated. Moreover, no human clinical studies on the potential chemopreventive effect have been done so far.

As discussed above, it appears that like the other biological functions of LF, its anti-tumour role is complex (Ward et al., 2005).

Several researchers suggested a direct effect of LF on tumour cell growth based on the fact that both LF and a splice variant are downregulated or absent in many cancer cell lines and in experimental tumours (Campbell et al., 1992; Panella et al., 1991; Penco et al., 1999; Teng et al., 2004; Benaissa et al., 2005; Siebert et al., 1997; Breton et al., 2004; Rossiello et al., 1984). Furthermore, Damiens and co-workers (1999) found that treatment of human breast carcinoma cells MDA-MB-231 with hLF induced growth arrest at the G1 to S transition of the cell cycle by modulating the expression and the activity of key G1 regulatory proteins, including the Cdk

inhibitors p21 and p27, which may be mediated in part by modulation of the Akt and MAPK pathways. The reported effects of LF occur through a p53-independent mechanism both in MDA-MB-231 cells and other epithelial cell lines such as HBL-100, MCF-7, and HT-29.The same conclusions were achieved in another study using head and neck cell lines (Xiao et al., 2004). *In vivo* studies also suggest that the inhibition of tumour cell growth by LF may be related to the ability of this protein to induce apoptosis of cancer cells by activating the FAS signalling pathway in cancerous cells (Fujita et al., 2004a, b). Fujita and co-workers (2004a) studied the effect of LF on the gene expression of 10 apoptosis-related molecules in colon mucosa of azoxymethane (AOM)-treated rats during early and late stages of colon carcinogenesis and found that FAS and pro-apoptotic BCL-2 members participate in the LF action and may contribute to suppressive effects on tumour development in the rat colon. Additionally, they studied (Fujita et al., 2004b) the influence of bLF on FAS-mediated apoptosis with regard to expression of FAS, activation of caspase-8 and caspase-3, and DNA fragmentation in the colon mucosa of AOM-treated rats and the results confirmed the suggestion that apoptosis caused by elevated expression of FAS is involved in chemoprevention by LF in colon carcinogenesis.

LF was also found to stimulate the production and activation of several immune cells, including lymphocytes and NK cells (Legrand et al., 2004; Horwitz et al., 1984; Shau et al., 1992), in addition to increasing the target cell sensitivity to NK lysis (Damiens et al., 1998), thus immunomodulation may be critical to the anti-tumour role of LF. Damiens and co-workers (1998) investigated LF involvement, at inflammatory concentrations, in cancer progression. They reported that LF has a significant effect on NK cell cytotoxicity against haematopoietic and breast epithelial cell lines. By pre-treatment of either NK cells or target cells with LF, it was demonstrated that the LF effect is due both to a modulation of NK cell cytotoxicity and the target cell sensitivity to lysis. Moreover, LF was found to exert an effect on target cells depending on the cell phenotype, i.e. it does not modify the susceptibility to lysis of haematopoietic cells such as Jurkat and K-562 cells, but does significantly increase that of the

breast and colon epithelial cells. Ultimately, it was demonstrated that LF inhibits epithelial cell proliferation by blocking the cell cycle progression.

Marked inhibition of tumour growth and reduced lung colonization by B16-F10 melanoma experimental metastasis were found in mice treated with hLF injected intraperitoneal as a result of an enhanced NK cell activity (Bezault et al., 1994). Moreover, it has been shown that the protective effect of oral administration of LF in several rodent cancer models is associated with enhancement of the local intestinal mucosal immune response. In this regard, upregulation and/or enhanced activation of NK cells, CD4+ T lymphocytes and CD8+ T-lymphocytes were observed upon LF administration (Wang et al., 2000; Iigo et al., 1999; Kuhara et al., 2000). A low absorption of LF from the intestine was reported, nevertheless an enhancement of the systemic immune response was also observed.

Some authors proposed that the mechanism implicated in the protective effect of LF against cancer cell development is mediated by IL-18 (Reddy, 2004), as LF was found to strongly regulate IL-18 expression in intestinal epithelium (Iigo et al., 2004; Varadhachary et al., 2004; Kuhara et al., 2000). Finally, LF was also shown to inhibit tumour-initiated angiogenesis *in vitro* and *in vivo*, which may relate to the anti-angiogenic properties of IL-18 (Shimamura et al., 2004; Yoo et al., 1997a; Cao et al. 1999; Norrby, 2004), LF was found to participate as a regulator of angiogenesis, possibly by blocking endothelial function and inducing IL-18 production.

PRODUCTION OF LACTOFERRIN AND ITS DERIVATIVES

Many methods of isolating LF from milk have been reported (Groves, 1960; Johansson, 1969; Law and Reiter, 1977; Wakabayashi et al., 2006), but it is quite difficult to scale up an experimental method to achieve industrial production. There are many problems involved in the manufacture of high-purity LF, including the need for mass production, reuse of the residual milk materials after isolation of LF, a comparatively simple manufacturing process, and high levels of stability and sanitation of the plant equipment. Nowadays, high-purity LF is obtainable

on a laboratory scale using gel filtration chromatography (Al-Mashikhi and Nakai, 1987), immobilized monoclonal antibodies (Kawakami et al., 1987), chelating chromatography (Al-Mashikhi et al., 1988), hydrophobic interaction chromatography, Cibacron Blue affinity chromatography (Shimazaki and Nishio, 1991), immunoaffinity chromatography (Noppe et al. 2006), carboxymethyl cation exchange chromatography (Yoshida and Ye, 1991; Elagamy et al., 1996), cation exchange membranes (Chiu and Etzel, 1997), adsorptive membrane chromatography (Plate et al., 2006), simulated moving bed technology (Andersson and Mattiasson 2006), semi-batch foaming process (Saleh and Hossain, 2001), and microfiltration affinity purification (Chen and Wang, 1991). However, none of these techniques has been used at commercial scales because of high processing costs (Zydney, 1998).

Recently, an approach of industrial isolation and purification of bovine LF (approximately 20-30 ton annually worldwide) from cheese whey and skim milk was reported (Tamura, 2004). The concentration of LF in cheese whey is roughly 100 mg/L and about 100-400 mg/l in bovine milk. Since LF exists as a cationic protein in milk and whey, it is readily adsorbed to a cation-exchange resin and then eluted using salt solutions. The eluted crude LF is then desalted and concentrated using ultrafiltration and diafiltration membranes, after which it is subjected to pasteurization. Purified LF powder with a purity of 95% or higher is finally obtained by freeze-drying (Tomita et al., 2002). In an alternative process, microfiltration and spray-drying are performed instead of pasteurization and freeze-drying, respectively. While making efforts to develop a practical method for the pasteurization of LF, Abe and co-workers (1991) found that LF is stable against heat treatment under acidic conditions, while heat treatment at a neutral pH causes denaturation of the protein. It is considered that heating at a pH above 4 and to a temperature of 90–100°C for 5–10 min as well as the UHT method are suitable and practical methods for the pasteurization of LF. This pasteurization process was patented, and it has been applied to the manufacture of a wide variety of commercial products containing LF. Additionally, LF was also isolated from bovine colostrum by ultrafiltration and then purified with a fast flow strong cation exchange chromatography system in a production scale (Lu et al., 2007). Processes involving chromatography have some limitations at industrial scale such as

high cost and relatively low throughputs. Membrane filtration could represent an interesting alternative to chromatography for LF fractionation as membrane processes are already well integrated in the dairy industry. However, fouling and poor selectivity in protein separation have been associated with such membrane filtration processes. Different strategies have been investigated to overcome limitations associated with LF separation by membrane filtration including variation of the hydrodynamic parameters (Chilukuri et al., 2001), modification of the physico-chemical environment (Chaufer et al., 2000; Rabiller-Baudry et al., 2001; Brisson et al., 2007), use of different membrane types (Mehra and Donnelly, 1993; Ulber et al., 2001) and alteration of the membrane surface properties (Rabiller-Baudry et al., 2001).

Regarding LF derivatives, a pepsin hydrolysate of LF was produced by treatment with porcine pepsin under acidic conditions (Saito et al. 1991). After hydrolysis has been completed, pepsin was inactivated by heat treatment. Then the reaction mixture was filtered and concentrated by reverse osmosis. Finally, the hydrolysate of LF was obtained by pasteurization and freeze-drying for use in infant formula. In addition, lactoferricin can be purified from this LF hydrolysate by two-step hydrophobic chromatography (Bellamy et al., 1992). The peptide is eluted with an acidic buffer, the eluted solution is concentrated by reverse osmosis, and finally, lactoferricin is produced by freeze-drying as a powder with over 95% purity. This production process for lactoferricin has also been patented. The toxicity of purified LF was judged to be extremely low in safety tests. From the results of single-dose, 4-week, and 13-week oral toxicity tests, the dose of LF that caused no adverse effects was found to be 2000 mg./kg/day for rats of both sexes (Yamauchi et al., 2000a). In addition, LF did not exhibit any mutagenic potential in a bacterial reverse-mutation test (Yamauchi et al. 2000b). Based on the results of these safety tests and the results of clinical studies, purified LF is considered to be a highly safe food additive.

Along with increased recognition of the biological effects of LF, as described below, the applications of LF have been expanded. LF-supplemented infant formula, follow-up milk, skim milk, yogurt, chewing gum, and nutritional supplements are being marketed. In addition to foods, LF is also used in skin care cosmetics, in special therapeutic diets for the relief of inflammation in dogs and cats, and in aquaculture feed (Wakabayashi et al., 2006).

CONCLUSION

Evidence that diet is a key environmental factor affecting the incidence of many chronic diseases is overwhelming. Indeed, several food derived compounds we eat are among the most promising chemopreventive agents being evaluated. To this regard, the use of naturally occurring compounds, like for instance LF, is advantageous because most of them are ingested routinely as food components and therefore they are safe. Nevertheless, the approval of a new chemopreventive agent requires the development of appropriate *in vivo* animal assay systems to provide reliable information regarding organotropism and adverse effects.

A host of interesting features of LF, namely its role on iron homeostasis, organ morphogenesis and host defence against infection, inflammation and cancer, have led to a wide range of potential applications in the medical field. Distinct mechanisms of action have been described for each LF's functions which possibly work in concert to potentiate its biological effect *in vivo*. LF presents the capacity to bind to a broad variety of epithelial and immune cells, which probably confers this protein the ability to regulate cellular signalling pathways and consequently exert its protective functions, for example against cancer. Nevertheless, further investigations on the cellular localization and downstream molecular events that follow LF engagement with these receptors are needed. The protective effect of LF against chemically induced carcinogenesis, tumour growth and/or metastasis have been demonstrated in an increasing number of animal model studies, therefore suggesting its great potential therapeutic use in cancer disease prevention and/or treatment. However, much available data has been derived from studies where high doses of non homologous LF of unknown purity were used and to which the tissues in our bodies may never be exposed, therefore all extrapolations require a careful interpretation of the results.

Finally, despite the evidence that LF possesses chemopreventive activity, its anti-cancer activity against established tumours, ability to potentiate chemotherapy as described with other

immunotherapeutics, and the immune mechanisms by which its anti-tumour activity is mediated, is still largely unknown.

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Table 1 Occurrence of lactoferrin (hLF – human lactoferrin; bLF – bovine lactoferrin) in biological fluids

	Biological fluid	Amounts reported (mg/ml)*
hLF	Colostral breast milk	> 7
	Mature breast milk	> 1-2
	Tear fluid	> 2.2
	Seminal plasma	> 0.4-1.9
	Synovial fluid	> 0.01-0.08
	Saliva	> 0.007-0.01
bLF	Colostral whey	> 1.5
	Milk	> 0.02-0.2

* Data adapted from Levay and Viljoen, 1995; Steijns and Van Hooijdonk, 2000; Babina et al., 2006; Shimazaki, 2000

Table 2 Lactoferrin (hLF – human lactoferrin; bLF – bovine lactoferrin) physico-chemical properties

	bLF	hLF	References
Amino acid residues	689	690	Pierce et al., 1991; Baker, 1994
Molecular weight	77 ± 1.5	76.8 ± 1.6	Querinjean et al., 1971; Spik et al., 1994; Castellino et al., 1970
Isoelectric point	8.5-9.0	8.4-9.0	Spik et al., 1994; Lampreave et al., 1990; Zakharova et al., 2000

Table 3 Reported *in vitro* and *in vivo* physiological roles of lactoferrin

Physiological role	Mechanism	References
Iron absorption	Increasing solubility and receptor mediated uptake	Kawakami et al., 1993; Mikogami et al., 1995; Levay and Viljoen, 1995; Ward et al., 2005
Antioxidant	Iron scavenger LF has the ability to bind to cell membranes enhancing its ability to prevent iron-mediated lipid peroxidation	Matsue et al., 1994; 1995; Konishi et al., 2006; Larkins, 2005
Antimicrobial	Growth inhibition by iron scavenging or membrane disintegration LF has a powerful iron-binding capacity and shows a strong interaction with other molecules and cell surface	Nemet and Simonovits, 1985 ; Yamauchi et al., 1993 ; Qiu et al., 1998 ; Weinberg, 2007 ; Santagati et al., 2005; Lönnerdal, 2003 ; Lee et al., 2005
Antiviral	Prevention of virus attachment, inhibition of virus replication, blocking of cell-virus interactions	Yi et al., 1997 ; Siciliano et al., 1999 ; Andersen et al., 2001 ; Ikeda et al., 2000 ; Giansanti et al., 2002 ; Longhi et al., 2006; Mistry et al., 2007; Ammendolia et al., 2007 ; Pan et al., 2006
Anti-inflammatory, immune modulating	LPS binding, stimulation of NK cells, reduction of pro-inflammatory cytokines, T-cell maturation. LF modulates the migration, maturation and function of the immune cells at the cellular level and at the molecular level	Zimecki et al., 1991, 1995 ; Hangoc et al., 1991 ; Crouch et al., 1992 ; Yamauchi et al., 1993 ; Adamik et al., 1998 ; Baveye et al., 1999 ; Dhennin-Duthille et al., 2000 ; Britigan et al., 2001; Fischer et al. 2006; Berlutti et al. 2006; Senkovich et al., 2007; Legrand et al., 2005; Kruzel et al., 2006
Anti-cancer	Regulation of NK cell activity, modulation of expression of G1 proteins, inhibition of VEGF(165)-mediated angiogenesis, enhancement of apoptosis	Bezault et al., 1994 ; Sekine et al., 1997 ; Yoo et al., 1997b ; Damiens et al., 1998, 1999 ; Norrby et al., 2001 ; Mohan et al., 2006a; McKeown et al., 2006; Kuhara et al., 2006 ; Baumrucker et al. 2006; Kim et al., 2006; Giuffre et al., 2006 ; Kawakami et al., 2006

Table 4 Effectiveness of orally administered bLF in humans.

Disease	Efficacy	Administrated agent and dose	References
Bacterial flora:			
Faecal flora in low birth weight infants	Increase of *Bifidobacterium*, decrease of *Clostridium*	bLF; 1mg/ml in infant formula	Wakabayashi et al., 2006
Faecal flora in infants	Increase of *Bifidobacterium*		Roberts et al., 1992; Logsdon et al., 2006
Infection (digestive tract):			
Gastric infection with *Helicobacter pylori*	Increase of eradication by triple therapy	bLF; 0.2g/body	Di Mario et al., 2003
Infection (other than digestive tract):			
Neutropenic patients	Decrease of incidence of bacteremia and severity of infection	bLF; 0.8 g/body	Trumpler et al., 1989
Chronic hepatitis C	Decrease of ALT and HCV RNA in serum	bLF; 1.8 and 3.6 g/body	Tanaka et al., 1999 ; Iwasa et al., 2002 ; Yamauchi et al., 2000c ; Konishi et al. 2006 ; Katsuaki, 2006
Influenza	Attenuation of pneumonia through the suppression of infiltration of inflammatory cells in the lung	62.5 mg per mouse	Shin et al., 2005

Table 5 Effectiveness of orally administered LF-related compounds on cancers

Cancer model	Efficacy	Animal	Administrated agent and dose	References
Carcinogen-induced tumour in colon, lung, esophagus, bladder, liver, tongue	Inhibition of tumour development	Rat	bLF 0.2 and 2% in diet	Sekine et al., 1997a, b; Ushida et al., 1999; Tanaka et al., 2000; Masuda et al., 2000; Fujita et al., 2002; Mohan et al., 2006b
Spontaneously developed intestinal polyposis	Inhibition of polyp development	Apc^{Min} mouse	bLF 2% in diet	Ushida et al., 1998
Tumour cell injection	Inhibition of lung metastasis	Mouse	bLF 0.3 g/kg	Iigo et al., 1999
Tumour cell injection	Inhibition of tumour development	Mouse	rhLF 1 g/kg	Varadhachary et al., 2004