International Dairy Journal 57 (2016) 1–6

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Review

Structure, function and nutritional potential of milk osteopontin

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ARTICLE INFO

Article history:
Received 28 October 2015
Received in revised form
3 February 2016
Accepted 6 February 2016
Available online 22 February 2016

ABSTRACT

Osteopontin (OPN) is a multifunctional protein present in most tissues and body fluids, with the highest concentrations found in milk. Processes for isolation of OPN from bovine milk for use in infant formula have been developed and studies have investigated the effects of oral administration of milk OPN. At the same time, plasma OPN levels have been shown to be elevated in some types of cancer, and OPN has been suggested as a potential diagnostic marker for cancer. OPN exists in several different isoforms in vivo, of which presumably only a minority is directly or indirectly implicated in cancer related events. In this article, we review the differences between milk-derived OPN and OPN derived from transformed cells and compare the structure of OPN from human and bovine milk. Furthermore, current knowledge about the function of OPN in milk and recent findings about the effect of orally presented OPN is discussed.

Contents

1. Introduction

Mammalian osteopontin (OPN) is an acidic highly phosphorylated glycoprotein containing the integrin binding Arg–Gly–Asp (RGD) and SVVYGLR sequences. OPN is present in most tissues and body fluids (Sodek, Ganss, & McKee, 2000), with the highest concentrations found in milk (Schack et al., 2009a). OPN is a key component in a variety of physiological processes such as inhibition of ectopic calcification, bone remodelling, cancer metastasis and immune modulatory functions (Anborgh, Mutrie, Tuck, & Chambers, 2011; Sodek et al., 2000; Wang & Denhardt, 2008). OPN can act as an opsonin, as it binds directly to several bacterial strains leading to enhanced phagocytosis by macrophages (Schack et al., 2009b). OPN is relatively resistant to proteolysis by neonatal gastric juice (Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004) and can induce Th1 type immunity by inducing interleukin-12 expression in macrophages (Ashkar et al., 2000). This indicates that milk OPN could be an important factor in the development of the immune system of infants.

Processes for isolation of OPN from bovine milk have been disclosed (Bertelsen, Wejse, & Trúgvason, 2014; Sørensen, Ostersen, Chatterton, Holst, & Albertsen, 2007). OPN is isolated from bovine whey using anion exchange technology and the final product contains ~78% protein of which 95% is OPN. OPN isolated by this method (Lacprodan OPN-10) contains ash (max. 9%), moisture

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http://dx.doi.org/10.1016/j.idairyj.2016.02.034

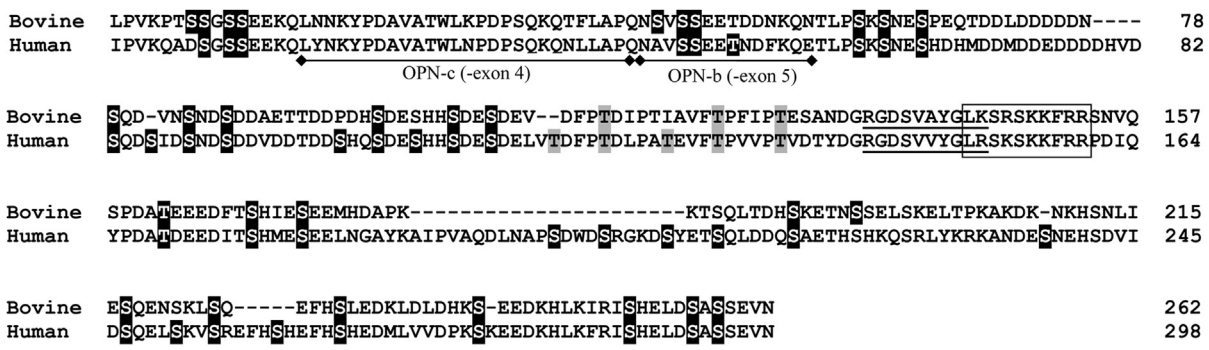

Fig. 1. Post-translational modification of bovine and human milk osteopontin (OPN). Phosphorylation and glycosylation sites are highlighted in black and grey, respectively (Christensen et al., 2005; Sørensen et al., 1995). The regions containing the identified cleavage sites in bovine and human milk are boxed (Christensen et al., 2010; Christensen & Sørensen, 2014). Exons 4 and 5 missing in OPN-c and OPN-b are indicated. The integrin binding RGD and SVVYGLR-motifs are underlined and introduced gaps are indicated by dashed lines.

(max. 5.5%) and less than 1% of fat and lactose (Kvistgaard, Matulka, Dolan, & Ramanujam, 2014). The bovine milk OPN (in the form of Lacprodan OPN-10) has been characterised by mass spectrometry and functional studies, which showed that it is highly phosphorylated and glycosylated (Addison, Azari, Sørensen, Kaartinen, & McKee, 2007). Milk OPN is very resistant to harsh conditions; e.g., heat treatment at 90 °C has no effect on the post-translational modifications decorating the protein (Sørensen, Højrup, & Petersen, 1995). Studies have investigated the beneficial effects of oral administration of milk OPN; however, at the same time, endogenously expressed OPN has for long been considered a factor in cancer related events (Anborgh et al., 2011; Bellahcène, Castronovo, Ogbureke, Fisher, & Fedarko, 2008; Gimba & Tilli, 2013). Here we review the differences between milk-derived OPN and OPN derived from transformed cells (cancer model) and compare the structure of OPN from human and bovine milk. Furthermore, we will review and discuss current knowledge about the function of OPN in milk and recent findings about the effect of orally presented OPN.

2. Structure and expression – comparison of human and bovine milk osteopontin

Human OPN comprises 298 amino acids whereas the bovine form only contains 262 residues, mainly because bovine OPN lacks a sequence of 22 residues corresponding to residues 188–209 in human OPN (Fig. 1). Comparison of the bovine and human OPN sequences reveals that 182 amino acids are identical (61%) and an additional 44 residues retain high structural similarity. OPN is encoded by a single copy gene, and during transcription human OPN can undergo alternative splicing generating two splice variants each lacking a single exon (Young et al., 1990). Moreover, OPN can be post-translationally modified by especially phosphorylation, *O*-glycosylation, and proteolytic processing (Kazanecki, Uzwiak, & Denhardt, 2007b; Qin, Baba, & Butler, 2004) leading to the existence of numerous OPN isoforms (Fig. 1). OPN isoforms are cell and tissue-specific and OPN expressed by different cell-types or under different conditions have been shown to be functionally different (Christensen et al., 2007; Christensen, Kläning, Nielsen, Andersen, & Sørensen, 2012; Kazanecki et al., 2007b).

We have performed PCR analysis on a bovine mammary gland cDNA library using gene-specific primers that revealed only a single transcript with a size of ~1045 base pairs (Fig. 2A), which is the expected size without alternative splicing. Similarly, we analysed the existence of differential RNA splicing from both a human mammary gland cDNA library and by reverse transcription-PCR of milk cells from two women. In all cases, only a single transcript was observed and no alternative splicing was detected (Fig. 2B) indicating that alternative splicing of OPN does not occur in bovine and human milk. These results are in accordance with other studies showing that alternative OPN splicing in both human and bovine milk is undetectable in normal specimens (Bissonnette, Dudemaine, Thibault, & Robitaille, 2012; Mirza et al., 2008).

In both bovine and human milk, OPN is very prone to proteolytic cleavage close to the RGD- and SVVYGLR sequences and hence a

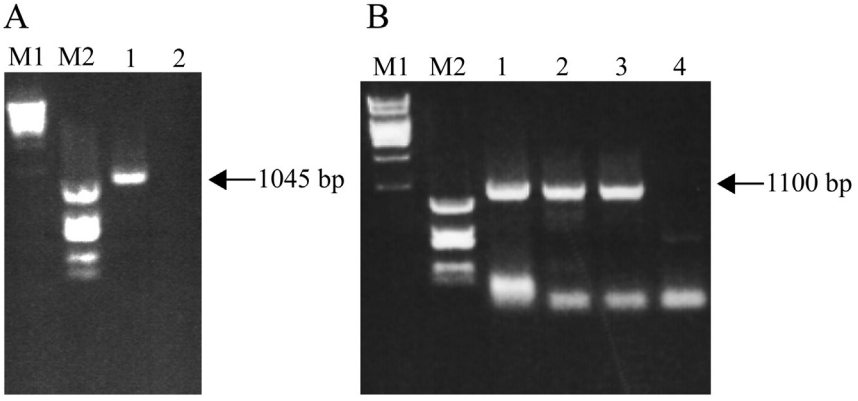

Fig. 2. Osteopontin (OPN) expression in mammary gland and human milk cells: PCR of mammary gland cDNA libraries using primers encompassing the coding sequence of bovine or human OPN, or reverse transcription PCR performed using total RNA from human milk cell transcripts. Panel A: lane M1, lambda marker; lane M2, pUC marker; lane 1, bovine mammary gland cDNA library; lane 2, no template control. Panel B: lane M1, lambda marker; lane M2, pUC marker; lane 1, human mammary gland cDNA library; lanes 2 and 3, cDNA from human milk cells; lane 4, no template control.

large fraction of milk OPN is found in a fragmented form with exposed integrin binding motifs (Fig. 1) (Bissonnette et al., 2012; Christensen, Schack, Kläning, & Sørensen, 2010; Christensen & Sørensen, 2014). Proteolytic cleavage close to the integrin binding motifs has been demonstrated to increase the integrin binding properties of OPN (Agnihotri et al., 2001; Christensen et al., 2010; Yokosaki, Tanaka, Higashikawa, Yamashita, & Eboshida, 2005).

OPN is highly phosphorylated in milk with ~25 phosphates distributed over 36 potential sites (34 serines and 2 threonines) in the human milk OPN (Christensen et al., 2012; Christensen, Nielsen, Haselmann, Petersen, & Sørensen, 2005) and ~22 phosphates distributed over 28 potential sites in bovine milk OPN (Boskey, Christensen, Taleb, & Sørensen, 2012; Sørensen et al., 1995). The phosphorylations are arranged in clusters of 3–5 phosphoresidues and are predominantly located in the target sequence of the kinase FAM20C (Tagliabracci et al., 2012). Bovine milk OPN contains three *O*-glycosylated threonine residues close to the integrin binding motifs (Fig. 1) (Sørensen et al., 1995). These threonine residues are well conserved among all analysed mammalian OPN sequences and are also glycosylated in human milk (Christensen et al., 2005). Human milk OPN contains two additional threonine-linked oligosaccharides on less well conserved threonines (Fig. 1). The glycan structures on human milk OPN have been shown to consist of large fucosylated N-acetyllactosamine units (Christensen et al., 2012), whereas the carbohydrates on bovine OPN consist of a disialylated GalNAc-galactose cores (Boskey et al., 2012; Christensen et al., 2007; Christensen, Petersen, & Sørensen, 2008). The functional role of OPN glycosylation in milk is not clear, but their location close to the integrin binding motifs could suggest a protective role of the carbohydrates against cleavage by endogenous milk proteases. Alignment of the amino acid sequences (Fig. 1) show that all structural important elements, like the integrin binding sequences, sites for post-translational modification and regulatory proteolytic cleavage sites are well conserved between human and bovine OPN.

3. Role of osteopontin in milk

OPN is present in many tissues and excretions, but by far the highest concentration of the protein is found in milk. Human milk contains ~138 μg mL^{-1} and bovine milk contains ~18 μg mL^{-1} (Schack et al., 2009a). In comparison, the OPN concentrations in adult human plasma and urine have been measured to be ~35 ng mL^{-1} and ~4 μg mL^{-1}, respectively (Kolbach et al., 2012; Schack et al., 2009a), and recently it was estimated that bovine plasma contains ~40 ng mL^{-1} OPN (Dudemaine, Thibault, Alain, & Bissonnette, 2014). The high level of OPN expression in human milk persists throughout the lactation period, and OPN, which possess known cytokine-like properties, was the most abundantly expressed when compared with the expression of 240 cytokine related genes in human milk (Nagatomo et al., 2004). Furthermore, the levels of OPN in the umbilical cord and infants' plasma are 7–10 times higher than in adult plasma (Schack et al., 2009a). Collectively, this suggests that OPN plays a role in the development of the infant and could provide an important immunological signal during development as will be discussed later.

OPN has been reported to be involved in mammary gland development and differentiation, as transgenic mice expressing OPN antisense-RNA have severe lactation deficiency and display a lack of mammary alveolar structures (Nemir et al., 2000). In contrast to these transgenic mice, OPN knockout mice are both fertile, lactating and produce a normal litter size (Rittling et al., 1998).

Milk OPN is a very acidic protein due to a high degree of negatively charged amino acids and the many phosphorylations decorating the protein. This intrinsic property allows OPN to bind and form soluble complexes with calcium ions, which together with especially the caseins, could inhibit unintentional precipitation of amorphous calcium phosphate in milk (Holt, Lenton, Nylander, Sørensen, & Teixeira, 2014; Holt, Sørensen, & Clegg, 2009; Kläning, Christensen, Sørensen, Vorup-Jensen, & Jensen, 2014). In vivo models using OPN-deficient mice have supported an inhibitory function of the protein in ectopic calcification (Ohri, Tung, Rajachar, & Giachelli, 2005; Steitz et al., 2002).

OPN can induce the expression of the Th1 cytokine interleukin-12 and inhibit the production of the Th2 cytokine, interleukin-10 (Ashkar et al., 2000). Hence OPN is a key cytokine in the regulation of the Th1/Th2 balanced immune response. Interestingly, phosphorylation of OPN is necessary for the induction of interleukin-12 expression (Ashkar et al., 2000). The generation of a Th1 response is essential for the clearance of intracellular pathogens, and OPN-deficient mice are more susceptible to both viral and bacterial infections than wild type mice (Ashkar et al., 2000; Nau et al., 1999). Thus, breast milk OPN could be hypothesised to protect infants against infections by inducing a Th1 immune response. This correlates with an observed difference in the induction of a Th1-like response in breast-fed, but not formula-fed, infants after immunisation against measles, mumps, and rubella (Pabst et al., 1997). Furthermore, milk OPN can induce the expression of interleukin-12 from intestinal mononuclear cells (Schack et al., 2009a). Human and bovine milk OPN have been shown to be resistant to proteolysis by neonatal gastric juices at pH 3.0 for 1 h at 37 °C (Chatterton et al., 2004) indicating that ingested milk OPN could contribute to polarise the immune system in a Th1 direction at the gut mucosal surface of infants.

OPN-knockout suckling mice are more susceptible to rotavirus infection and show more intense and prolonged diarrhoea than wild-type suckling mice (Maeno et al., 2009). OPN has also been shown to bind directly to bacteria and to enhance phagocytosis of these (Schack et al., 2009b). Likewise, OPN reduces dental biofilm formation by binding to the surface of bacterial cells (Schlafer et al., 2012). This suggests that OPN in milk can also play a more direct role in the immune defence by interacting directly with invading pathogens.

In milk, OPN can form complexes with lactoferrin, lactoperoxidase and IgM through electrostatic or affinity interactions (Azuma, Maeta, Fukuchi, & Kanno, 2006). OPN has therefore been hypothesised to act as a transporter of these immunomodulating and antimicrobial proteins to their site of action and to protect them from proteolysis (Azuma et al., 2006; Yamniuk, Burling, & Vogel, 2009). Thus, OPN can in several different ways contribute to the defence against pathogens in milk and thereby also in the neonate or infant.

4. Osteopontin and cancer – same gene but different protein

Human OPN has been associated with cancer development (Anborgh et al., 2011; Bellahcène et al., 2008). Clinically, OPN has been suggested as a potential biomarker in some cancer types, where its expression has been associated with disease progression in several cancer forms (Bellahcène et al., 2008; Shevde & Samant, 2014). In vitro and in vivo, OPN has been reported to influence the behaviour of cancer cells in a manner that promotes malignancy, including cell survival, proliferation, invasion, angiogenesis and metastasis at distant sites (Bellahcène et al., 2008; Shevde & Samant, 2014). Plasma OPN levels in different cancer patients are elevated to 115–198 ng mL^{-1} (Bramwell et al., 2006; Hotte, Winquist, Stitt, Wilson, & Chambers, 2002; Ramankulov et al., 2007; Singhal et al., 1997). This is significantly higher compared with the OPN level in healthy adults of ~30–45 ng mL^{-1} (Ramankulov et al., 2007; Schack et al., 2009a; Singhal et al., 1997). Though, the OPN concentrations in the umbilical cord (263 ng mL^{-1}) and infants' plasma (342 ng mL^{-1})

are even higher (Schack et al., 2009a). This clearly demonstrates that high OPN plasma levels cannot exclusively be connected with cancer processes. These plasma levels also indicate that OPN plays a role in the normal developmental processes taking place in the growing infant.

It is not clear whether the elevated OPN expression observed in cancer patients is part of the disease process or whether it is an immunological response to the cancer. Neither is it clear whether the OPN expressed by the transformed cells in the tumour is the same as the OPN expressed by the normal cells in the body. It has been suggested that the two forms of OPN have different structure and function, as host-derived OPN has been observed to act as a macrophage chemoattractant, whereas tumour-derived OPN enhanced growth and survival of metastases (Crawford, Matrisian, & Liaw, 1998). Post-translational modification can regulate the function of OPN in physiological and pathological processes, and most tumour cells appear to express hypophosphorylated OPN (Anborgh et al., 2011). For instance, normal rat kidney cells secrete both phosphorylated and non-phosphorylated OPN (Nemir, DeVouge, & Mukherjee, 1989). However, after transformation of the cells the expression of non-phosphorylated OPN was significantly increased and expression of phosphorylated OPN was decreased. The OPN gene is responsive to *ras* (Guo, Zhang, Mitchell, Denhardt, & Chambers, 1995), which is an oncogene, and *ras*-mutations are frequently observed in cancer cells (Downward, 2003). Therefore *ras*-transformed cells can be used as a model for cancer cell modification of OPN. We have compared the expression of OPN in *ras*-transformed fibroblast with the expression in the parental non-transformed cells using different monoclonal antibodies recognising sites that potentially can be phosphorylated. It is clear that the OPN produced by the two versions of the cells are structurally different, and that the OPN produced by the *ras*-transformed cells are not phosphorylated to the same degree as the OPN produced by the normal cells (Fig. 3). This is in line with previous characterisations of OPN forms (Kazanecki, Kowalski, Ding, Rittling, & Denhardt, 2007a) and in particularly with an analysis of OPN from lactating mammary glands of mice compared with OPN from mammary tumours arising from *ras*-transformation. This analysis showed a significant difference between the two OPN forms (Rittling & Novick, 1997), which most likely could be attributed to a difference in phosphorylation of the OPN isoforms. In a more direct analysis, only four phosphate groups were identified on OPN expressed by *ras*-transformed fibroblasts making it the least phosphorylated OPN form described so far (Christensen et al., 2007). This low level of phosphorylation of OPN from transformed cells is in strong contrast to milk OPN which is highly phosphorylated and contains 22–25 phosphate groups (Boskey et al., 2012; Christensen et al., 2012).

OPN pre-mRNA consists of 7 exons (Young et al., 1990); however, two splice variants with deletions of exon 4 (OPN-c) or exon 5 (OPN-b) have been described in humans (Fig. 1). Though the expression and functional roles of the different isoforms is unclear, OPN-C is specifically expressed in different tumours such as ovarian, prostate, breast cancer tissues where this isoform has shown malignancy-promoting effects (Gimba & Tilli, 2013). However, alternative splicing does not occur in the OPN expressed in milk as mentioned earlier.

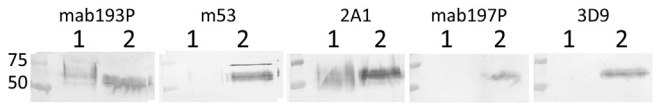

Fig. 3. Monoclonal antibody recognition of osteopontin (OPN) from fibroblasts and *ras*-transformed fibroblasts. Western blotting results showing monoclonal antibody recognition of OPN from non-transformed murine 3T3 fibroblasts (lane 1) or from *ras*-transformed murine fibroblasts (lane 2). The *ras*-transformed cells were derived from the parental line 3T3-275 by transformation with ras^{val12} (Wu, Denhardt, & Rittling, 2000). The epitopes for the antibodies are $P^{190}VA^{192}$ for 2A1, K^{283}FRISHELDSASSEVN^{298} for 3D9, and Y^{149}GLRSKS^{154} for m53 (Anborgh et al., 2009; Kazanecki et al., 2007a), and mab193P and mab197P have unspecified epitopes in the N- and C-terminal parts of osteopontin, respectively (Plumer et al., 2008).

In conclusion, though OPN is coded by a single gene, many isoforms exists and milk OPN and cancer derived-OPN seem to be structurally different. There is no indication that milk OPN should be involved in cancer development. On the contrary, in a recent study mice were injected with *ras*-transformed fibroblastic tumour cells, and it was demonstrated that oral intake of water containing 300 mg L^{-1} bovine milk resulted in a significant reduction in the growth rate and size of the tumours (Rittling, Wejse, Yagiz, Warot, & Hui, 2014). The mechanism for this suppression of tumour growth was related to angiogenesis, as tumours from OPN fed mice had abnormally large blood vessels which were speculated to be a consequence of insufficient nutrient transfer of the vessels or that they could be inherently unstable (Rittling et al., 2014).

5. Oral administration of milk osteopontin

Human breast milk contains ~138 mg L^{-1} OPN, which corresponds to ~2.1% (w/w) of the total protein in human milk. Bovine milk contains ~18 mg L^{-1} and standard infant formula contains on average only ~9 mg L^{-1} OPN (Schack et al., 2009a). Bovine and human milk OPN are structurally very similar with regard to amino acid sequence and phosphorylation pattern, and both proteins are *O*-glycosylated in the same region of the protein. Therefore it is suggested to use bovine OPN in infant formulas to close the concentration gap to human milk (Boskey et al., 2012; Christensen et al., 2012, 2005; Sørensen et al., 1995).

As a natural occurring milk protein and as such part of the natural optimal diet for infants, it seems evident that oral intake of milk OPN should not be harmful in any way. Though, as it is a new component proposed for use in infant formula, it has been subjected to safety evaluations. A proprietary whey-based protein product (Lacprodan OPN-10) that contains approximately 95% bovine milk OPN has been tested and found not to be genotoxic in vitro and in vivo (Kvistgaard et al., 2014). Lacprodan OPN-10 has been used as a source of bovine milk OPN in several studies investigating the effect of oral intake of OPN. For instance, studies showed that intake of a diet with up to 2% Lacprodan OPN-10 for 91 d was not toxic nor did it affect general health, growth or was teratogenic when administered to pregnant Wistar rats (Kvistgaard et al., 2014).

Recent studies have shown that supplementing formulas with Lacprodan OPN-10 have beneficial effects on formula fed offspring and infants. In a study with infant rhesus monkeys, OPN was shown to have a large impact on the genes expressed in the intestine (Donovan et al., 2014a). From birth to 3 months of age, infant rhesus monkeys were exclusively breastfed, fed normal formula without OPN or formula containing 125 mg L^{-1} bovine milk OPN. Addition of OPN to the formula did not result in any difference in overall neonatal growth, body composition or blood immune cell composition. However, microarray analysis of the small intestinal transcriptome showed that 1017 genes were differently expressed between the formula-fed and breastfed rhesus infants, but addition of OPN to the formula reduced the difference to 217 genes (Donovan et al., 2014a). This demonstrates that bovine milk OPN at a concentration comparable with what is found in human milk shifts the gene expression in the intestine to be more similar to that found in breast-fed infants.

The effect of adding bovine milk OPN (Lacprodan OPN-10) to formula has also recently been investigated in human infants

(Lönnerdal, Kvistgaard, Peerson, Donovan, & Peng, 2015). In a randomised controlled trial, mothers either breast- or formula-fed their infants from 1 to 6 months of age. The formula-fed infants received either standard formula or formula with 65 mg L^{-1} or 130 mg L^{-1} OPN (double-blinded). The study showed that addition of OPN changed the plasma levels of several amino acids and cytokines in formula-fed infants to be more similar to what was observed in breast-fed infants, though not all changes were statistically significant. Interestingly, addition of OPN to formula significantly lowered the levels of the pro-inflammatory cytokine TNF-α but significantly increased levels of interleukin-2 that plays key roles in oral tolerance (Lönnerdal et al., 2015). Importantly, there were no differences in appetite, growth, weight or height among the different infant groups (Lönnerdal et al., 2015). Furthermore, the groups that received OPN containing formulas had significant less fever days than the group that received standard infant formula (Lönnerdal et al., 2015). Supplementing infant formula with 65 mg L^{-1} OPN was also indicated to support improved immune development in infants, as it significantly shifted the gene expression of peripheral blood mononuclear cells to be more similar to breast-fed infants (Donovan et al., 2014b).

A considerable amount of ingested OPN is likely to be digested by duodenal and gastric proteases, however a concentration of ~1050 ng mL^{-1} bovine milk OPN has been measured in plasma of mice that had received 200 μg of bovine milk OPN by gavage (da Silva et al., 2009). In another study, 50 mg OPN dissolved in chocolate milk was fed to OPN knockout mice, and after 4 h ~5000 ng mL^{-1} OPN could be measured in their plasma (Rittling et al., 2014). By immunohistochemistry, it has also been demonstrated that orally ingested OPN can be found in the colon mucosa of mice (da Silva et al., 2009). Thus, it is strongly indicated that some ingested OPN survives passage though the digestive system, and can potentially exert its function at the gut mucosal surfaces. In fact, administration of exogenous bovine milk OPN, and not unphosphorylated OPN, in the drinking water had a protective effect on several disease parameters in a mouse model of colitis (da Silva et al., 2009). This is in line with a study showing exacerbated tissue destruction and reduced repair in OPN-deficient mice compared with wild-type mice in an acute colitis model (da Silva et al., 2006). In addition, oral intake of a physiological concentration of bovine milk OPN protected against liver injury during chronic alcohol feeding of mice (Ge, Lu, Leung, Sørensen, & Nieto, 2013). The mechanism involved OPN binding to lipopolysaccharide preventing lipopolysaccharide translocation from the gut which is important for the onset of alcoholic liver disease, as it promotes macrophage infiltration and activation and hence liver injury (Ge et al., 2014).

6. Conclusion

OPN is complex protein expressed in many tissues and body fluids, with the highest concentrations in milk. OPN is involved in both pathological and normal physiological processes and the protein exists in many different isoforms resulting from alternative splicing and post-translational modification. Human and bovine milk OPN are expressed as single transcripts without alternative splicing and both are highly phosphorylated. In contrast, cancer-derived OPN often undergoes alternative splicing and are not or poorly phosphorylated. Oral administration of bovine milk OPN has shown promising results in infant nutrition and has been shown to attenuate colitis and alcohol-induced liver injury.

Acknowledgement

This work was partly supported by Arla Foods Ingredients Group P/S, Sønderhøj 10, 8260 Viby J, Denmark. The company has had no involvement in the content or conclusions of the article.

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