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Lactoferrin induced neuronal differentiation: A boon for brain tumours

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ABSTRACT

The cumulative treatments of bovine lactoferrin (bLf) and iron saturated lactoferrin (Fe-bLf) in the neuroblastoma cells showed neuronal differentiating actions evident with the expression of specific differentiating markers, β-tubulin III and neurofilaments. The protein treatments also showed lowered endogenous survivin that is responsible for cell proliferation and the miRNA 584 and miRNA214-3p, required for differentiation. Further, bLf adopted the PI3K signalling predominantly, while Fe-bLf involved both the PI3K and ERK signalling for inducing differentiation. In conclusion, this is the first study to report the neuronal differentiating effects of milk proteins and future studies are warranted for clinical application.

1. Introduction

Lactoferrin (Lf) is an iron binding single chain glycoprotein molecule containing 690 amino acid residues with an approximate size of 70–80 KDa and exists in body secretions as a line of natural defence. Unveiling its structure, Lf is folded in the homologous N- and C-terminal lobes, with independent domains enclosing the binding sites for iron (Kanwar et al., 2008; Kanwar and Kanwar, 2013). The most noticeable physicochemical feature of Lf is its ability and affinity to bind iron and as such, it binds two Fe^{3+} ions tightly, though reversible. Presumably, because of this function, the biological activity of Lf was assumed to be in the iron absorption (Baker and Baker, 2005). However, it has multifunctional activities ranging from the intestinal iron absorption, intestinal cell growth augmentation, regulation of myelopoiesis and immune responses (Kanwar et al., 2008). Lf also has a defensive role in the body as its levels were found to be profoundly elevated in the pathological conditions such as the neurodegeneration (Leveugle et al., 1994), inflammatory diseases (Uchida et al., 1994), asthma and arthritis (Decoteau et al., 1972). In addition to this, bovine Lf (bLf) also exhibited potent anti-cancer activities in the xenograft mice model including the inhibition of tumour metastasis (Kanwar et al., 2008). Iron is an essential co-factor for regulating the oxygen transport, energy metabolism and DNA synthesis in the cells. This is attributed to the unique co-ordination and redox reactivity of iron that allows it to bind with oxygen, mediate electron transfer and catalysis (Aisen et al., 2001). The major proportion of body iron is present in the haemoglobin of red blood cells facilitating the oxygen transport.

In the case of neurons, iron is majorly transported via the transferrin receptor endocytosis and divalent metal transporter (DMT-1) protein from the blood stream and has no scope for storing the excess iron (Moos et al., 2000). Both the proteins used, bLf and Fe-bLf has iron content and therefore their influence on the iron metabolism was also studied. In the current study, we used the SK-N-SH continuous human cancer cell line that is regarded as an important neuronal cell line for studying the mechanisms of brain tumours (Biedler et al., 1973) and neurodegeneration (Dang et al., 2010; Baratchi et al., 2011). Though several studies have showed that the bLf and its iron saturated form Fe-bLf have potent anti-cancer activities (Gibbons et al., 2011; Kanwar and Kanwar, 2009, 2013; Kanwar et al., 2009, 2012), the exact physiological and biological functions of bLf in the neuron cells are yet to be disclosed. Here, we examined the effect of cumulative doses of the natural bLf and Fe-bLf, which were way lower than the threshold chemotherapeutic dose used. In addition, the effect of these proteins against the drug efflux protein, P-glycoprotein (P-gp) has been studied. The role of multidrug-efflux transporters has attracted significant research attention and amongst them, P-gp or ABCB1 is well studied (Bauerschmitz et al., 2004; Potschka et al., 2002; Rizzi et al., 2002; Tishler et al., 1995). On the whole, we tried to evaluate the dif-

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ferentiating potential of bLf and Fe-bLf in the tumourous SK-N-SH neuroblastoma cell line along with their role in iron metabolism and P-gp inhibitory activity. The results showed that both the proteins have a potential future application in differentiating the tumour cells and could open up new avenues in treating the neurological disorders.

2. Materials and methods

(Refer Supplementary material.)

3. Results

3.1. Preparation, iron estimation and purity of iron saturated bovine lactoferrin

The prepared iron saturated lactoferrin was evaluated for its iron content and purity. As observed from the Supplementary Fig. S1A and B, presence of 80 KDa bands confirmed the purity of both the proteins, bLf and the iron saturated bLf. The percentage iron saturation in Fe-bLf was found to be 91.4% as determined spectrophotometrically (Fig. S1C).

3.2. bLf internalization

Preliminarily, the internalization of bLf in the SK-N-SH cells was evaluted. Post incubations with bLf treatments for 4 h, all the cells seemed to uptake and internalize the bLf corresponding to 84%. As observed, some of the cells showed the periplasmic bLf that is evident from the green fluorescence, indicating the initial interaction of protein with the cell surface. The protein was also observed to interact with the DNA and was confirmed with the nuclear localization of bLf with increasing time (Fig. S2).

3.3. Morphology changes

In order to determine the differentiating potential of bLf and Fe-bLf, the morphology changes were observed on a daily basis. As observed from Fig. 1A and B, bLf and Fe-bLf were treated in a cumulative fashion at a lower dose starting from 1 μg to a max of 1 mg and morphological changes were observed. 1 and 3 μg have not showed any significant effect and were not included in the results. However, concentrations starting from 10 μg has started to show differentiation evident from the cell shape and elongation of the neurite processes from the day 2 corresponding to 15.4 and 17% expression.

The bLf and Fe-bLf at 30 μg had the maximum differentiating effect post day 5 treatments compared to the other doses used and this conclusion was drawn based on the number of alive cells showing neurite processes. The percentage differentiation of bLf and Fe-bLf at 30 μg dose was observed to be 46.8% and 43.2%, respectively, and was the maximum with all the doses compared.

Bovine Lf at 100, 300 and 1000 μg showed only 24.8%, 18.2% and 17.6% of differentiation while Fe-bLf showed 25.6%,18.8% and 19.6%, respectively. Though doses above 30 μg showed signs of differentiation, the relative ratio of live cells to cells with neurite processes reduced, implicating the 30 μg as the optimal dose of differentiation. Hence, the 30 μg dose was used for the rest of the study and the differentiating effects of bLf and Fe-bLf were compared against the standard retinoic acid induced differentiation.

3.4. CyQUANT assay

The bLf and Fe-bLf induced changes in terms of proliferation was evaluated using the CyQUANT assay. The principle of this assay is that CyQUANT GR dye emits strong fluorescence upon binding to the nucleic acids which is a reflective of the proliferating cells. As observed from Fig. 1C, the percentage proliferation of SK-N-SH

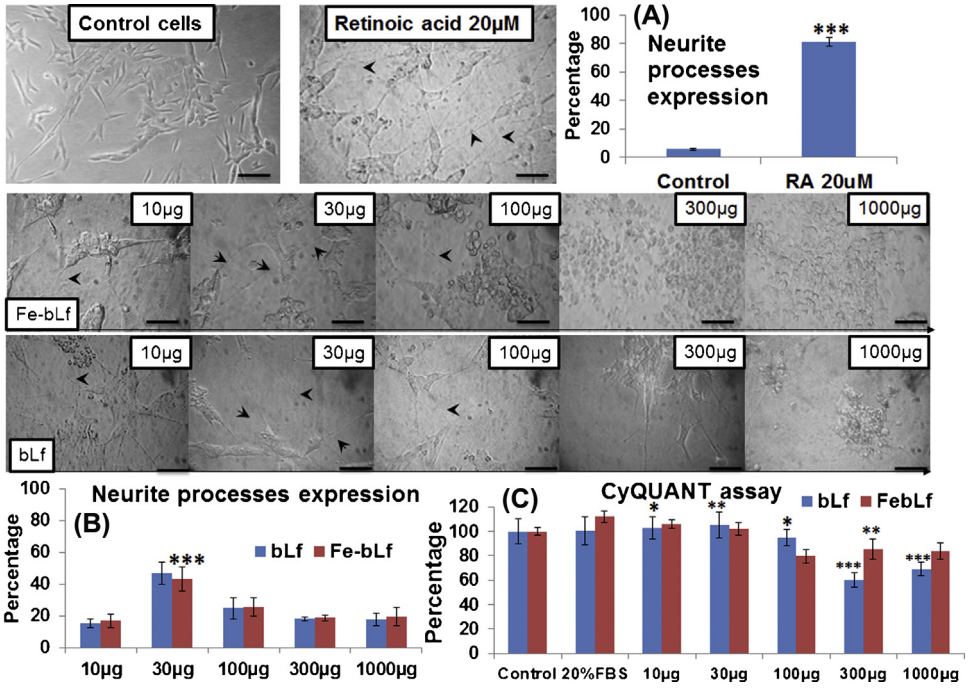

Fig. 1. Effect of bLf and Fe-bLf on the morphology and proliferation in SK-N-SH cells.
Treatments following 30 μg/ml doses of both bLf and Fe-bLf were optimum in inducing differentiation and doses above this range showed apoptotic effect and hence not included in the rest of the study. Retinoic acid induced differentiation served as the positive control. Data are represented as avg ± std, with cells counted in at least 5 different fields. (A) Histogram for neurite processes post RA and (B) post bLf and Fe-bLf treatments. (C) CyQUANT assay for percentage proliferation. bLf and Fe-bLf showed no changes in the proliferative status up to 30 μg but a reduced cell growth was evident from 100 μg and above doses. All images taken at 40× objective and data is represented as avg ± SD, $n = 3$; scale bar = 10 μm and $^{***}p < 0.001$ is considered statistically significant.