In vitro evaluation of bovine lactoferrin potential as an anticancer agent

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ABSTRACT

Bovine lactoferrin (bLF) was shown to efficiently inhibit the growth of MCF-7, T-47D, MDA-MB-231 and Hs578T breast cancer cells in a concentration-dependent manner. However, apoptosis was only induced in MCF-7 cells, which was associated with the mitochondria membrane depolarisation and a decrease of Bcl-2 levels. bLF led to the cycle arrest of MCF-7 cells at G1/G0 phase, as well as a significant decrease in the expression of CDC25c. The possibility that the observed anticancer effects could be due to the high exogenous bLF concentrations in the culture media was excluded. Moreover, bLF was shown to restrain the colony formation of MCF-7 cells, although it promoted cell migration. This later effect was unspecific and related to the presence of a high protein concentration in the culture or medium. The results gathered in this work provide valuable insights for the evaluation and further study of the potential of bLF in cancer therapy.

1. Introduction

Due to safety concerns on the use of pharmaceuticals, as well as their regulatory limitations, dietary agents are becoming attractive alternatives as cancer chemo-preventive agents (Korhonen & Pihlanto, 2006; Wakabayashi, Yamauchi, & Takase, 2006). Some dietary agents, such as selenium (Yamada et al., 2008), vitamin D and green tea polyphenols have been described as interesting chemo-preventive agents for prostate cancer (Syed, Khan, Afaq, & Mukhtar, 2007).

Lactoferrin (LF), a milk-derived glycoprotein, is widely distributed in several secretion fluids of mammalian cells, being extremely abundant in milk (Kappeler, Ackermann, Farah, & Puhan, 1999). This protein has been assigned with multiple biological functions, including antibacterial, antiviral, antifungal and immune regulatory activities (Adlerova, Bartoskova, & Faldyna, 2008; Wakabayashi et al., 2006). Iron chelation by LF is generally believed to be responsible for these functions, especially for the antibacterial effect (Jenssen & Hancock, 2009). More interestingly, it has been found that down-regulation of the LF gene could be associated with higher incidence of breast cancers (Furmanski, Li, Fortuna, Swamy, & Das, 1989). On the other hand, the exogenous supply of LF and its variants were reported to efficiently inhibit the cancer growth both in vitro and in vivo (Damiens et al., 1999; Xu et al., 2010; Yamada et al., 2008).

Despite its great potential, the mechanisms underlying the LF and its variants cytotoxicity against cancer cells are still relatively unknown. The genetically-induced overexpression of human lactoferrin (hLF) in MCF-7 cells was reported to promote cellular apoptotic activities (Liao, Du, & Lönnerdal, 2010). Also, recombination adenovirus-mediated hLF cDNA led to cell cycle arrest and stimulated apoptosis in MCF-7 cells associated with a decrease of Bcl-2 (Wang et al., 2012). Furthermore, the restoration of delta-lactoferrin (ΔLF) in HEK 293 cells resulted in cell cycle arrest and growth retardation (Breton et al., 2004). Importantly, exogenous hLF could efficiently inhibit in vitro the growth of cancer cells and induce cell cycle arrest at G1 phase (Yamada et al., 2008; Zhou et al., 2008). This effect occurred through a p53-independent pathway, and the key G1 regulatory proteins played an important role in the process (Yamada et al., 2008). Additionally, bovine lactoferrin (bLF) was found to induce apoptosis of SGC-7901 human stomach cancer cells by inhibiting the Akt activation and regulating its downstream signals (Xu et al., 2010).

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However, hLF-induced apoptosis in PC12 cells (rat pheochromocytoma cells) was observed to be associated with a decrease in the expression of the phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2) and Bcl-2 (Lin, Chiou, Chen, & Kuo, 2005). In addition, hLF was reported to induce apoptosis of Jurkat T cells via the regulation of c-Jun N-terminal kinase (JNK) activity (Lee et al., 2009). Furthermore, the main bLF-derived peptide, lactoferricin (LfcinB), also showed similar effects as its parent protein. Nevertheless, LfcinB inhibited the proliferation of cancer cells mainly by activating apoptosis-inducing pathways (Furlong, Mader, & Hoskin, 2006; Onishi, Roy, Juneja, Watanabe, & Tamai, 2008; Roy, Kuwabara, Hara, Watanabe, & Tamai, 2002; Sakai, Banno, Kato, Nozawa, & Kawaguchi, 2005). JNK/SAPK (stress-activated protein kinase) activation, down-regulation of Bcl-2, mitochondria swelling and release of cytochrome c were believed to be the mechanisms underlying the LfcinB cytotoxicity against cancer cells (Mader, Salsman, Conrad, & Hoskin, 2005; Sakai et al., 2005).

All these in vitro reports suggest that the mechanisms behind LF and LfcinB cytotoxicity against cancer cells are greatly dependent on the cell type (Duarte, Nicolau, Teixeira, & Rodrigues, 2011; Furlong et al., 2006; Lin et al., 2005; Xu et al., 2010). Additionally, the potential of LF for application in cancer therapy was further confirmed in vivo. In those experiments, the anticancer effect of LF was shown to be mainly exerted through activation of both the innate and adaptive immune responses, as well as through the stimulation of the proliferation and differentiation of T helper cells (Th) into type 1 helper cell (Th1) or type 2 helper cell (Th2) phenotypes, which release tumour-killing cytokines (tumour necrosis factors (TNF), interferon-γ (IFN-γ), caspase-1 and interleukin-18 (IL-18)) in the intestine and tumour (De la Rosa, Yang, Tewary, Varadhachary, & Oppenheim, 2008; Fischer, Debbabi, Dubarry, Boyaka, & Tome, 2006; Kuhara, Yamauchi, & Iwatsuki, 2012).

Considering the established correlation between LF and breast cancer, in the present work we studied the bLF cytotoxicity against several breast cancer cells and proposed the possible mechanisms involved in such anticancer effect. Evidence provided by the in vitro experiments will be of great value towards a future clinical use of bLF in chemoprevention and cancer therapy.

2. Materials and methods

2.1. Cell lines and lactoferrin

T-47D and MDA-MB-231 breast cancer cell lines were kindly provided by IPATIMUP and Medical School of University of Porto (Portugal), respectively. Hs578T cells were donated by the Life and Health Sciences Research Institute, University of Minho (Portugal). MCF-7 was bought from the American Type Culture Collection. The cells were cultured at 37 °C in a humidified incubator with a 5% CO_2 atmosphere in L-glutamine-containing DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 10% foetal bovine serum (FBS) (Biochrom AG) and 100 U mL^{-1} penicillin/streptomycin (Sigma−Aldrich, Steinheim, Germany). Bovine lactoferrin was purchased from DMV (Veghel, The Netherlands). The protein purity is about 80% with 3.5% moisture and 21% iron-saturated according to the manufacturer.

2.2. Effects on cell growth and death

Trypan blue staining was used to estimate cell growth and cell death induced by the bLF treatment. Breast cancer cells (MCF-7, T-47D, MDA-MB-231 and Hs578T) were seeded in 24-well plates overnight. Afterwards, bLF at several concentrations (12.5 μM, 50 μM, 125 μM and 175 μM) was added to each well. After a 48 h of treatment, both floating and attached cells were collected and resuspended in medium to obtain an adequate cell concentration. A 50 μL volume of suspended cells were mixed with 50 μL of trypan blue 0.04% (w/v) (Sigma−Aldrich) and were incubated for 5 min before counting viable and non-viable cells using a haemocytometer under a light microscope. The cells able to exclude trypan blue (viable cells) and stained in blue (dead cells) were used to estimate cell growth. The control (without bLF treatment) was set as 100%. Cell viability was expressed in percentage and calculated according to the following equation: Number of viable cells/total number of cells × 100.

2.3. Nuclear condensation assay

The ability of bLF to induce cell death by apoptosis was estimated by the nuclear condensation assay as previously described (Dias, Duarte, Lima, Proença, & Pereira-Wilson, 2013). In brief, cells at exponential phase were collected and seeded in 6-well plates. After 24 h of incubation, the cells were treated for 72 h with bLF at several concentrations (12.5 μM, 50 μM, 125 μM and 175 μM). Then, both floating and attached cells were collected, fixed and attached to a polylysine-treated slide using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA). Nuclei were stained with Hoechst (5 μg mL^{-1}) fluorescent dye for 10 min in the dark and observed under a fluorescent microscope (Olympus IX71, Hamburg, Germany). The apoptosis rate was calculated as the number of cells presenting nuclear condensation divided by the total number of cells (stained in blue) from a count higher than 400 cells per slide.

2.4. Annexin V/propidium iodide (PI) staining

Annexin V/PI staining was also used to estimate cell death by apoptosis. MCF-7 cells were seeded in 6-well plates for 24 h. Subsequently, cells were treated with 12.5 μM, 50 μM, 125 μM and 175 μM of bLF. After 48 h of bLF treatment, the cells were collected and washed twice with ice-cold PBS and resuspended in 1× Binding Buffer (0.01 M Hepes, pH 7.4, 0.14 M NaCl, 2.5 M $CaCl_2$) to get a final cell concentration of 1×10^6 cells mL^{-1}. A 100 μL volume of each sample was then stained with 5 μL FITC Annexin V (BD Pharmingen, Franklin Lakes, NJ, USA) and 10 μL PI (50 μg mL^{-1}). After a 15 min incubation at room temperature, cells were then analysed by flow cytometry using a Coulter Epics XL flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

2.5. Cell cycle analysis

MCF-7 cells were seeded on 100 mm petri dish and cultured for 24 h. Fresh medium with 12.5 μM, 50 μM, 125 μM and 175 μM of bLF was added to the cells and incubated for 24 h. In the end, the cells were collected and processed for cell cycle analysis as previously described (Dias at al., 2013).

2.6. Mitochondrial membrane potential assay

MCF-7 cells were incubated with different concentrations of bLF (12.5 μM, 50 μM, 125 μM and 175 μM) for 2 h, 24 h and 48 h. A positive control was included, containing carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma−Aldrich) at a 50 μM and incubated for 30 min at 37 °C before cell collection. Subsequently, cells were resuspended in 1 mL of warm-PBS, and 10 μL of 200 μM JC-1 (Molecular Probes, Life Technologies, Eugene, OR, USA) was added to each sample and incubated for 15 min under cell culture conditions. At last, the samples were analysed by flow cytometry (Beckman Coulter Inc.).

2.7. Histone extraction

MCF-7 cells were incubated with 12.5 μM and 125 μM bLF for 1 h, 6 h and 12 h. Incubation of cells with 50 μM etoposide was used as positive control for DNA damage. Cells were collected and washed with ice-cold PBS. Next, cells were resuspended in Triton Extraction Buffer (TEB: PBS with 0.5% Triton X-100 (v/v), 2 mM phenyl-methylsulfonyl fluoride, 0.02% (w/v) NaN$_3$) at a cell density of 1×10^7 cells mL^{-1}. The cell suspension was left on ice for 10 min with gentle stirring; nuclei were centrifuged at $6500 \times g$ for 10 min at 4 °C and washed once with half volume of TEB. Subsequently, the nuclei pellet was resuspended in 0.2 N HCl at a density of 4×10^7 nuclei mL^{-1} to perform an acid extraction of histones overnight at 4 °C. After centrifugation, the supernatant containing histone protein was used for further analysis. The histone protein extracts were quantified using the Bradford reagent from Sigma—Aldrich and analysed by western blot. The antibody against phospho-histone H2A.X (Ser139, clone JBW301) was bought from Millipore, while anti-β-actin was from Sigma—Aldrich.

2.8. Western blot assay

MCF-7 cells were seeded in 6-well pates 24 h before incubating cells with bLF at the concentrations of 12.5 μM, 50 μM, 125 μM and 175 μM for 48 h. After cell washing with PBS, total protein was extracted using RIPA (Radio-Immunoprecipitation Assay) buffer. Protein from the samples were loaded onto a 10% SDS-PAGE gel and transferred into PVDF membranes (GE Healthcare, Buckinghamshire, UK). Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-Actin was used as loading control. The antibodies anti-CDC25c, anti-Bcl-2 and anti-p53 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.9. Colony formation assay

MCF-7 cells were trypsinised and pipetted repeatedly to disperse them until single cells account for 95% of the total number of cells. Next, the cells were counted and then seeded in 6-well-plates to a concentration of 1×10^3 cells per well and incubated for 24 h. Subsequently, the cells were treated with bLF at several concentrations (12.5 μM, 50 μM, 125 μM and 175 μM), while DMEM without FBS was used as a positive control. After 24 h incubation with bLF, the media in all wells was replaced with fresh media (DMEM with 10% FBS). After 7–10 days, the cells were fixed with 70% methanol for 15 min and air dried. Giemsa was used to stain cells for 10 min and was subsequently washed carefully with water. Colonies containing at least 50 cells were counted. Colony formation was calculated as the ratio between the number of colonies in the experimental samples and the number of colonies in the control sample.

2.10. Migration assay

The bottom of 6-well plates was marked with 5–10 straight lines with 0.2 cm interval among the lines. Subsequently, 5×10^5 MCF-7 cells per well were seeded in 6-well plates for growing until cell confluence reached over 85%. Then, a scratch wound across each well of the 6-well plates was made using a pipette tip. Next, cells were washed twice with PBS and FBS-free DMEM containing 175 μM bLF or BSA was added. Cells were photographed at the exact same point (marked with straight line) in the 6-well plates after 0 h, 6 h, 12 h and 24 h. The cell migration ratio was calculated by comparing the wound area at the different time points with the wound area at 0 h in each group.

2.11. Statistical analysis

Statistical significance of the experimental results was determined by the Student's _t_ test. For p-values below 0.05 the differences between experimental groups were considered significant.

3. Results

3.1. Lactoferrin inhibits the growth of breast cancer cells

To test whether bLF was able to inhibit the growth of the breast cancer cells, the trypan blue staining method was firstly used since it provides information on growth inhibitory activities and total cell death. Considering the total number of cells in each well, we observed that bLF significantly inhibited the growth of breast cancer cells after 48 h of treatment in a concentration-dependent manner (Fig. 1). The magnitude of growth inhibition was identical in all cell lines, which indicates that this effect is not cell type specific. Cell growth inhibition reached about 70%–80% for an exposure to 175 μM bLF. For these conditions, in the case of the Hs578T cell line, cell death increased about 30% as compared with non-treated cells. However, few dead cells were observed in the experiments conducted with the other cell lines (data not shown). These results suggest that the inhibitory effect of bLF is mostly associated with the inhibition of cell proliferation.

3.2. Lactoferrin selectively induces apoptosis in MCF-7 cells

Based on the observation that some cell death occurred in the experiments with Hs578T cells, we then performed a nuclear condensation assay to estimate the potential induction of apoptosis. However, at 48 h no evident nuclear condensation could be observed in any of the cell lines used (data not shown), which corroborates the lack of massive cell death shown above. Regarding this phenomenon, we hypothesise that the cell death by apoptosis could be a more delayed event. Therefore, induction of apoptosis was evaluated 72 h after incubation of cells with bLF. The results demonstrated that bLF failed to induce apoptosis in all breast cancer cell lines under study (data not shown), except for the MCF-7 cells (Fig. 2). Cells presenting nuclear condensation are shown with arrows in Fig. 2A. Nuclear condensation was found to increase in a dose-dependent way in MCF-7 cells, reaching a ratio of about 35% after 72 h of treatment with 175 μM bLF (Fig. 2B). This assay indicated that bLF could selectively induce apoptosis in MCF-7 cells among the tested breast cancer cells.

To confirm the induction of apoptosis by bLF in the MCF-7 cells, the annexin V/PI assay was performed after 40 h of incubation, since the translocation of phosphatidylserine for the outer leaflet of plasma membrane is an apoptotic event that occurs earlier than the DNA fragmentation (Elmore, 2007). The results showed that the amount of non-apoptotic cells (Q4 region: annexin V negative/PI negative) decreased in a concentration-dependent manner compared to the non-treated group (Fig. 2C). In contrast the number of cells in the early stage (Q3 region: annexin V positive/PI negative) and later stage of apoptosis (Q2 region: annexin V positive/PI positive) was considerably increased by bLF in a concentration-dependent manner. Although the percentage of apoptotic cells in bLF-treated groups estimated by the Annexin V/PI assay was less than the one determined by the nuclear condensation assay, it was possible to confirm the ability of bLF to induce apoptosis in MCF-7 cells.
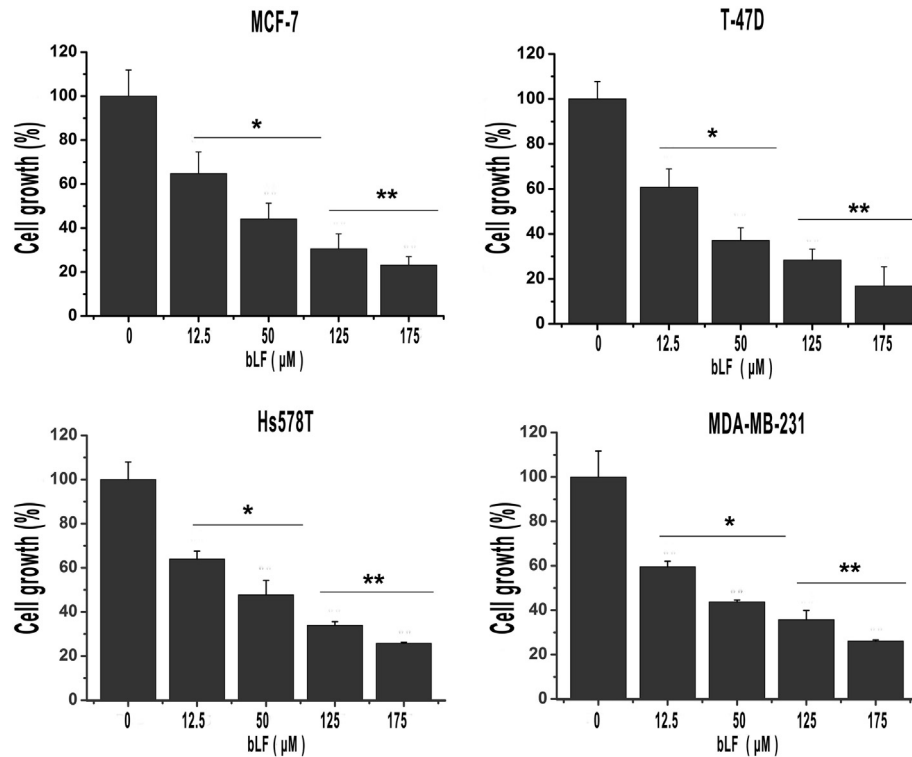
Fig. 1. Effect of bovine lactoferrin (bLF) on growth of breast cancer cells (MCF-7, T-47D, Hs578T and MDA-MB-231): values correspond to the mean ± SD of three independent experiments; $^*p < 0.05$ and $^{**}p < 0.01$ when compared with the control.

3.3. Lactoferrin inhibits cell cycle progression in MCF-7 cells

Considering that apoptotic cell death was only observed in MCF-7 cells, in this experiment we only tested this cell line to infer the effects of bLF in the cell cycle progression. As shown in Fig. 3A, bLF clearly induced cell cycle arrest of MCF-7 cells at the G1 phase after 24 h of treatment, and this effect increased in a dose-dependent manner. This event was accompanied with a remarkable decrease in the percentage of cells at the S phase (cells synthesising DNA).

Since the concentration of bLF being used was relatively high (175 μM), reaching a quantity of 14 g L^{-1} in the culture media, we questioned whether the observed effects could be due to this considerable amount of exogenous protein added, which would disturb the cellular microenvironment and prevent cell division. Therefore, a parallel assay using bLF and bovine serum albumin (BSA) at similar amounts (14 g L^{-1}) on the MCF-7 cell cycle progression was conducted. As shown in Fig. 3A, BSA also induced cell cycle arrest of MCF-7 cells at the G1 phase. However, this effect was found to be much lower when compared to bLF. Moreover, cells incubated with bLF presented a distinct morphology, while the morphology of the BSA treated group was very similar to the control one (Fig. 3B). This suggests that bLF could significantly inhibit the MCF-7 cell growth through cell cycle arrest due to a specific biological function.

DNA damage is one of the main events that lead to cell cycle arrest. Since it was shown that cells can internalise bLF, it is plausible to consider that bLF may arrest the cell cycle by inducing DNA damage. A very early step in the cellular response to DNA double-strand breaks (DSBs) is the phosphorylation of a histone H2A variant, H2A.X, at the sites of DNA damage. Therefore, the phosphorylation levels of histone H2A.X was used as a marker of DNA damage. As shown in Fig. 3C, the exposure of MCF-7 cells to 12.5 μM and 125 μM bLF did not induce phosphorylaton of histone H2A.X, contrarily to the positive control etoposide (50 μM). Therefore, these results demonstrate that the induction of cell cycle arrest by bLF was not due to DNA double-strand breaks.

3.4. Lactoferrin decreases mitochondrial membrane potential

In the apoptotic cascade, mitochondrial depolarisation is usually associated to the intrinsic apoptosis pathway (Ly, Grubb, & Lawen, 2003). In this study, we used the JC-1 probe to assess the modification of the mitochondrial membrane potential in MCF-7 cells after treatment with bLF for 2 h, 24 h and 48 h. After 2 h of bLF treatment at several concentrations (12.5 μM, 50 μM, 125 μM and 175 μM), it could be observed a significant decrease of the red to green ratio in the fluorescence, which represents the decrease of the mitochondrial membrane potential. Furthermore, the depolarisation effects of bLF on mitochondria increased in a dose-dependent way (Fig. 4A). The mitochondrial depolarisation occurred in all bLF-treated groups after 24 h incubation, although not in a dose-dependent manner. Contrarily, after 48 h of bLF treatment, mitochondria depolarisation was found to be more pronounced and to be dependent on the bLF concentration. From the cell distribution in the dot plot graph, it was observed that treatment of MCF-7 cells with 175 μM bLF for 48 h led to a significant shift of the number of cells with red to green fluorescence, as compared with the non-treated cells (0 μM bLF) (Fig. 4B). In fact, the percentage of cells with depolarised mitochondria after 48 h of bLF treatment (30%) is similar to the apoptosis rate observed at 72 h (35%). When cells were observed under a fluorescent microscope it was confirmed that bLF induced the depolarisation of mitochondria in a significant part of the cell population, in contrast to the positive control CCCP that basically depolarised the mitochondria of all cells. As a control condition for protein load, 175 μM BSA treatment for 48 h was also tested, but no significant differences from the control were observed (data not shown). This shows that bLF-induced mitochondria depolarisation is a specific effect of this protein.
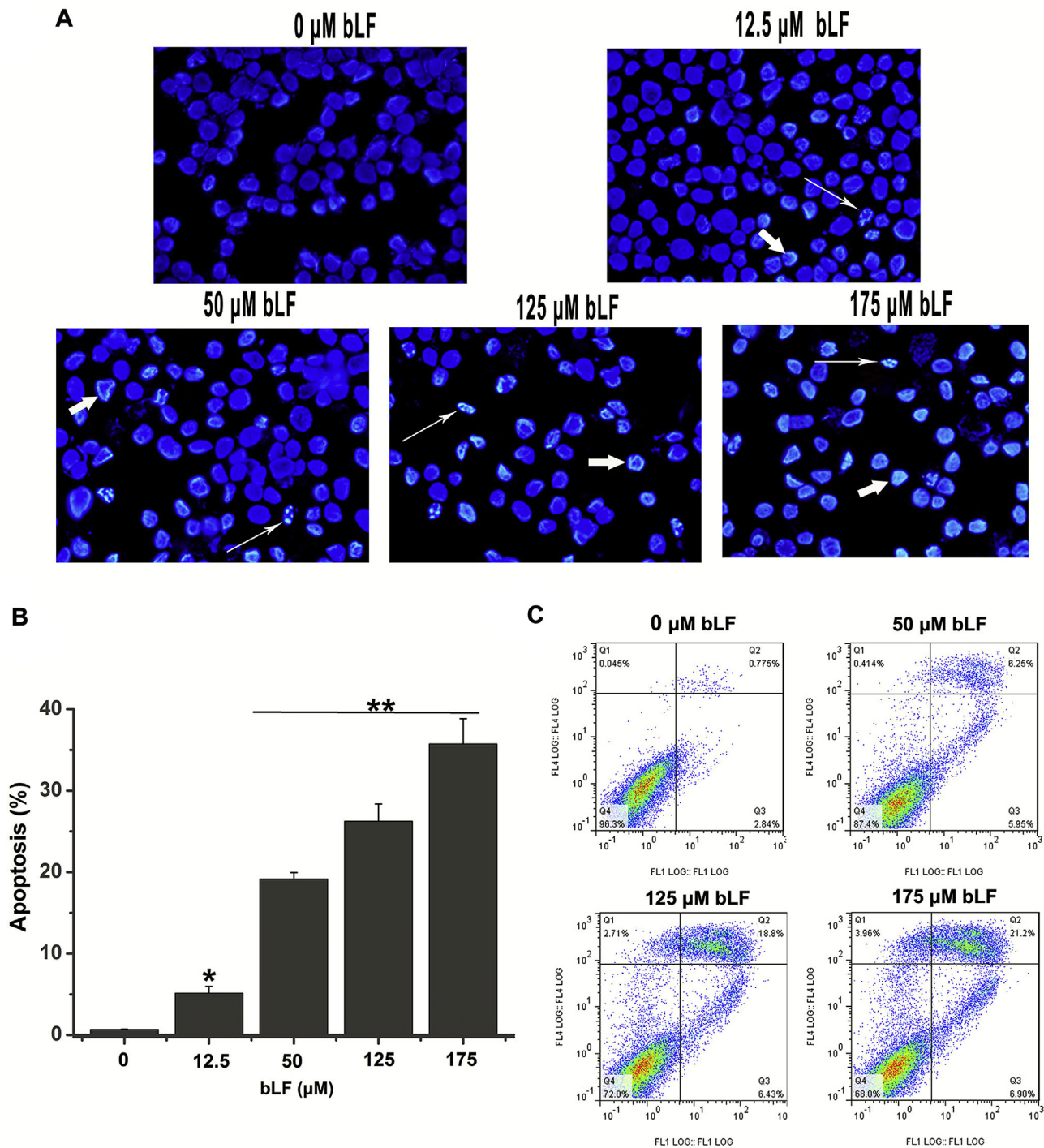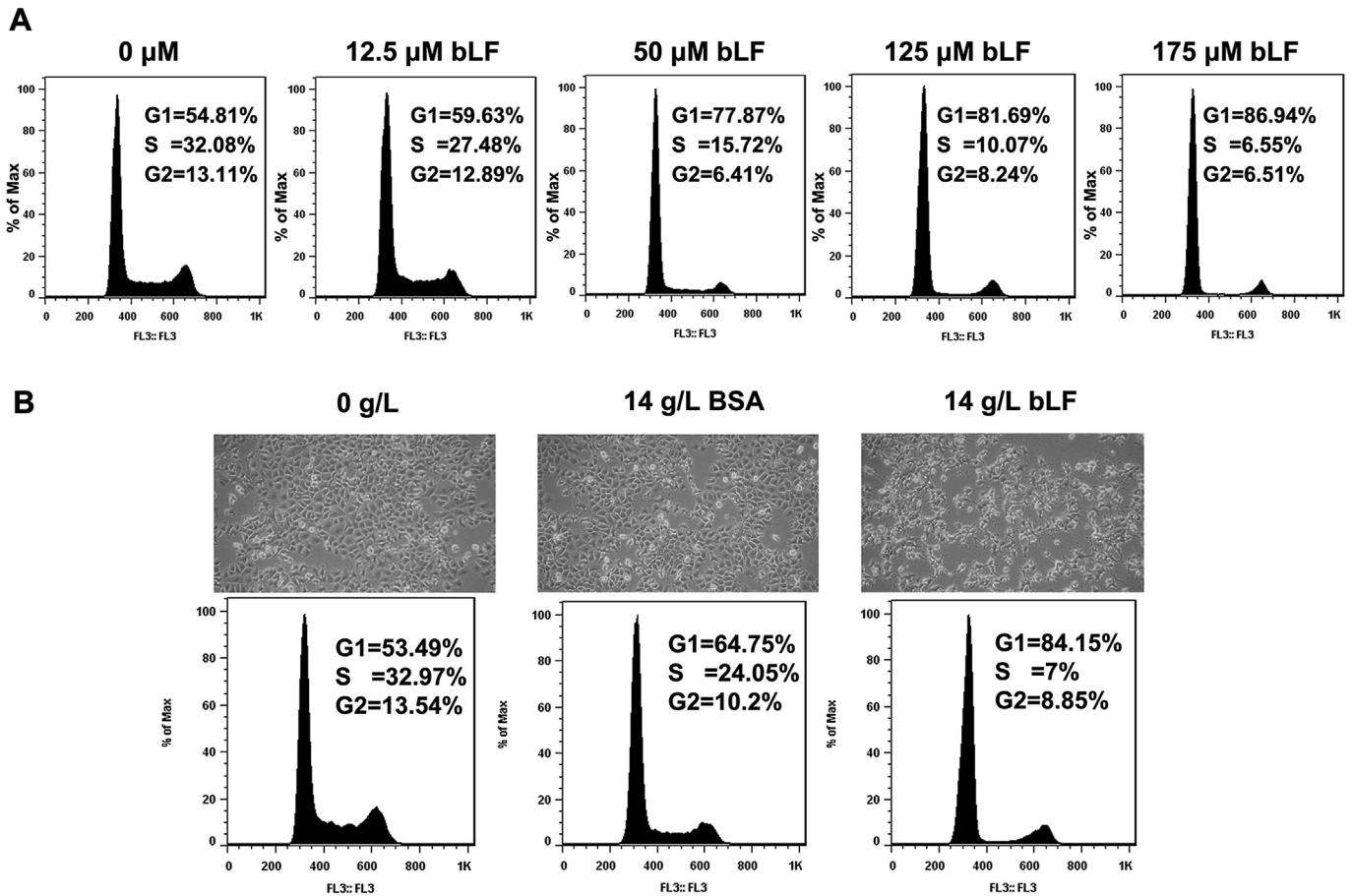
Fig. 2. Effect of bovine lactoferrin (bLF) in the apoptosis of MCF-7 cells. Panel A: representative images of the nuclear condensation assay where apoptotic cells are shown with arrows: thick arrows correspond to examples of early apoptotic cells (condensed DNA); slim arrows correspond to examples of late apoptotic cells (condensed and fragmented DNA). Images are provided with a magnification of $400\times$. Panel B: apoptosis rate induced by bLF as mean $\pm$ SD of three independent experiments, as estimated by the nuclear condensation assay: $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with the control. Panel C: apoptosis assay by Annexin V/PI; results are representative of two independent assays.

3.5. Downregulation of Bcl-2 was associated with the lactoferrin-induced apoptosis

Considering the apoptosis and mitochondria membrane depolarisation observed in MCF-7 cells treated with bLF, the expression of Bcl-2 was also studied by western blot. As shown in Fig. 5A, bLF significantly decreased in a concentration-dependent manner the expression of the anti-apoptotic protein Bcl-2. Moreover, it is

known that the tumour suppressor gene p53 has a critical role in the regulation of Bcl-2 and other proteins of the Bcl-2 family (Meulmeester & Jochemsen, 2008). However, by western blot analysis we did not observe significant changes in the total levels of p53 in the bLF-treated groups after 48 h of treatment (Fig. 5A).

Related with inhibition of cell proliferation, the levels of CDC25c were measured by western blot and shown to clearly decrease after bLF treatment for 48 h (Fig. 5A). Although it is not a key regulator in

Fig. 3. Effect of bovine lactoferrin (bLF) on the cell cycle progression of MCF-7 cells. Panel A: cells were incubated with bLF at several concentrations for 24 h and cell cycle was analysed by flow cytometry; results correspond to a representative experiment from three independent assays. Panel B: comparison between the effect of bLF and bovine serum albumin (BSA) at the same concentration on the cell cycle progression of MCF-7 cells after 24 h of treatment; the top image is representative of cell morphology for each condition (magnification of the images: 40×).

the G1/S transition of cell cycle, the downregulation of CDC25c suggests that bLF induced cell cycle arrest through the regulation of key factors in the cell cycle progression.

3.6. Lactoferrin significantly inhibits colony formation

Considering that bLF inhibited cell growth of MCF-7 cells due to both cell cycle arrest and induction of apoptosis, we then performed an anchorage-dependent colony formation assay as a functional assay to determine the effectiveness of the bLF anti-cancer effect. This assay essentially tests the ability of a single cell to grow into a colony by undergoing cell division. By comparing with the control condition (0 μM bLF), the bLF-treated cells exhibited a much lower cloning efficiency (Fig. 5B and C). The number of colonies in the treatment with 175 μM bLF could only reach 2.6% of the control (***$p < 0.001$). Additionally, DMEM media without FBS used as a positive control also inhibited the colony formation.

3.7. Induction of cell migration by bovine lactoferrin in MCF-7 cells is not specific of this protein

Metastasis is the primary cause of mortality in most cancer patients. The repression of the metastatic process has been used as an important strategy in cancer therapy. Cell migration and invasion are the two key factors involved in the metastasis formation (Valster et al., 2005). LF was previously described as a promoting

factor for the migration of MCF-7 cells (Ha et al., 2011). Herein, we also tested the effect of bLF in cell migration, and we indeed observed that this protein promotes the migration of MCF-7 cells at 175 μM (Fig. 6). To test if this effect was only due to the presence of a higher concentration of protein in the extracellular milieu, we also compared the results with an experiment conducted with the same concentration of another protein, namely BSA. Cell migration was greatly increased when cells were treated with BSA. Actually, BSA appeared to be more efficient in promoting cell migration than the bLF after 6 h of treatment. However, no significant differences were found between BSA- and bLF-treated groups after 12 h and 24 h of incubation. This result showed that the ability of bLF to induce cell migration is an unspecific property and it may result from a positive change of cell microenvironment due to the presence of a high concentration of exogenous protein.

4. Discussion

In this study, we demonstrated that bLF at several concentrations significantly inhibited the growth of the four breast cancer cell lines, but it induced apoptosis only in MCF-7 cells. The different genetic profiles among the used cells may explain this selectivity in the apoptosis induction. For example, the p53 gene that plays an important role in this type of cell death (Meulmeester & Jochemsen, 2008) may be associated with the present finding. Whereas T-47D, MDA-MB-231 and Hs578T cells harbour a mutated
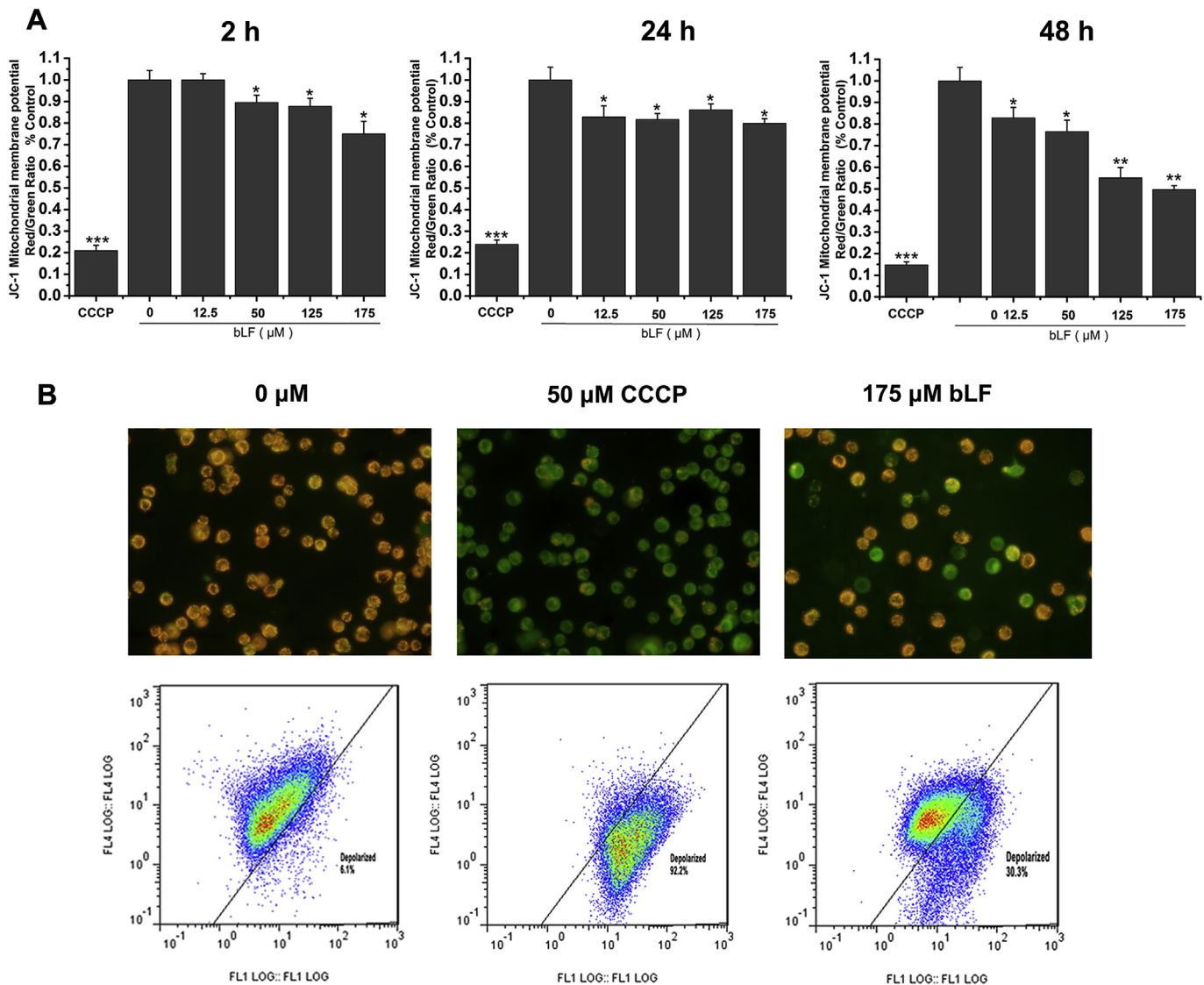
Fig. 4. Effect of bovine lactoferrin (bLF) on the mitochondrial membrane potential. Panel A: MCF-7 cells were incubated with bLF at several concentrations for 2 h, 24 h and 48 h and mitochondrial membrane potential was analysed by flow cytometry after labelling cells with JC-1 fluorescent probe. The red/green ratio was calculated from the respective median fluorescent value, where a decrease of this value in comparison to the control is indicative of mitochondrial membrane depolarisation. Values correspond to the mean ± SD of three independent experiments: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.01$ when compared with the control. Panel B: the top image is representative of cells labelled with JC-1 probe, where red cells contain polarised mitochondria and green cells contain depolarised mitochondria (magnification of the images: 400×); the bottom image corresponds to JC-1-labelled MCF-7 cells after 48 h of treatment with positive control (50 μm CCCP) or 175 μm bLF analysed by flow cytometry. The graphs exhibit the percentages of cells with depolarised mitochondria (green positive/red negative). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

p53 gene, MCF-7 cells possess a wild-type p53 (Jänicke, 2009; Xu et al., 2007). Therefore, the resistance of T-47D, MDA-MB-231 and Hs578T cells to undergo apoptosis in the presence of bLF may be due to the lack of the normal functions of p53. Previous studies reported the ability of LF to induce apoptosis in cancer cells (Damiens et al., 1999; Wang et al., 2011; Xu et al., 2010). The current study shows that this effect may depend on the genetic background of the cell line used, such as the presence of a functional p53 protein. Previously, we have reported the ability of bLF to inhibit cell growth of T-47D and Hs578T cells, and to increase the activity of caspases by 2-fold as a marker of apoptosis (Duarte et al., 2011). However, as demonstrated here, this mild induction of caspases activity might not be enough to trigger cell death by apoptosis. Herein, we showed that bLF at higher concentrations induced selectively apoptosis in MCF-7 after 72 h of treatment.

Additionally, the bLF inhibitory effect on the MCF-7 cells growth was also shown to be associated with the cell cycle arrest at the G1

phase and a decrease of CDC25c levels. This activity has been confirmed for hLF in some studies (Damiens et al., 1999; Wang et al., 2011). Regarding to MCF-7 cells, previous studies also demonstrated that re-expression of LF or ΔLF using several delivery vectors induced cell cycle arrest (Breton et al., 2004; Mader et al., 2005). In the current study we demonstrated that exogenous bLF could also induce cell cycle arrest in these cells. We also excluded that these anticancer effects triggered by bLF could be due to DNA damage.

Furthermore, the mitochondrial intrinsic death pathway has been reported as the main mechanism of LfcinB-induced apoptosis (Bi et al., 1996; Eliassen et al., 2006). Our results demonstrated that mitochondrial depolarisation was also associated in the bLF-induced apoptosis. This effect was also specific to bLF since no mitochondria depolarisation was observed when cells were treated with BSA. Depolarisation of the mitochondria membranes was kept during the time period ranging from 2 h to 48 h, and no reversible
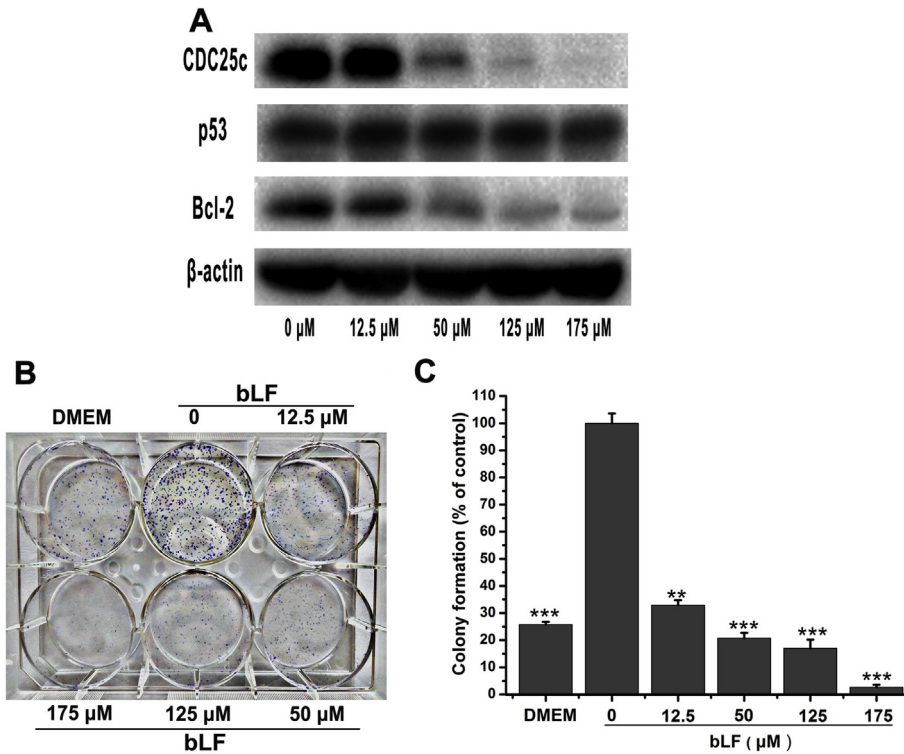
Fig. 5. Levels of Bcl-2, p53 and CDC25c in MCF-7 cells after treatment with bovine lactoferrin (bLF) at several concentrations for 48 h, as measured by western blotting with β-actin used as loading control (A) and effect of bLF on the MCF-7 cells ability for anchorage-dependent colony formation (B, C). Panel B: cells were seeded at low density and incubated with several concentrations of bLF and DMEM (FBS-free medium) for 24 h. Then, cells were left for 1 week to grow in complete medium to form colonies from single cells. Colonies were fixed, stained and images were captured. Panel C: quantification of colony formation as mean ± SD of three independent experiments; **$p < 0.01$ and ***$p < 0.001$, when compared with the control (0 μM bLF).

change could be observed. The changes in the membrane potential presumably stand for the opening of the mitochondrial permeability transition (MPT) pore, which allows the release of cytochrome c from the mitochondria. This is a key event in the initiation of apoptosis cascade (Gottlieb, Armour, Harris, & Thompson, 2003). Therefore, these findings suggest that depolarisation of mitochondria membrane potential is an important factor in the bLF-induced apoptosis of MCF-7 cells.

In addition to the mitochondrial depolarisation, bLF significantly decreased the anti-apoptotic Bcl-2 protein, which confirms that the intrinsic death pathway may be involved in the apoptosis induced by this protein. This is in a good agreement with former studies in other cancer cell lines that report LF- and LfcinB-induced apoptosis associated with a down-regulation of Bcl-2 (Mader et al., 2005; Wang et al., 2012; Xu et al., 2010). The decrease of Bcl-2 is considered as an important event to the opening of the MPT pore and loss of the mitochondrial membrane potential, for the release of cytochrome c and other pro-apoptotic proteins characteristic of the intrinsic apoptosis pathway (Lin et al., 2005). It is known that the tumour suppressor gene p53 has a critical role in the regulation of Bcl-2 and other proteins of the Bcl-2 family (Kuhara et al., 2012). As we previously discussed, the p53 has probably a key role in the apoptosis selectively induced in MCF-7 cells as compared with the other used breast cancer cells. Although we did not find changes in the total p53 levels, this does not exclude that p53 may have an important function in the bLF-induction of apoptosis in MCF-7 cells, for example through its phosphorylation. However, a previous study with hLF showed that induction of apoptosis in cancer cells occurred through a p53-independent mechanism (Adlerova et al., 2008). The role of p53 in the apoptosis induction by bLF needs therefore to be further elucidated.

Finally, the bLF anticancer potential against MCF-7 cells was confirmed by its pronounced ability to inhibit the colony formation. These findings are in accordance with other reports. For instance, the hLF was previously shown to significantly inhibit the colony formation of nasopharyngeal carcinoma cells (Gottlieb et al., 2003). The efficient inhibition the colony formation of MCF-7 cells by bLF suggests its potential antitumour effect in vivo. Although bLF promoted the migration of MCF-7 cells, this effect was shown to be an unspecific effect of this protein. Therefore, this is probably not relevant in the in vivo context in view of the high concentration of proteins present in the biological fluids.

Interestingly, several studies have shown that bLF exhibits similar biological activities to hLF (Liao, Jiang, & Lonnerdal, 2012; Lonnerdal, Jiang, & Du, 2011). Although other LF sources could also be considered (e.g., camel, sheep or goat; Rodrigues, 2013), these have not been widely evaluated for their anti-cancer potential. Also, the mechanism by which bLF exerts its effect against MCF-7 cells in this study was found to be similar to the one reported for hLF (Adlerova et al., 2008; Fischer et al., 2006; Yamada et al., 2008). Therefore, bLF could be considered as a suitable substitute for hLF with the advantage that it can be produced in great amounts from bovine milk. On the other hand, the oral administration of bLF has been suggested as a good choice for chemoprevention strategies (Kuhara et al., 2000; Norrby, Mattsby-Baltzer, Innocenti, & Tuneberg, 2001). New nanoparticles with bLF encapsulated have been developed for possible applications in the food and biopharmaceutical industries (Balcão et al., 2013; Hu et al., 2011). Additionally, the absence of adverse effects of bLF for humans at the proposed levels of consumption has been approved by European Food Safety Authority (EFSA). Therefore, we anticipate that bLF could be considered in the

A

B
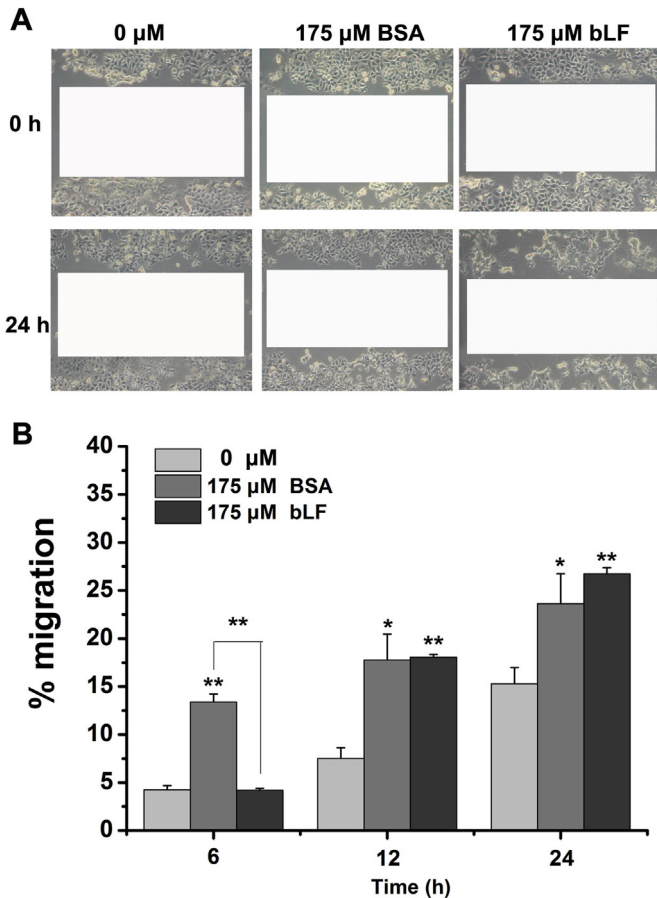
Fig. 6. Effect of bovine lactoferrin (bLF) on the migration of MCF-7 cells; cells were incubated with the same concentration of bLF and bovine serum albumin (BSA) for 6 h, 12 h and 24 h. Panel A: representative images of cell migration into the wounded area after 24 h of incubation (magnification: 40×). Panel B: relative wound closure as measured by Image J software; results correspond to the mean ± SD of three independent experiments; $^*p < 0.01$ and $^{**}p < 0.01$ when compared with the control or among each other.

future as a potential nutraceutical for chemoprevention and/or cancer treatment.

5. Conclusion

In summary, the bLF effect against four breast cancer cell lines was evaluated in a concentration range from 12.5 μM to 175 μM. The protein was found to selectively induce apoptosis in MCF-7 cells, as a delayed event that could only be observed at 40–48 h with mitochondria depolarisation and the presence of phosphatidylserine in the outer leaflet of plasma membrane, as well as at 72 h with DNA condensation and fragmentation. Furthermore, bLF significantly decreased the levels of the anti-apoptotic Bcl-2 protein that, in addition to the mitochondrial membrane depolarisation, suggests that the intrinsic pathway may be involved in the apoptosis induction. The inhibitory effect of the milk protein on MCF-7 cells growth was also shown to be associated with the cell cycle arrest at the G1 phase. Lastly, the bLF anticancer potential against breast cancer cells was confirmed by its pronounced ability to inhibit colony formation.

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