Lactoferrin Down-Regulates G₁ Cyclin-Dependent Kinases during Growth Arrest of Head and Neck Cancer Cells

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ABSTRACT

The molecular mechanism of lactoferrin-induced cell growth inhibition is incompletely understood. Studying head and neck cancer cells treated with human lactoferrin, we observed growth arrest in three of four cell lines tested. This growth arrest was caused by cell cycle inhibition at the G₀-G₁ checkpoint. Lactoferrin-induced growth inhibition was associated with a large increase in p27 protein, accompanied by decreased phosphorylation of retinoblastoma protein, and suppression of cyclin E. Decreased levels of phospho-rylated Akt were also observed in lactoferrin-sensitive cell lines after treatment. These findings suggest that in head and neck cancer cells the growth inhibitory effects of lactoferrin are mediated through a p27/cyclin E-dependent pathway that may be modulated in part by changes in Akt phosphorylation.

INTRODUCTION

Lactoferrin is an iron binding glycoprotein that is mainly found in external secretions such as breast milk. Lactoferrin is a component of the nonspecific immune system, having antimicrobial properties against bacteria, fungi, and several viruses (1). Recently, some studies have suggested chemopreventive and antitumor activity for lactoferrin as well. In rat models, lactoferrin are mediated through a p27/cyclin E-dependent pathway that may be modulated in part by changes in Akt phosphorylation.

MATERIALS AND METHODS

Reagent. Recombinant human lactoferrin was obtained from Agennix, Inc., and stored in solution (100 mg/mL) at −20°C.

Cell Lines. Four head and neck cancer cell lines (011, 012, 019, and 022) were previously established at the Johns Hopkins Hospital from head and neck cancers treated definitively by surgical resection and deposited at the American Type Culture Collection (Manassas, VA). These cell lines were mainly by surgical resection and deposited at the American Type Culture Collection (Manassas, VA). These cell lines were maintained in a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and 100 μg/mL penicillin/streptomycin.

Flow Cytometry for Cell Cycle Analysis. Cells grown to 70–80% confluency in 25-cm² flasks were treated with 10 μmol/L lactoferrin for 8, 17, 25, 32, 40, 48, 56, or 65 hours. Adherent cells were trypsinized and fixed in 70% ethanol at 4°C. Before analysis, cells were washed once in PBS and resuspended in 1 mL of propidium iodide working solution (PBS, 0.5 mg/mL RNase, and 0.1 mg/mL propidium iodide). Cell cycle distribution was determined by flow cytometry using a FACScan cytofluorimeter and the CellQuest Software program (Becton Dickinson Immunocytometry Systems, San Jose, CA).
**RESULTS**

**Analysis of Cell Cycle Distribution.** Four head and neck cancer cell lines were treated with lactoferrin for various times ranging from 8 to 42 hours. Compared with untreated cells, treatment with lactoferrin (10 μmol/L) for 25 hours induced growth arrest at the G0-G1 phase of the cell cycle in 011 (from 59 to 72%), 012 (from 45 to 75%), and 022 cells (from 48 to 63%), with a comparative drop in the S-phase from 30 to 18%, 45 to 16%, and 30 to 16%, respectively. However, lactoferrin had no effect on 019 cells (from 48 to 63%), with a significant decrease in the phosphorylation of the Rb protein was also clearly seen after 24-hour lactoferrin treatment in the 012 and 022 cells but not in 019 cells (Fig. 1A–C). These data are consistent with the flow cytometry data showing no arrest in the latter cell line.

Given these results, we next searched for changes in CDK inhibitor protein levels. We found that after lactoferrin treatment there was a 7-fold increase in p27 protein levels in 022 cells (Fig. 1C) and an almost 5-fold increase in 012 cells (Fig. 1A). On the other hand, p27 expression decreased between 10 and 65% in both treated and untreated 019 cells at all treatment time points as compared with the start of the experiment (Fig. 1B). In keeping with the absence of growth arrest observed in these cells. Slight increases in p21 and p57 protein levels were also observed in both 012 and 022 cells but not in the resistant 019 cells. Conversely, lactoferrin treatment decreased levels of the INK4 CDK inhibitor p19 by ~50% in 012 and 022 cells (Fig. 1A–C). CDK4 protein levels were unchanged in all cell lines after treatment (data not shown).

Investigating p53 protein expression we found that 019 cells expressed a mutant p53 allele, but we did not observe any change in p53 abundance in any of the cell lines tested after lactoferrin treatment (Fig. 2) to account for the observed changes in CDK inhibitors. Because p27 abundance can be affected by changes in mitogenic growth factors, as well as the phosphatidylinositol 3’-kinase/Akt pathway, we also examined TGF-β, PTEN, Akt, and phosphorylated Akt protein levels. After 24 hours of lactoferrin treatment, when growth arrest was observed, we found no consistent change in TGF-β in 012 and 022 cells. Total Akt protein levels in 012, 019, or 022 cells were unchanged as was PTEN expression in 012 and 022 cells. However, PTEN expression was absent in 019 cells. A significant decrease in pAkt was observed in the 012 and 022 cell lines (~2- and 6-fold, respectively), both of which experienced G0-G1 growth arrest (Fig. 3).

**DISCUSSION**

Chemoprevention and chemotherapy with naturally occurring compounds such as lactoferrin have become increasingly important strategies in inhibiting carcinogenesis and tumor growth. To better understand one possible mechanism of lactoferrin-mediated antitumor activity in head and neck cancer cells, we investigated how the cell cycle is affected by lactoferrin. To address the question of how lactoferrin-induced cell cycle arrest, we tested the expression of a number of molecules that regulate the transition from G0-G1 to S phase in two lactoferrin-sensitive cell lines (012 and 022) and one resistant cell line (019). Molecules tested included cyclin E, CKD4, various CDK inhibitors, and phosphorylated Rb.

Compared with control cells, lactoferrin treatment (10 μmol/L) resulted in a strong reduction in the expression of cyclin E in both 012 and 022 cells (Fig. 1A, B, and C) but had no effect on cyclin E levels in 019 cells (Fig. 1B). A significant decrease in the phosphorylation of the Rb protein was also clearly seen after 24-hour lactoferrin treatment in the 012 and 022 cells but not in 019 cells (Fig. 1A–C). These data are consistent with the flow cytometry data showing no arrest in the latter cell line.

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head and neck cancer cell lines studied. This arrest was associated with a decrease in the S phase of these cells. Given lactoferrin’s low bioavailability (5), we expect that the concentration of lactoferrin required in our studies to induce growth arrest would not be achieved after oral administration of the protein. In addition, this concentration is somewhat higher than that used by Damiens et al. (8) to induce G₁ arrest in breast cancer cells. However, this concentration is within the range of concentrations reached in urine after oral administration (3) or in serum after i.v. dosing (10).

To additionally examine the molecular mechanisms underlying lactoferrin-induced cell cycle arrest, we tested the levels of key regulatory proteins required for transition past the G₁ restriction point of the cell cycle. Accumulating evidence suggests that cyclins and their antagonists, the CDK inhibitors, play a key role in the induction and inhibition of cell cycle progression (11–16). In mammalian cells, the active complexes in G₁ contain cyclin D–CDK4/6, cyclin A, and cyclin E–CDK2 (12). Cyclin D functions upstream of the Rb protein by binding to CDK4 or CDK6 leading to Rb phosphorylation. The phosphorylation of Rb in mid-to-late G₁ releases the transcription factors bound by Rb, resulting in their subsequent binding to the promoter regions of various genes leading to DNA synthesis (17). This is followed by assembly and activation of cyclin E and CDK2. Cyclin A and CDK2 then modulate additional steps required for DNA synthesis in S phase (18–21). CDKs also interact with a family of CDK inhibitors. To date, CDK inhibitors have been classified into two groups, based on similarities in sequence and action. One group, the CIP/KIP family, includes the inhibitors p21, p27, and p57, which appear to bind and inhibit the kinase activities of cyclin D/CDK4, cyclin D/CDK6, cyclin E/CDK2, and cyclin A/CDK2 (22–24). A second group, the INK4 family, includes p15, p16, p18, and p19 (25). These CDK inhibitors specifically bind CDK4 and CDK6 and prevent the association of the CDKs with their cyclin partners.

In 012 and 022 cells, treatment with lactoferrin for 24 hours resulted in large increases in p27 protein, along with substantially smaller increases in p21 and p57. This small increase in p21 contrasts with the findings of Damiens et al. (8), who attributed lactoferrin-induced growth arrest in human breast cancer cells to a...
p53-independent increase in p21 protein. Although the observed increases in all three CIP/KIP family members, p21, p57, and p27, may have contributed in some part to the decreased phosphorylation of Rb and reduction in cyclin E, given that the largest change was seen in p27 protein, we focused further attention there.

Although transcriptional regulation of p27 is seen, p27 regulation occurs predominantly at the levels of translation and protein stability (26). This is consistent with our observation that p27 mRNA levels were unchanged in treated compared with control cell lines (data not shown), suggesting that lactoferrin treatment blocked p27 degradation by inhibiting proteolysis. p27 protein levels can be altered by changes in a number of pathways, including the mitogen TGF-β pathway and the phosphatidylinositol 3’-kinase/Akt pathway. TGF-β down-regulates the steady-state levels of p27 protein (27). Decreases in phosphorylated Akt cause decreased p27 phosphorylation and increased resistance of the protein to proapoptosomal degradation (28). Increased p27 protein levels were not associated with a change in TGF-β protein in either 012 or 022 cells. However, we did see a substantial decrease in pAkt in 012 and 022 cells. Furthermore, lactoferrin treatment did not induce cell cycle arrest nor alter p27 protein levels in the PTEN(−/−) 019 cell line in which Akt would be expected to remain constitutively active (phosphorylated; ref. 29). Our findings, therefore, are most consistent with a decrease in pAkt inducing an increase in p27 protein in our lactoferrin-sensitive cells.

In conclusion, in select head and neck cancer cell lines, lactoferrin induces G0-G1 cell cycle arrest that is likely mediated by a decrease in pAkt, causing a marked increase in p27 and a reduction in cyclin E and pRb protein levels. Lactoferrin has been shown to bind to insulin-like growth factor-binding protein-3 (30). Hence, it may act by altering the availability or stability of insulin-like growth factor, a known regulator of the phosphatidylinositol 3’-kinase/Akt pathway (31). Delineation of the role of these, as well as additional potential upstream mediators, will require additional exploration.

REFERENCES

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