Polymorphism and Altered Methylation of the Lactoferrin Gene in Normal Leukocytes, Leukemic Cells, and Breast Cancer

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ABSTRACT

Human lactoferrin has been found to be decreased or absent in most breast cancer and leukemia cells. In order to examine the lactoferrin gene for both structural alterations and the degree of methylation, we isolated a 2117-kilobase complementary DNA from human breast tissue. This complementary DNA was used to probe DNA extracted from normal peripheral blood, leukemia cells from patients, leukemia cell lines, and breast cancer cell lines. Immunocytochemical staining of these cells confirmed the decreased production of lactoferrin in malignancy. MspI restriction enzyme fragment patterns demonstrated genetic polymorphism which occurred in DNA from both normal and malignant cells. Polymorphism was also noted with XbaI. In this case, there were two fragment patterns that were only found in DNA from malignant cells. The degree of DNA methylation was also evaluated. The methylation pattern of DNA extracted from malignant cells was highly variable and generally less methylated than DNA extracted from normal WBCs. It is possible that the decrease in lactoferrin associated with cancer is multifactorial and includes gene structural changes as well as altered regulation. Further study is needed to determine whether the changes found in this study are the result of the malignancy or contribute to its onset or maintenance.

INTRODUCTION

Lactoferrin is a single polypeptide molecule (M, 76,000) with sites where two oligosaccharide chains can attach (1). This protein shares significant homology with transferrin, however, its role in iron transport is limited, since it binds iron stronger than transferrin (1). Two and possibly three isoforms of lactoferrin have been isolated using affinity chromatography (2, 3). Lactoferrin has been shown to inhibit bacterial growth by chelating iron and directly attacking the cell wall (4), contribute to the anemia of chronic disease (5), improve intestinal absorption of iron in infants (5), inhibit myelopoiesis (6), and degrade mRNA (2, 7, 8). Large quantities of lactoferrin are found in breast milk (9), estrogen-stimulated uterine epithelium (10), and in neutrophilic granulocytes (11) with smaller amounts in tears, saliva, serum, and seminal fluid (12).

While normal breast ductal epithelium and neutrophilic granulocytes contain lactoferrin, their malignant counterparts frequently do not (13, 14). This has been evaluated at the protein level and in a few samples at the mRNA level (14). Analysis at the genomic level has not been performed. DNA variations, which are detected in the coding regions, may lead to abnormal protein structure and loss of normal function. Variations, such as mutations, deletions, or changes in methylation, at the promoter regions could lead to altered regulation of the gene. Evaluation of the lactoferrin gene may provide interesting insight concerning the production of lactoferrin in malignant cells.

An inverse relationship between DNA methylation and gene expression has been demonstrated for many housekeeping genes (15). In malignancy, overall decreases in the total amount of methylated DNA consistently have been found, independently of gene expression (16). Using a lactoferrin cDNA clone isolated from human breast tissue, we have evaluated restriction fragment length changes and DNA methylation patterns in DNA from the WBCs of 10 normal controls, ANLL cells from 7 patients, T-cell acute lymphocytic leukemia from one patient, 3 leukemia cell lines, and 7 breast cancer cell lines. This report is a comparative study of the lactoferrin gene in these different cell types.

MATERIALS AND METHODS

Human Tissue. Heparinized blood (150 ml) or heparinized bone marrow (5 ml) were obtained from normal paid donors after informed consent was obtained. Informed consent and leukemia cells were obtained from seven patients with acute leukemia undergoing emergent leukapheresis. The FAB classifications were: M2, two patients; M7, two patients; and M4, ANLL not further specified and T-cell acute lymphocytic leukemia, one patient each. Nucleated cells were obtained from 80 ml of blood from normal donors after first incubating cells at 37°C for 30 min in 1:20 diluted methylcellulose (30 g/500 ml Hanks' balanced salt solution) to sediment the RBCs. The leukocyte-rich fraction was removed and centrifuged into a pellet at 500 x g for 10 min at 4°C. Cells from patients with leukemia were used either fresh or diluted in RPMI 1640 containing 20% fetal calf serum and 10% dimethyl sulfoxide and then frozen at -70°C until use. Human leukocyte antigen typing, cytogenetic analysis, and bone marrow biopsy results were available for all but one patient who died soon after leukapheresis. All cell lines were originally obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37°C, 93% humidity, and 5% CO₂. Breast cancer cell lines and HBL 100 (a cell line derived from a lactating breast) were maintained and provided by Dr. J. Dirk Iglehart (Department of Surgery, Duke University). Cells were grown to confluence and separated from dishes with trypsin 0.05%/EDTA (Gibco), washed, and centrifuged. For all samples, DNA was isolated according to standard methodology (17).

Isolation of cDNA. A Clonetech cDNA library from normal human breast tissue (HL 1037b) was plated in host cells Y1090, filter lifted, and probed with mouse lactoferrin cDNA T267 (10). Positive clones were plaque purified and the inserts subcloned into the EcoRI site of Bluescript II SK+ (Stratagene). The recombinant clones were transformed into XL1 Blue cells (Stratagene). A 2.1-kilobase insert (HLF 1212) was isolated and sequenced using the dideoxy nucleotide termination reaction and ³²P-DATP label under contract by Lark Sequencing Company.

Southern Analysis. Ten μg of DNA was digested at 37°C for 3 h with EcoRI, BamHI, HindIII, PvuII, PstI, MspI, XbaI, HpaII, Mbol, or Sau3A1 under conditions specified by the manufacturer (BRL). HpaII and Sau3A1 will not cleave DNA when specific bases within their

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Recall, therefore, that the DNA in this tissue is highly methylated (Figs. 4, 5, and 3-9). There was one patient (M2 leukemia) with a restriction fragment analysis. A few large bands in normal DNA were present in DNA obtained from all 3 leukemia and all breast cancer patients. In pattern C, an extra 9.0-kilobase band together with the 3.5, 5.0, and 6.6-kilobase and unchanged bands (Fig. 6, lanes 3-6, 8-10, 13, and 14; Fig. 7, lane 2). The last patterns were only seen in DNA obtained from malignant tissue. In pattern C, an extra 9.0-kilobase band together with the 3.5, 5.0, and 6.7-kilobase bands along with the three light 2.0-kilobase bands and the unchanged bands (Fig. 6, lanes 3-6, 8-10, 13, and 14; Fig. 7, lane 2). The last patterns were only seen in DNA obtained from malignant tissue. In pattern C, an extra 9.0-kilobase band together with the 3.5, 5.0, and 6.6-kilobase and unchanged bands were observed in 3 leukemia patient samples (Fig. 6, lanes 11 and 12, insert, and 16). Also noted is the absence of the light 2.0-kilobase bands. Pattern D contained only the 4 unchanged and the three light 2.0-kilobase bands and was present in DNA obtained from all 3 leukemia and all seven breast cancer cell lines (Fig. 6, lanes 17-19; Fig. 7, lanes 3-9). There was one patient (M2 leukemia) with a restriction pattern like that of the cell lines (Fig. 6, lane 15). There were no chromosomal abnormalities, FAB categories, or phenotypic types associated with any polymorphic XbaI pattern.

Methylation Changes. Mbol and Sau3A restriction fragments are identical in both normal and cancer cells (Figs. 2 and 3), indicating that there is no difference in methylation in the restriction sites recognized by these enzymes. The Hpall recognition site contains a CG pair and is more frequently methylated in humans. Hpall produces a few large bands in normal DNA when compared with its isoschizomer, Mbol, indicating that the DNA in this tissue is highly methylated (Figs. 4, 5, and 6).
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Fig. 1. Immunocytochemical staining of normal bone marrow (A, x 400) and the breast cancer cell line SKB R3 (B, x 680) using anti-lactoferrin antibody at 1:1500.

8). Leukemic cells are also methylated but to a lesser and more variable degree (Fig. 8, lanes 10–19). Cell lines KG 1 and HL 60 were the least methylated, the latter being more myeloid and considered by some to be promyelocytic. Breast cancer cell line methylation pattern is also variable and overall is the least methylated of all cells tested (Fig. 9). MDAMB 468 and SKB R3 fragments were the closest to MspI, and as noted above, SKB R3 is the only cell line that contained some stainable lactoferrin.

DISCUSSION

Lactoferrin is a multifunctional protein that exists as more than one isoform. Of particular interest to oncology is its ability to degrade RNA and inhibit myeloid growth. For several years, lactoferrin has been known to specifically inhibit myelopoiesis, and thus, it may function as a feedback inhibitor of myeloid growth. Inhibition occurs at concentrations as low as $10^{-17}$ M, and lactoferrin can suppress the release of colony-stimulating activity or interleukin-$\beta$ from monocytes, fibroblasts, and endothelial cells (6, 21–23). RNase activity was noted in human milk in 1976 (7) and was ascribed to lactoferrin by Furmanski et al. in 1989 (2). This isoform has now been isolated from granulocytes (8) and is identical to iron-binding lactoferrin by peptide mapping, isoelectric point, and antigenicity. The two forms, whose major differences are functional, are separable by affinity chromatography. While the purpose of RNase lactoferrin is unknown, several properties of lactoferrin that may be related to RNase activity are (a) binding to a receptor-like DNA molecule on B-cell membranes (24) and (b) prolongation of the survival of mice infected with Friends leukemia virus (25).

In addition to the many normal functions of lactoferrin, several disease states have been caused by or have been associated with decreased lactoferrin. Several cases of neutrophil-specific granule deficiency have been reported with symptoms of frequent pyogenic infections (26). In these cases, neutrophils are deficient in secondary granules which contain lactoferrin. Nasal secretions of several such patients contain normal concentrations of lactoferrin, indicating that this gene can be abnormally regulated in specific tissues (26). Lactoferrin has been found in the malignant counterparts of tissues that normally secrete it, such as the salivary glands (27), stomach (28), bone marrow (29), and breast (30). In most malignancies, the tendency is for less lactoferrin to be found in the most immature tumors. This has more directly been found in AML and breast cancer. The presence of lactoferrin has been proposed as a marker of M2 leukemia, M4 leukemia (29), and low-grade breast cancer (30). In these instances, the decreased production of lactoferrin could reflect the dedifferentiated state of the cell which normally does not produce lactoferrin; however, a more direct role in the malignant process cannot be ruled out. Several studies have shown the presence of lactoferrin receptors on human tumor cells such as colon cancer HT29-D4 (31) and several leukemia cell lines (32). The significance of this finding is unknown. Finally, Das et al. (7) evaluated breast milk for inhibitors of reverse transcriptase in ethnic groups with different risks of breast cancer. They discovered that RNase activity was lowest in Parsi women, an inbred group which has a high risk of breast cancer. Forty percent of the milk in Parsi women did not normally inhibit avian myeloblastosis virus reverse transcriptase. Since viral synthesis may begin on the cell membrane (33), this function may be an important defense mechanism. A lack of RNase may lead to increased viral invasion and subsequent malignant transformation. Furthermore, a lack of RNase activity in any specific organ might increase the risk of malignancy in that particular organ.

Human lactoferrin is located on chromosome 3 in the q21-qter region, which is adjacent to transferrin (34). Lactoferrin cDNA was first isolated from the mouse uterus where mRNA and protein are estrogen inducible (10). Subsequently, a partial cDNA fragment covering the 3' end of the lactoferrin cDNA was isolated from human bone marrow (14) and a complete cDNA from breast tissue (19, 20). Our human lactoferrin cDNA isolated from breast tissue agrees with the modified AA sequence of iron-binding lactoferrin in all areas except the 3 sites in the N-terminal region. This area corresponds to exon 2 in
Differences between the published protein derived AA sequence and our cDNA derived sequence are indicated by underlining in the extra AA in our sequence or indicating substitutions beneath the area of substitution. Nucleotide differences based on published sequence data are indicated above our sequence. Nucleotide changes resulting in a different AA are typed below the area of substitution.

* C. T. Teng and Y. Liu, unpublished data.
of methylated DNA consistently have been found to occur in malignancy (16). Focal areas of hypermethylated DNA, especially in the 5' areas of specific genes, have been observed in cancer. DNA methylation has been postulated to contribute to the malignant phenotype by (a) blocking the binding of transcriptional factors to target sequences, (b) causing configurational changes in chromatin, (c) binding to negatively acting factors, and (d) promoting allelic deletions (16). Specific genes that have been found to be abnormally methylated in ANLL and breast cancer are the calcitonin (37) and estrogen receptor genes (38), respectively.

The methylation changes found in the lactoferrin gene elucidate several points. First, they confirm that CG pairs are more commonly methylated in humans, since there are no changes
Fig. 4. Restriction fragments produced usingMspI and lactoferrin cDNA (HLF 1212) as the probe. Lanes 1-9, DNA from normal donors; lanes 10-16, DNA from leukemia cells from patients; lane 17, cell line K562; lane 18, KG 1; lane 19, U937. Ordinate, kilobases.

Fig. 5. Restriction fragments produced usingMspI and lactoferrin cDNA (HLF 1212) as the probe. Lanes 1 and 2, DNA from normal donors; lanes 3-9, DNA from breast cancer cell lines: lane 3, MDAMB 468; lane 4, BT 474; lane 5, HBL 100; lane 6, MDA 175; lane 7, SKB R3; lane 8, ZR 75-1; lane 9, ZR 75-30. Restriction fragment patterns as discussed in the text are in the following lanes: lane 1, pattern A; lane 2, pattern B; and lanes 3-9, pattern D. Ordinate, kilobases.

In summary, Southern analysis indicated that the human lactoferrin gene is polymorphic when tested usingMspI and Xbal restriction enzymes. MspI restriction is associated with deletions of a 3.5-kilobase band, independently of whether the DNA was from normal subjects, leukemia cells from patients, leukemia cell lines, or breast cancer cell lines. Xbal restriction was associated with 4 patterns. The most striking change was between Sau3A1 and MboI. Also noted is the observation that lymphocytes, which do not typically produce lactoferrin, have this area of DNA highly methylated. Malignant cells are less methylated than normal peripheral WBCs, as can be seen in our leukemia and breast cancer cells. It is of interest that the only cancer cell line (SKB R3) to produce any stainable lactoferrin was also the least methylated. Further information could be obtained by evaluating normal breast and uterine epithelium at various stages of estrogen stimulation. Since there is evidence that the 5' region of a gene is focally hypermethylated in tissues that do not produce the protein, this region of the lactoferrin gene requires additional scrutiny.

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the deletion of many bands found in all cell lines whether derived from leukemia or breast cancer. The variability of methylation in breast and leukemic cells provides further evidence that methylation differs from normal patterns and is a likely component of regulatory control. The explanation and the significance of these changes will require gene cloning and sequencing, transfection experiments, and evaluation of the population.

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