Isolation of Lactoferrin cDNA From a Human Myeloid Library and Expression of mRNA During Normal and Leukemic Myelopoiesis

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Lactoferrin is a major constituent of polymorphonuclear leukocyte granules and is present in mature neutrophils but not in blasts or promyelocytes. We have isolated a cDNA probe for lactoferrin and used it to study the synthesis of lactoferrin mRNA by normal and leukemic myelocyte precursors. The probe pHL-41 has been subcloned in phage M13 and characterized by restriction endonuclease analysis and nucleic acid sequencing. pHL-41 contains approximately 40% of the coding sequence of the lactoferrin gene. The 3’ untranslated region includes a stop codon and a possible polyadenylation signal. There is a >98% agreement between the cDNA-deduced amino acid sequence and that determined by analysis of the protein.

Myeloid cells from normal bone marrow and circulating leukocytes from patients with chronic granulocytic leukemia contain lactoferrin mRNA transcripts that are indistinguishable in size and relative quantity. The human promyelocytic leukemia cell line HL-60 contains no lactoferrin mRNA. Induction of monocytic or granulocytic differentiation fails to induce the synthesis of detectable lactoferrin message. Similarly, studies with the human myeloblastic leukemia cell line PLB-985 reveal the inability of these cells to produce lactoferrin mRNA even under conditions that bring about morphologically demonstrable granulocytic differentiation. These data suggest that granulocytic differentiation in the leukemic cell lines is incomplete or defective. The presence of lactoferrin may play a role in the orderly expression of the genetic program leading to the development of the normal mature granulocyte.

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MATERIALS AND METHODS

Cell lines and granulocytes. The HL-60 cell line, originally isolated from a patient with APL[28] as a kind gift of Dr Hal Broxmeyer. PLB-985 is a new human myeloid leukemia cell line that is characterized by a less mature phenotype than HL-60 and the ability to undergo granulocytic differentiation in the presence of chemical inducing agents. Peripheral granulocytes from leukemia patients were obtained from routine diagnostic specimens. Granulocytes from CGL peripheral blood were prepared from leukapheresis specimens obtained with patients’ informed consent.

HL-60 and PLB-985 cells were propagated in RPMI 1640 medium containing 5% Nu-Serum (Collaborative Bioresearch, Bethesda, MD). Induction of granulocytic maturation was accomplished with dimethyl sulfoxide (DMSO) or dibutyryl cyclic adenosine monophosphate (Bt2 cAMP) as previously described.[19,20] Monocytic maturation of the cells was induced with phorbol myristate acetate (PMA). Induction of differentiation was routinely assessed by morphological examination of the cells under Wright’s stain, determination of the percentage of adherent cells in the population, and demonstration of decreased transcription of the myc oncogene[21] as determined by Northern blot analysis.

RNA extraction. White cells from human blood and leukapheresis specimens were purified from buffy coat by a single wash with

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Fig 1. Sequencing strategy for clone pHL-41. Significant enzymes are indicated above the map. Restriction fragments were purified on polyacrylamide gels and subcloned into M13mp18 and M13mp19. Arrow heads indicate the direction of sequencing. Each fragment was sequenced in at least two independent reactions. \( \rightarrow \). Dideoxy sequence; \( \rightarrow \) bidirectionally sequenced (Maxam-Gilbert).

![Fig 1](image)

Fig 2. Nucleotide sequence of clone pHL-41 and deduced amino acid sequence. Nucleotides are numbered from the 5' end of the clone. Amino acid residues are numbered according to the sequence of Metz-Boutigue et al. Discrepancies between the deduced and published amino acid sequence are indicated by squares, with the published residue (or its absence) given below. A possible polyadenylation signal begins at nucleotide 984.
phosphate-buffered saline (PBS). Erythrocytes were removed by treatment of the cell pellet with 10 vol of 0.2% NaCl for 30 seconds at 0°C followed by the addition of an equal volume of 1.6% NaCl. Centrifugation at 1,000 g for ten minutes provided a pellet that was composed entirely of nucleated cells. Cultured cells in suspension were washed once with ice cold PBS and pelleted as before. Adherent cells (eg, HL-60 and PLB-985 grown in the presence of PMA) were lysed directly in their culture flasks.

Total cellular RNA was extracted from cultured cells and human neutrophils with rapid lysis of frozen material in guanidinium isothiocyanate and was further purified by pelleting through 5.7 mol/L cesium chloride. Polyclonally labeled DNA probes were prepared by two cycles of chromatography over oligo-DT-cellulose (Pharmacia Fine Chemicals, Piscataway, NJ).

cDNA synthesis and library screening. Double-stranded cDNA was synthesized by the method of Gubler and Hoffman by using as a template 10 μg of poly-A RNA from human CGL granulocytes. EcoRI linkers were added, and the cDNA was ligated into λgt10 and packaged in vitro by using commercial packaging extract (Stratagene, San Diego, CA) and the instructions of the manufacturer. The library prepared in this manner contained >10^6 independent clones. The unamplified library was plated at high density on Escherichia coli C600 hflA-Plaques were transferred to nylon membranes (GeneScreen Plus, NEN-DuPont, Boston) coated with bacteria for in situ signal amplification. The membranes were hybridized to an oligonucleotide probe, with sequence 5'-d(GAYTGTTCCNGAYAARTT)-3' corresponding to the amino acid sequence of residues 638 to 643 of human colostrum lactoferrin. The filters were washed at high stringency under conditions that make DNA strand dissociation (T_D) independent of guanine plus cytosine (GC) content. Sixteen independent clones were isolated and subcloned into M13mp18 and M13mp19 for nucleotide sequencing.

DNA sequencing and analysis. Cloned inserts were sequenced in multiple directions by using the dideoxy chain termination method of Sanger with Klenow fragments of DNA polymerase I and avian myeloblastosis virus (AMV) reverse transcriptase. The sequencing reactions were modified for the use of 32P-labeled nucleotides and buffer gradient gels. In addition, some regions were bidirectionally sequenced using the chemical method of Maxam and Gilbert. The sequencing strategy is shown in Fig 1. Sequence data were analyzed by using the UWCG computer program.

Hybridization analysis of RNA. RNA was analyzed by Northern blotting from formamide/formaldehyde gels and hybridized to 32P-labeled DNA probes. The probes included the internal EcoRI-PstI fragment of pHL-41 (Fig 1), a cDNA probe for γ-actin kindly provided by L. Kedes, and a cDNA probe for c-myc. The probes were labeled by the random oligonucleotide technique.

RESULTS

Isolation and sequence of pHL-41. Sequence analysis (Fig 2) of the lactoferrin inserts obtained on this screening of the library revealed that one clone, pHL-41, contained 1,023 bases, including the coding sequence for amino acids 428 to 703, a stop codon, a possible polyadenylation signal, and a poly-A tail. The amino acid sequence deduced from the cDNA differed in three residues from the protein sequence determined.

Expression of lactoferrin mRNA by myeloid cells. The ability of myeloid cells to express lactoferrin mRNA transcripts was determined by Northern blot analysis of polyadenylated RNA from various sources. Normal human bone marrow granulocytes and granulocytes from CGL peripheral blood synthesized transcripts of the same mol wt. The intensity of hybridization of the lactoferrin band and a concurrently hybridized γ-actin band has been found to be comparable in CGL and in normal bone marrow–derived granulocyte precursors (Fig 3, lanes 1 and 2).

Interestingly, cells from the same CGL patient that had been obtained after blast transformation (myeloid type) had occurred displayed no hybridization to the lactoferrin probe (Fig 3, lane 3). In addition, AML and APL blasts also synthesized no lactoferrin mRNA (data not shown).

The HL-60 and PLB-985 cell lines were studied because of their capacity to undergo maturation in a granulocytic or monocytic direction under chemical stimulation. We have previously shown that lactoferrin translation product is undetectable in DMSO- or cis-retinoic acid–induced HL-60 cells. These studies have now been extended to include a determination of the ability of these cells to express lactoferrin transcripts. Poly-A RNA from uninduced and induced cells was hybridized to a lactoferrin probe as described earlier. In no instance was normal- or aberrant-sized transcript detected. In all observed instances, synthesis of actin mRNA was constitutive as predicted (Fig 3, lanes 4 to 8). The γ-actin hybridization acted as control to rule out the possibility that the absence of lactoferrin signal (lanes 4 to 8) was due to technical factors affecting the efficiency of mRNA transfer; it also acted as a standard against which the intensity of the lactoferrin band (lanes 1 and 2) could be compared.
DISCUSSION

We report here the successful cDNA cloning of human neutrophil lactoferrin. The clone has been characterized by nucleotide sequence analysis and encompasses approximately 40% of the coding region as well as the 3' terminus. The clone was used as a probe to study the expression of the gene in normal and leukemic cells and in two leukemia cell lines capable of limited granulocytic differentiation. The results reported here indicate that myeloid cell populations containing precursors more mature than promyelocytes synthesize lactoferrin mRNA. Thus, normal bone marrow is indistinguishable from CGL peripheral blood with respect to the abundance and size of the major lactoferrin mRNA transcript. In concurrence with the absence of morphologically recognizable differentiation, the lactoferrin transcript was not found in the peripheral white cells of CGL blast crisis or in AML and APL cells.

The absence of lactoferrin transcripts in undifferentiated and chemically induced (differentiated) HL-60 and PLB-985 cells indicates that in vitro maturation of these cells is defective. These lines clearly display some characteristics of maturation such as morphological increases in nuclear pyknosis, expression of the fMet-Leu-Phe receptor, and decreased myc transcription. Their inability to express a developmentally modulated gene that characteristically begins expression at the myelocyte stage with progressive increases to the fully mature myeloid elements suggests substantial arrest of at least portions of the orderly sequence of genetic modulation integral to cell maturation.

Delineation of the mechanisms responsible for this persistent defect should provide insights into the process of maturation arrest that typifies malignant transformation. The successful cloning of this cDNA probe will provide the basis for exploring this gene and its control mechanisms.

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MOLECULAR CLONING OF HUMAN NEUTROPHIL LACTOFERRIN

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