Correlation of Messenger RNA Levels With Protein Defects in Specific Granule Deficiency

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Neutrophil specific granule deficiency (SGD) is a rare congenital disorder of unknown cause associated with an impaired inflammatory response and an absence of neutrophil secondary granules. Reduced levels of several neutrophil proteins have led to the suggestion that the defect may lie at the level of transcription, a hypothesis that is supported by abnormally low levels of lactoferrin message in the bone marrow of two SGD patients. We have examined the level of seven granule protein RNAs in one SGD patient and have compared them with reported protein levels. We have found the RNA levels for all of these genes to be reduced in proportion to the decreased levels of their respective proteins. These data further support the hypothesis that the reduced protein levels reflect a defect in transcriptional control.

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Granulocyte differentiation progresses through several well-defined stages marked by characteristic morphologic changes. These changes include progressive nuclear segmentation as well as the acquisition of stage-specific granules. As the cell progresses to the myelocyte stage, secondary (specific) granules are acquired and the cell becomes committed to terminal neutrophil differentiation. Secondary granules and the expression of secondary granule content protein genes, therefore, provide good markers for commitment to terminal granulocytic maturation.

Specific granule deficiency (SGD) is a rare congenital disorder in which patients present with recurrent infections attributable to functional neutrophil abnormalities. Microscopic examination of patient neutrophils shows morphologic abnormalities of nuclear lobulation and membrane configuration, as well as a near absence of secondary granules. The cells display abnormal cell motility, defective bactericidal activity, and a heightened state of activation. Biochemical studies have shown several neutrophil proteins to be present at lower than expected levels, including but not limited to the full range of secondary granule content proteins. Protein levels for both transcobalamin and lactoferrin, two of the secondary granule content proteins, have been shown to be reduced in patient plasma but normal in saliva from at least one individual. This finding suggests that the defect is restricted to neutrophils.

Strauss et al. described a patient who presented with recurrent bacterial infections and unique neutrophil abnormalities. When lactoferrin RNA levels were studied in the bone marrow (BM) from this patient and one other with SGD, a decrease in lactoferrin RNA was seen. RNA from lacrimal tissue was also tested and lactoferrin levels were shown to be normal, suggesting that the defect was restricted to hematopoietic tissue. Reduced levels of lactoferrin RNA suggested that the defect may be at the level of transcription.

We describe here our analysis of BM RNA from one of these patients looking at the levels of steady-state RNA encoding a wide range of affected proteins. To determine if the RNA defect is specific to lactoferrin, we have examined the BM RNA levels for transcobalamin I, neutrophil collagenase, neutrophil gelatinase, and defensins. All of these RNAs are shown to be produced at a reduced level when compared with normal BM. We have also examined the RNA levels of CD18, an adhesion protein that is a component of both the plasma membrane and the secondary granule membrane. Flow cytometric analysis of CD18 has shown its presence on the SGD neutrophil membrane, but it fails to undergo the increase in membrane expression normally seen upon neutrophil activation. The RNA level for CD18 is shown to be unaffected.

MATERIALS AND METHODS

Patient information. Normal BM (NBM) was obtained from anonymous discarded samples retrieved after clinical procedures were performed. The SGD patient sample was obtained for this study after informed consent. The patient has been described previously. Briefly, MF is a 35-year-old man clinically free of any infection at the time of study. The patient's BM aspirate showed normal cellularity, a myeloid:erythroid (M:E) ratio of 15:1, and the following cellular differential: 19% neutrophils, 1% band forms, 15% myeloblasts, 10% promyelocytes, 8% myelocytes, 5% monocytes, and 3% eosinophils. This is comparable to previous reports of this patient.

RNA isolation. White blood cells (WBCs) were separated from patient and normal BM using sedimentation of the RBCs followed by RBC lysis in isotonic solution. After adding 0.15 M NaCl, theuffy coat was centrifuged at 200 g for 10 minutes at 4°C and then resuspended in ice-cold distilled H2O for 30 seconds and reincubated at room temperature for 45 minutes. The DNA was functional in suspension at 200 g for 10 minutes at 4°C and the pellet was resuspended in ice-cold distilled H2O for 30 seconds and then brought to 0.9% NaCl. After repeat centrifugation, the pellet

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was resuspended in 0.2% NaCl for 30 seconds and then brought to 0.9% NaCl. The WBCs were pelleted again and resuspended in 3 mL guanidine and spun over a CsCl cushion to separate the RNA.11

Northern blotting. Ten micrograms of total RNA was size separated on a formaldehyde gel and then transferred by standard Northern technique to nitrocellulose filters. cDNA fragments were labeled with 32P using nick-translation. Hybridization was performed overnight at 42°C in 50% formamide. The filter was then washed at 55°C for 30 minutes in 0.1% sodium dodecyl sulfate (SDS) and 0.1× SSC and exposed to x-ray film. Probes were removed from the filter by heating the wash solution to the boiling point and then agitating the filter in the solution until cooled to room temperature.

The cDNA fragments used as probes consisted of a 1.6-kb EcoRI fragment of transcobalamin I (TCI),12 a 2.4-kb fragment of lactoferrin12a a 2-kb fragment of myeloperoxidase13 (kindly provided by Dr Susan Weil, University of Pennsylvania, Philadelphia), a 2.4-kb EcoRI fragment of neutrophil collagenase,14 a 2.4-kb EcoRI fragment of neutrophil gelatinase (Devarajan et al, submitted), an 800-bp EcoRI fragment of CD1815 (provided by Dr Daniel Tenen, Beth Israel Hospital, Boston, MA), and a polymerase chain reaction (PCR) fragment from defensins isolated using 20-bp oligonucleotides to nucleotides 8-27 and 322 to 341.16

Densitometry. Films were scanned using a densitometer from Molecular Dynamics (Sunnyvale, CA). Densities are reported in Table 1 as ratios of SGD RNA:NBM RNA. All ratios are corrected for loading with myeloperoxidase (MPO) expression, which was defined as 100%. Protein ratios have also been computed for comparison. MPO, known to be unaffected at

## RESULTS

Total RNA was isolated from the BM leukocytes from an SGD patient and a control individual, as well as from the peripheral blood leukocytes from an individual with chronic myelogenous leukemia (CML). Figure 1 shows a Northern blot of these RNA samples probed for neutrophil collagenase, neutrophil gelatinase, lactoferrin, transcobalamin I, defensins, CD18, and MPO transcripts. Lane 1 contains SGD patient BM RNA, lane 2 contains NBM RNA, and lane 3 contains CML peripheral blood RNA. Neutrophil collagenase, neutrophil gelatinase, lactoferrin, transcobalamin I, and defensins are all detected at reduced levels in lane 1 as compared with lane 2. The level of reduction varies between genes, but corresponds to the levels seen for the individual proteins (Table 1). Results from densitometry are included in Table 1 and are reported as ratios of SGD to normal RNA, normalized to MPO expression, which was defined as 100%. Protein ratios have also been computed for comparison. MPO, known to be unaffected at
the protein level in SGD, is not reduced at the RNA level in the SGD leukocytes, CD18 is actually increased at the RNA level in SGD as compared with the control. All transcripts are detected in the CML RNA.

DISCUSSION

Specific granule deficiency is a syndrome characterized by numerous defects in patient neutrophils. Along with an apparent absence of secondary granules, there is a reduction in the level of numerous proteins including, but not limited to, secondary granule content proteins. Lactoferrin and TCI are produced at normal levels in other tissues, suggesting that the defect is restricted to neutrophils. Furthermore, only a subset of neutrophil proteins are affected, which suggests that the defect is not general.

Defects in both gene expression and protein processing have been suggested as mechanisms that might account for the abnormal reduction in protein levels in SGD neutrophils. Abnormal intracellular transport to the granule or defective movement of proteins into abnormal granules could lead to rapid degradation of free granule content proteins within the cytoplasm. However, normal production and packaging of myeloperoxidase into primary granules, suggests that this is not the case in SGD. Furthermore, the absence of the primary granule defensins, despite otherwise apparently normal primary granules, further suggests that the defect is a primary defect of protein production.

Our data, in conjunction with the previous experiments by Lomax et al looking at lactoferrin RNA levels, strongly suggest that the defect in SGD has an impact on steady-state levels of messenger RNA. We have studied the RNA levels for several genes encoding proteins known to be affected in SGD to determine if their RNA levels also correspond with their reduced protein levels. We have shown that the steady-state RNA levels for neutrophil collagenase, neutrophil gelatinase, TCI, and defensins are all reduced in the BM from one SGD patient. CD18, a protein present on the membrane of the secondary granule, has been shown to be present at normal levels on SGD neutrophils. The RNA level is increased in the SGD sample as compared with normal, with a ratio of 149%. Because increased surface expression of CD18 in stimulated neutrophils results from fusion of secondary granules with the plasma membrane, the failure of SGD neutrophils to exhibit increased CD18 after f-Met-Leu-Phe (FMLP) stimulation presumably reflects the absence of secondary granules in those cells.

These data suggest that the most likely explanation for the observed protein defects is a primary failure of RNA accumulation. Such a lesion could be the result of either a transcriptional defect or a lesion affecting RNA stability. Parallel changes in RNA stability of multiple genes would be expected to be dependent on shared sequences determining RNA metabolism. Analysis of the cDNA sequences of these proteins shows no such homologous regions predicting common RNA degradation pathways. Consequently, we feel that this is an unlikely explanation for the observed protein deficit. We postulate that SGD reflects a primary defect in gene expression at the level of transcription, possibly in a shared transcription factor common to the affected subset of proteins. Definitive confirmation of this would be best provided by nuclear run-on assays. However, BM cells are unavaiable for such studies.

SGD is the first disease syndrome in which a seemingly unrelated group of proteins have been found to be affected at the RNA level. As such, it presents a unique opportunity to study the coordinate expression of a group of diverse genes related by the time of their appearance during myeloid differentiation. This has led us to begin studying the promoters of these genes to find regulatory regions shared between them in hopes of identifying a common transcription factor affected in SGD.

REFERENCES


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