Ultrastructural Cytochemical Analysis of Blastic Transformation of Chronic Myelocytic Leukemia

By Pimpa Palakavongs, Saul Teichberg, Vincent Vinciguerra, Thomas J. Degnan, and Pairoana Sinlaratana

Ultrastructural cytochemical changes occurring during the blast phase of chronic myelocytic leukemia (CML) are described. Normal developing promyelocytes contain myeloperoxidase (MPO)-positive rough endoplasmic reticulum, nuclear envelope, and Golgi apparatus. All secretory granules of normal promyelocytes are also MPO-positive. In this study we have found abnormal promyelocytes with MPO-positive as well as MPO-negative secretory granules in blast phase CML patients which contrast with the normal pattern of MPO distribution in most CML patients not in the blast phase or in nonleukemic controls. Alkaline phosphatase activity was found in the nuclear envelope of blasts and promyelocytes of one of the blast transformation patients who had a markedly increased leukocyte alkaline phosphatase score. The cytochemical changes in the distribution of MPO suggest that immature leukemic cells may alter their patterns of secretory granule production. Such processes may reflect the emergence of an abnormal clone of cells during the blastic transformation of CML.

Despite current methods of therapy, the median survival of patients with chronic myelocytic leukemia (CML) remains between 3 and 4 yr,1 due primarily to the transition from a chronic to a blastic phase which occurs in 80% of these patients.2 Although it is clinically and morphologically similar to acute myeloblastic leukemia, the blastic phase remains one of the most refractory forms of leukemia to treat. Most patients die within 1 yr.3 The blastic crisis is characterized by alterations that precede its onset. These include additional chromosomal abnormalities besides the Philadelphia chromosome4 and an elevation of the leukocyte alkaline phosphatase (LAP).5 The cause of the latter is unknown.

Previous studies of ultrastructural cytochemical changes occurring during the blast transformation have largely concentrated on peripheral blood examination.6-7 Ulliot and Bainton6 have noted three types of defective mature polymorphonuclear leukocytes (PMN) with abnormal distributions of azurophilic and specific secretory granules, suggesting multiple clones of abnormal cells. Bizarre, mature granulocytes containing basophilic and eosinophilic granules have also been seen.7 A variety of morphological anomalies have been reported in granulocytes from patients with different stages of CML, including large

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bundles of microfilaments, excessive nuclear pouching, and annulate lamellae. Cawley and Hayhoe have reported an apparent nuclear–cytoplasmic asynchrony in CML involving production of azurophilic granules in cells with later stage (metamyelocyte) nuclei.

The present study concerns an ultrastructural cytochemical analysis of developing granulocytes in CML, focusing on the blastic transformation phase of the disease. Our preliminary analysis of a limited number of cases suggests that during the blastic transformation of CML variable numbers of immature leukemic promyelocytes produce abnormal secretory granule populations. The appearance of such cytochemically distinct promyelocytes could reflect the emergence of an abnormal cell clone.

MATERIALS AND METHODS

Patients
Specimens from 16 patients consisted of bone marrow aspirates and/or peripheral blood buffy coats prepared according to the method of Ullyot and Bainton. Four patients were in the blastic transformation of CML using Acute Leukemia Group B criteria as described by Karanas and Silver. These patients had significant bone marrow replacement by blasts and promyelocytes, and their clinical features are outlined in Table 1. One patient whose entire clinical course was rapid, lasting 16 mo, was Ph chromosome negative, while three patients were Ph' positive. The LAP score was elevated in three patients who had received previous chemotherapy, including busulfan, hydroxyurea, prednisone, 6-mercaptopurine and vincristine, prior to being studied. One patient had not received chemotherapy. Specimens were also examined from five patients with active CML (four of whom were untreated and newly diagnosed), three patients in remission (Table 2), and four nonleukemic patients with anemia as controls.

Routine Fixation and Electron Microscope Preparation
For routine morphological preparations, specimens were fixed in 1.5% glutaraldehyde (Fisher Chemical Co., Fairlawn, N.J.) with 1% sucrose for 1 hr, rinsed in cold 0.1 M cacodylate buffer (pH 7.4) with 7% sucrose, postfixed in cold buffered 1% OsO4 for 1 hr, rinsed with cold 7.5% sucrose, and soaked en bloc with veronal acetate buffered uranyl acetate for 30 min at room temperature. They were then rinsed in cold 7.5% sucrose, dehydrated in a graded series of ethanols, and embedded in Epon.

Cytochemical Preparations
For cytochemical studies, cells were fixed for 10 min in cold cacodylate-buffered 1.5% glutaraldehyde with 1% sucrose and then rinsed in cold cacodylate-buffered sucrose for up to 24 hr.

To demonstrate myeloperoxidase (MPO) activity, tissue was first soaked in the medium of Graham and Karnovsky at pH 7.6 without substrate (H2O2) for 10-15 min at room temperature and then incubated in the full cytochemical medium for 60 min at room temperature. Sucrose (5%) was added to all incubations.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Ph</th>
<th>LAP (50-100)</th>
<th>Source</th>
<th>Secretory Granules in Abnormal Promyelocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68/M</td>
<td>Neg</td>
<td>250</td>
<td>Bone marrow</td>
<td>MPO + AG, MPO -</td>
</tr>
<tr>
<td>2</td>
<td>59/M</td>
<td>Pos</td>
<td>181</td>
<td>Bone marrow</td>
<td>MPO + AG, MPO -</td>
</tr>
<tr>
<td>3</td>
<td>58/F</td>
<td>Pos</td>
<td>1</td>
<td>Peripheral blood</td>
<td>MPO + AG, MPO -</td>
</tr>
<tr>
<td>4</td>
<td>65/F</td>
<td>Pos</td>
<td>160</td>
<td>Bone marrow</td>
<td>MPO + AG, MPO -</td>
</tr>
</tbody>
</table>

AG, azurophilic granule.
Table 2. Summary of Results in CML Patients: Clinical Data and Ultrastructural Cytochemical Analysis

<table>
<thead>
<tr>
<th>Case</th>
<th>Phase</th>
<th>Lap</th>
<th>Source</th>
<th>MPO Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Active treated</td>
<td>93</td>
<td>Bone marrow</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>Active untreated</td>
<td>6</td>
<td>Bone marrow</td>
<td>Normal</td>
</tr>
<tr>
<td>7</td>
<td>Active untreated</td>
<td>2</td>
<td>Peripheral blood</td>
<td>Abnormal</td>
</tr>
<tr>
<td>8</td>
<td>Active untreated</td>
<td>3</td>
<td>Bone marrow</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>Active untreated</td>
<td>22</td>
<td>Bone marrow</td>
<td>Normal</td>
</tr>
<tr>
<td>10</td>
<td>Remission</td>
<td>20</td>
<td>Bone marrow</td>
<td>Normal</td>
</tr>
<tr>
<td>11</td>
<td>Remission</td>
<td>121</td>
<td>Bone marrow</td>
<td>Normal</td>
</tr>
<tr>
<td>12</td>
<td>Remission</td>
<td>15</td>
<td>Bone marrow</td>
<td>Normal</td>
</tr>
</tbody>
</table>

We found that when the entire bone marrow chips were incubated in the Graham and Karnovsky medium, reaction product was confined to the periphery of the chips used in the incubation, while cells in deeper regions of the tissue were unreactive. This difference was probably due to an artifact of incubation, wherein the substrate (H₂O₂) was depleted by the enzymatic activity of cells at the periphery of the chip, including erythrocytes and their precursors containing peroxidatic hemoglobin. (Recently we noted a similar cytochemical artifact in cultured spinal cord explants exposed to exogenous peroxidase.13) In order to maximize the regions we could usefully study, bone marrow chips were carefully minced in cold buffer under a dissecting microscope prior to incubation. In all cases we studied regions of minced bone marrow chips showing clearly demonstrable strong cytochemical MPO reaction product.

In some cases cells were incubated for alkaline phosphatase activity in a modified Gomori medium using the calcium-lead method with 1,3-propanediol buffer (pH 9.2) for 30-90 min at 37°C.

For all cytochemical studies controls consisted of substrate-free media. All controls showed no demonstrable reaction product.

Following incubations cells were briefly rinsed in cold 7.5% sucrose, postfixed in buffered OsO₄ and processed for electron microscopy as described above.

Electron Microscopy

Silver to grey thin sections were cut on a Porter-Blum MT2-B ultramicrotome, stained with lead citrate15 or uranyl acetate and lead citrate,16 and were examined on a JEOL JEM-100 electron microscope operated at 60 kV. Electron micrographs were taken at initial magnifications of 4000-20,000.

RESULTS

Myeloperoxidase

Most of our attention was focused on immature developing granulocytes in preparations derived from bone marrow aspirates and incubated for the localization of MPO activity. In two cases, where leukocyte counts were elevated, we studied peripheral blood samples. It has previously been demonstrated that in humans, MPO localization is very useful for the identification of azurophilic granules and for the analysis of the stages of granulocyte development.17 In general, cells in our preparations showed the ultrastructural development and cytochemical characteristics that have been described in detail for normal developing human PMN.17 Briefly, myeloblasts consisted of undifferentiated cells with a large nucleus, prominent nucleolus, sparse rough endoplasmic reticulum (RER), and no secretory granules; they were without cytochemically demonstrable MPO activity, reflecting the absence of MPO synthesis. Promyelocytes (Fig. 1) had large nuclei, distinct nucleoli, a well-developed Golgi apparatus, and RER; they showed MPO reaction product in their entire secretory ap-
Fig. 1. Promyelocyte from a patient with anemia, incubated for MPO activity. Reaction product is seen in the endoplasmic reticulum (E), Golgi apparatus (G), and all the secretory granules (A). Nucleolus is at N, mitochondria at M. x 16,000.

paratus (RER and Golgi apparatus), as well as in all their azurophilic secretory granules that had been produced at this stage. MPO reaction in the RER was indicative of MPO synthesis at this stage of development. In the myelocyte, MPO reaction product was normally confined to the azurophilic granules; none was found in the secretory apparatus. During this stage a second set of secretory granules, the specific granules, are formed. During later developmental stages (metamyelocyte, band and mature PMN) nuclear chromatin was condensed, nucleoli lost, and MPO confined to diminishing numbers of azurophilic granules.

Blastic transformation. Bone marrow aspirates of three patients and the peripheral blood of one patient in blastic transformation phase of CML contained both normal developing granulocytes and variable numbers of “promyelocytes” (15%–50%) with an abnormal distribution of reaction product for MPO. These
abnormal promyelocytes had typically distinct nucleoli and diffuse nuclear chromatin, and they showed MPO in their entire secretory apparatus. In contrast to normal promyelocytes, in which all secretory granules were MPO-positive azurophils, these cells showed membrane-delimited secretory granules both with and without demonstrable MPO (Figs. 2–4). The MPO-negative secretory granules in these abnormal promyelocytes appeared to be of two morphological types (Figs. 2–4): one had a fairly dense granular matrix and a distinct electron-lucent halo; the other granule type was more electron lucent, smoother in texture, and often smaller in diameter. In some cells both MPO-positive and MPO-negative large granules were seen closely associated with Golgi membrane systems, suggesting that they may have formed from Golgi saccules (Fig. 3).

In all four of the cases of blastic transformation that we examined, myelocytes, metamyelocytes, bands and mature PMN did not show demonstrable MPO activity in their secretory apparatus. These cells did contain both MPO-

Fig. 2. Promyelocyte from a patient with blast-phase CML, incubated for MPO activity. Reaction product is seen in the nuclear envelope (N), endoplasmic reticulum (E), and a few secretory granules (A). Many secretory granules show no reaction product (arrows). Mitochondria are at M, nucleolus at NU. × 21,600.
Fig. 3. Portion of a promyelocyte from a patient with blast phase CML, incubated for MPO activity. Reaction product is seen in the nuclear envelope (N), endoplasmic reticulum (E), some Golgi saccules (G), and some secretory granules (A). Two morphological types of granules without demonstrable MPO activity are seen (arrows). Some contain a granular matrix with an electron-lucent halo, whereas other MPO-negative granules appear smoother and relatively electron lucent. Some MPO-positive and MPO-negative granules are seen in images suggesting that they arise by budding from Golgi membrane systems (arrowheads). x 30,000.

positive and MPO-negative granules. Mature PMN in the patients we studied usually contained MPO-positive azurophils and MPO-negative specific granules. Occasionally we encountered mature PMN with both MPO-positive and MPO-negative granules that appeared to be large azurophilic granules. It should be borne in mind that since we usually were looking at bone marrow aspirates from blastic phase CML patients the proportion of more mature cells was quite small.

Active CML. Developing granulocytes in the bone marrow aspirates or peripheral blood of five patients with active CML, four of whom were newly diagnosed and untreated, were also studied. In four patients developing cells showed an entirely normal distribution of MPO activity during all stages of
development. In one case, however, promyelocytes, metamyelocytes, and bands contained MPO-positive as well as MPO-negative secretory granules in addition to an MPO-positive secretory apparatus (Fig. 5). The later stage cells were identified as metamyelocytes and bands by the absence of nucleoli and condensed nuclear chromatin. In the mature PMN of this patient, MPO reaction product was entirely confined to a few secretory granules and was not seen in the RER or Golgi apparatus. This patient therefore had a similar morphological abnormality to that seen in the blast transformation patients with the addition of persistent MPO synthesis into the later myelocytic stages. After the patient entered a clinical and laboratory remission with busulfan, the bone marrow was reexamined. At that time all developing promyelocytes, myelocytes, metamyelocytes, and bands had an entirely normal distribution of MPO activity.

**Treated CML in remission.** Reaction product for MPO was found in an entirely normal distribution in developing granulocytes in the bone marrows of three patients in remission from CML during chemotherapy.

**Nonleukemic controls.** Reaction product for MPO was also normally distributed in the developing granulocytes of four control nonleukemic patients with anemia.

**Alkaline Phosphatase**

Alkaline phosphatase activity was difficult to demonstrate in the developing granulocytes from either bone marrow aspirates or buffy coat preparations using the Gomori medium, and morphological preservation after incubation was poor. However, in one case of blastic transformation phase CML in which the clinical LAP score was very elevated (240 compared to a normal of 100), we
Fig. 5. Cell in later maturational stage from patient with untreated active CML, incubated for MPO activity. Reaction product is seen in the endoplasmic reticulum (E) and several granules (A). Many MPO-negative granules are also seen at arrows. Mitochondria are at M, nucleus with condensed chromatin at N. x 15,000.

found strong cytochemical reaction product for alkaline phosphatase in the nuclear envelope (Fig. 6) and occasionally in the Golgi apparatus of several very immature cells.

Morphological Studies

The morphological abnormalities in developing promyelocytes and myelocytes we observed in remission, active, and blastic transformation phases of CML were entirely comparable to those reported in detail elsewhere. They consisted primarily of excessive nuclear folds and bundles of microfilaments. It may be noteworthy that the bundles of microfilaments in our preparations were confined to cells from peripheral blood buffy coat preparations and were never seen in developing bone marrow granulocytes. Microfilament bundles were seen in patients whether or not they were receiving chemotherapy.
DISCUSSION

The present findings in blastic transformation of CML suggest that there is an alteration in the normal processes of secretory granule production in leukemic promyelocytes. Both MPO-positive and MPO-negative secretory granules were found in variable numbers in the promyelocytes of the blastic phase CML patients we studied. The same cells also contained MPO-positive RER and Golgi apparatus. Previous work on human neutrophil development showed that normal promyelocytes with an MPO-positive RER and Golgi apparatus always contained only MPO-positive azurophilic granules, indicative of the formation of these primary granules in the promyelocyte. We think it unlikely that the variable numbers of MPO-negative granules we see in the blast phase promyelocytes are merely the result of some cytochemical or preparative artifact. Using identical incubation conditions and identical substrates, MPO was found in an entirely normal distribution (RER, Golgi apparatus, and all secretory granules) in nonleukemic controls with anemia and most active phase and remission CML patients. Furthermore, in the blast phase cases many promyelocytes showed an entirely normal pattern of MPO localization. Our analysis of MPO-negative granules was always carried out on the same cells that showed strong reaction product for MPO in the RER, Golgi apparatus, and some secretory granules. As pointed out above, we only chose for study areas from our minced bone marrow chips where MPO reaction product was clearly demonstrable. Thus it is unlikely that our results are due to artifacts alone.

The appearance of a population of abnormal developing promyelocytes coexisting with normal promyelocytes in the blast phase CML patients lends support to the notion of Ullyot and Bainton that these enzyme cytochemical techniques may reveal the emergence of abnormal clones of cells. Previous work has focused on mature PMN in patients with terminal phase CML and has revealed three types of abnormal PMN with either MPO-negative azurophilic granules, no azurophilic granules, or no specific granules. Because we have concentrated on bone marrow preparations of blast transformation patients with very high levels of immature cells, we have not examined a large enough population of mature PMN to come to a confident conclusion regarding the developmental relationships between our abnormal promyelocytes and mature
PMN in these patients. Our limited analysis suggests that some PMN have both MPO-positive and MPO-negative azurophilic granules. They may therefore be derived from the promyelocytes that also contain two types of granules.

While most patients with CML not in the blast phase demonstrated a normal pattern of MPO distribution, we did observe a developmental anomaly in one patient with active, untreated CML. This patient showed MPO activity in the RER and Golgi apparatus of promyelocytes, myelocytes, metamyelocytes and bands. In addition, MPO-positive and MPO-negative secretory granules were present in all these stages. The later developmental stages of these cells showed distinctly condensed nuclear chromatin. These observations of demonstrable MPO in the secretory apparatus of later developmental stages (bands) suggested that MPO synthesis continued in these cells well beyond the myelocyte stage where it normally terminates. When this patient was reexamined after 3 mo of a chemotherapy-induced (busulfan) remission, all the cells were normal in their MPO distribution with MPO reaction product confined only to azurophilic granules in the myelocytes, metamyelocytes, and bands. Similar processes of nuclear–cytoplasmic asynchrony have been suggested by other morphological studies. Obviously, we cannot yet ascertain whether the morphological abnormalities seen in this patient represented changes similar to those in blast transformation patients. The significance of the findings in this patient remains unclear.

The exact nature of the MPO-negative secretory granules in promyelocytes is not entirely clear. In humans, azurophilic and specific granules have been usefully classified on the basis of the presence or absence of MPO activity, although MPO-negative azurophils have been noted in both acquired and congenital disease. While the MPO criterion is extremely useful and avoids certain morphological preparative pitfalls, the two granule types do have additional differentiating characteristics: azurophils tend to be larger and have a more granular matrix, whereas specific granules are generally smaller and have a more electron-lucent smooth matrix. Based on this identification our evidence suggested that there are three populations of secretory granules in many of our abnormal promyelocytes: MPO-positive azurophils, MPO-negative azurophils, and MPO-negative specific granules. We do not believe that any appreciable number of the MPO-negative granules in these promyelocytes are the microperoxisomes that have been described as containing peroxidatic activity at pH 9.7 and are present in neutrophils of patients with congenital MPO deficiency. Most of the MPO-negative granules we see are considerably larger than microperoxisomes and lack their typical granular matrix.

Our observations of demonstrable alkaline phosphatase in immature cells of the one patient with a very elevated LAP score (240) may be of interest. During blastic transformation of CML there frequently is an elevated LAP; precocious synthesis of LAP might explain this characteristic elevation of the LAP score. Previous work by Malashova and Fuksova supports this notion. It should be borne in mind that in reading an LAP score one cannot clearly differentiate between the various granulocyte development stages; thus, a positive reading could well include numerous immature cells in addition to the more mature cells that one usually associates with a positive LAP.
We cannot yet determine whether there is a relationship between the elevated LAP score and the early appearance of secretory granules that resemble specific granules. Considerable controversy still exists as to whether or not alkaline phosphatase in humans is in the specific granule or in another small morphologically undefined granule.28 Because of the unreliability of the Gomori technique for the electron microscopic localization of alkaline phosphatase, these questions cannot as yet be resolved. Furthermore, we cannot be certain that the MPO-negative granules we see in these leukemic promyelocytes are identical in their biochemical content to those found in normal cells.

Another problem concerns the process by which MPO-negative secretory granules might have been formed in promyelocytes with an MPO-positive RER and Golgi apparatus. It could turn out, for example, that the MPO-negative granules in these promyelocytes are formed prior to the MPO-positive granules in very immature cells before MPO synthesis is initiated. If this is the case, we should find some immature cells with MPO-negative granules and MPO-negative RER. Unfortunately, we are reluctant to draw any conclusions from such cells in our preparations since they are subject to a cytochemical artifact due to lack of substrate penetration. Breton-Gorius et al.29 have recently reported the presence of MPO-positive and MPO-negative azurophilic granules in promyelocytes of a “preleukemic” patient. In their preparations, the RER and Golgi apparatus are MPO-negative, and they have suggested that MPO synthesis terminates prematurely in these cells. This situation is not the case in the present study, since the secretory apparatus was MPO-positive in the same cells with MPO-negative secretory granules. On the other hand, our morphological evidence does suggest that in at least some promyelocytes both MPO-positive and MPO-negative granules may form at the same time from distinct Golgi saccules in a manner similar to that seen in the rabbit.30

Despite the caution that is clearly warranted because of the limited number of blastic phase cases we have examined, this type of cytochemical study may eventually be of some value in the management of CML. Current concepts of treatment include attempts to avert the onset of the blastic transformation using more aggressive chemotherapy31 or immunotherapy.32 If it turns out on the basis of studies with larger numbers of patients that the kind of metabolic abnormalities our cytochemical observations reflect are indeed indicative of clonal activity during blastic transformation, we may be able to detect blast crisis in remission patients earlier, permitting more rapid intervention and possibly delay of the onset of this refractory stage. Clearly our data are too premature at this stage for us to be confident of their predictive value.

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Ultrastructural cytochemical analysis of blastic transformation of chronic myelocytic leukemia

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