In Vitro Modulation of Alkaline Phosphatase Activity in Neutrophils From Patients With Chronic Myelogenous Leukemia by Monocyte-Derived Activity

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To clarify the underlying mechanism of low neutrophil alkaline phosphatase (NAP) activity in chronic myelogenous leukemia (CML), CML neutrophils were cultured in liquid medium with different numbers of monocytes. Alkaline phosphatase activity in CML neutrophils, assessed cytochemically, increased with the numbers of monocytes. NAP activity was not induced by the interaction between neutrophils and monocytes, but by the presence of a monocyte-derived soluble activity. NAP activity in normal neutrophils was also lowered by depletion of monocytes from culture medium. Under such monocyte-depleted conditions, both CML and normal neutrophils proliferated and differentiated to produce mature neutrophils. Thus induction of NAP activity can be modified in vitro by changing the amount of NAP-inducing activity released from monocytes. However, whether a reduction of NAP-inducing activity in CML neutrophil is the cause of low NAP activity in vivo remains uncertain.

EUTROPHIL ALKALINE PHOSPHATASE (NAP) activity is uniformly low in patients with chronic myelogenous leukemia (CML) at the time of diagnosis and is known to increase during an infectious or inflammatory complication or clinical remission with chemotherapy. Recently, CML neutrophils have been demonstrated to recover NAP activity in vitro and in vivo. NAP activity of CML neutrophils also increases after transfusion in febrile patients. Low NAP activity of CML neutrophils in vivo has been considered to be a result of some still unidentified environmental influence. In the present study, whether in vitro NAP activity of CML neutrophils could be maintained at a low level under liquid culture conditions was examined.

MATERIALS AND METHODS

Patients. Fifteen untreated patients in the chronic phase of CML were studied. Five were male and ten, female. They ranged from 23 to 76 years old (mean, 50.2). The peripheral blood leukocyte count ranged from 11.4 to 259.0 x 10^9/L (mean, 113.4 x 10^9/L). Ph1 chromosome was detected in 14 patients. Three healthy volunteers were included as controls. Informed consent was obtained from all subjects.

Cell preparations. Bone marrow and peripheral blood were obtained with heparinized syringes. Light-density (LD) cells were separated from each sample by Ficoll-Conray gradient centrifugation (1.077 g/mL). Nonphagocytic (NP) cells were obtained by depleting LD cells of phagocytes using iron-phagocytosis. Briefly, LD cells (5 x 10^6) were incubated in 1 mL of α-medium (Flow Laboratories, Irvine, Scotland) with 40 mg/mL carbonyl iron powder (GAF Corp., New York) and 20% fetal bovine serum (FBS; Flow Laboratories, North Ryde, Australia) for 30 minutes at 37 °C. LD cells (1 x 10^6) were plated in a plastic Falcon Petri dish (35-mm in diameter) containing 1.0 mL of α-medium with 0.88% methyl cellulose, 10% GCT-CM, and 20% FBS and then incubated at 37 °C in a humidified atmosphere of 5% CO2 in air for seven days. The dishes were assessed under an inverted microscope for colonies containing 20 or more cells. Fifty individual colonies were aspirated from each sample by a finely drawn out micropipette, and smears of each colony were prepared by a Cytospin (Shandon Southern Products, Cheshire, England).

Liquid culture. Cells (1 x 10^6) in 1 mL of α-medium with 20% FBS were incubated in Falcon plastic Petri dishes (35-mm in diameter) in duplicate for three to seven days at 37 °C in a humidified atmosphere of 5% CO2. Both adherent and nonadherent cells were completely harvested by a Pasteur pipette after scraping the dishes gently with a rubber policeman. Average volume recovery was 0.9 mL per dish. Cell viability was at least 90% by trypan blue dye exclusion. Three slides were prepared from each dish by cyto centrifugation and subjected to alkaline phosphatase staining, and May-Grünwald-Giemsa staining for the analysis of cell composition and dual esterase staining for the detection of the number of monocytes and macrophages.

Granulocyte-macrophage colony assay. The granulocyte-macrophage (GM) colony assay was performed according to the method of Iscove et al. A giant cell tumor-conditioned medium (GCT-CM; GIBCO, Grand Island, NY) was used as the exogenous colony-stimulating activity (CSA). LD cells (1 x 10^6) were plated in a plastic Falcon Petri dish (35-mm in diameter) containing 1.0 mL of α-medium with 0.88% methyl cellulose, 10% GCT-CM, and 20% FBS and then incubated at 37 °C in a humidified atmosphere of 5% CO2. Both adherent and nonadherent cells were completely harvested by a Pasteur pipette after scraping the dishes gently with a rubber policeman. Average volume recovery was 0.9 mL per dish. Cell viability was at least 90% by trypan blue dye exclusion. Three slides were prepared from each dish by cyto centrifugation and subjected to alkaline phosphatase staining, and May-Grünwald-Giemsa staining for the analysis of cell composition and dual esterase staining for the detection of the number of monocytes and macrophages.

Alkaline phosphatase stain and scoring system for enzyme activity. The naphthol AS-MX phosphate/fast blue RR-staining has high cytochemical sensitivity and reveals sharp localization of enzyme activity. The correlation coefficient between enzyme activities estimated by this method and those by colorimetric assessment was very high (r = .87). Smears were fixed for five seconds at ~3 °C with 10% formalin in methanol. After washing in running water, slides were mounted with 2 mL of reaction medium and incubated for two hours at 37 °C. The reaction medium was made by adding 1 mg of fast blue RR (Sigma Chemical Company, St Louis) to 1 mL of stock solution, which was prepared by dissolving 10 mg of naphthol AS-MX phosphate (Sigma) in 4 mL of dimethyl formamide and adding 120 mL of water and 76 mL of 0.2 mol/L propanediol buffer. This stock solution was kept refrigerated and was thoroughly mixed before use. After washing in running water, the slides were counterstained with 1% safranine-O for three minutes. NAP activity appeared as blue granules.
The enzyme activity in each neutrophil was graded according to the number of blue granules in the cytoplasm: type 0 (no granules), type I (less than five granules), type II (less than 30 granules), type III (more than 30 granules, unevenly distributed), type IV (many granules, evenly distributed with small gaps), and type V (numerous granules, densely distributed). The sum of rating in granules, evenly distributed with small gaps), and type V (numerous granules, densely distributed). The sum of rating in 100 mature neutrophils (restricted to band and segmented forms) was considered as the NAP score for a particular specimen. The normal range of peripheral blood NAP score was from 158 to 295 (n = 20). In the liquid culture experiments, 400 mature neutrophils (band and segmented forms), 200 from each of the duplicated liquid culture dishes were graded and the mean NAP score for 100 cells was calculated in each experiment.

Preparation of LD cell conditioned medium. LD cells (5 × 10⁶) containing monocytes were suspended in 1 mL of α-medium with 20% FBS. They were incubated for five days at 37°C in a humidified atmosphere of 5% CO₂. The supernatant was harvested by centrifugation (3,000 rpm) and filtering with 0.45-μm Millipore filter (Millipore Corp, Bedford, Mass).

Neutrophil count. The absolute number of nucleated cells harvested from liquid cultures was counted using a hemacytometer. Neutrophil numbers were calculated from each differential count on May-Grunwald-Giemsa-stained smear.

Statistics. Student’s t test used to assess the statistical significance between comparable experimental groups.

RESULTS

NAP activity of granulocyte-macrophage colony cells. There was no colony in which all mature neutrophils lacked NAP activity. The NAP score for cells in 50 colonies in each experiment was always high. There was no significant difference between peripheral blood and bone marrow samples (Fig 1).

NAP activity of CML light-density cells in liquid culture. The NAP score of band and segmented forms of neutrophils in the LD cell preparation was regarded as the preincubation NAP score. The NAP score began to increase on day -3 of culture and reached the maximum between days 5 and 7 (Fig 2A). The maximum NAP score in this culture condition seldom exceeded the normal upper limit. Similar results were obtained from both peripheral blood and bone marrow samples.

NAP activity of CML nonphagocytic cells in liquid culture. The CML NP cells were cultured in liquid medium to avoid the effect of monocytes, which are known to produce various biological substances, including CSF. The NAP score of band and segmented forms of neutrophils in the NP cell preparations was regarded as the preincubation NAP score. The NAP activity did not increase during the seven-day incubation period (Fig 2B). Similar results were obtained from both peripheral blood and bone marrow samples.

Relationship between the induction of NAP activity and monocyte number. Isolation of monocytes from CML peripheral blood or bone marrow was difficult because of their relative scarcity. Therefore, mixtures of CML LD cells and CML NP cells were incubated in liquid culture at various ratios to ascertain the monocyte number at which NAP activity was induced. The mean percentage of monocytes estimated using dual esterase stain were 1.6%, 0.9%, 0.4%, and 0.2%, when the percentage of LD cells in the mixture was 100%, 50%, 20%, and 0% (all NP cells), respectively. The NAP activity began to increase when the mixture contained 20% LD cells and reached a plateau when the mixture contained 50% LD cells (Fig 3). The results obtained with peripheral blood samples were similar to those obtained with bone marrow samples.

Dependency of NAP activity induction on monocyte-derived soluble activity. To investigate whether the increase of NAP activity in LD cells depended on the

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Fig 1. Neutrophil alkaline phosphatase (NAP) score in band and segmented forms of neutrophils derived from granulocytemacrophage colony (CFU-GM) in five patients with chronic myelogenous leukemia. The vertical line indicates mean ± SD (n = 5).

Fig 2. Change in neutrophil alkaline phosphatase (NAP) score in a liquid culture system using light-density cells (A) and nonphagocytic cells (B) depleted of monocytes from the bone marrow of four patients with chronic myelogenous leukemia. The mean ± S.D. (n = 4).

Fig 3. Neutrophil alkaline phosphatase (NAP) score in coculture of light-density cells and nonphagocytic cells obtained from the bone marrow of four patients with chronic myelogenous leukemia at four mixing ratios of monocytes. The mean ± S.D. (n = 4).
interaction between neutrophils and monocytes or on the soluble factor produced by monocytes. NP cells were cultured as target cells in liquid medium over the agar underlayer that contained various mixtures of LD cells and NP cells. The NAP activity of CML NP cells in the upper liquid increased proportionately to the CML LD cell percentage of the agar underlayer (Fig 4).

**NAP-inducing activity and CSA in CML LD cell-conditioned medium.** Autologous LD cell-conditioned medium was added to the liquid culture of NP cells to induce NAP activity, and to the CFU-GM assay as a CSA. As shown in Fig 5, the LD cell-conditioned medium from case A had strong NAP-inducing activity as well as measurable CSA. The conditioned medium from case B showed weak NAP-inducing activity but no CSA. Neither NAP-inducing activity nor CSA were observed in the conditioned medium from case C.

**NAP-inducing activity of exogenous CSA sources.** Various amounts of CSA sources were added to the liquid culture of CML NP cells. Preliminary tests disclosed that the human placental conditioned medium (HPCM), GCT-CM, and CSF-Chugai18 (Chugai Pharmaceutical Co, Tokyo) stimulated normal bone marrows to form a similar number of CFU-GM. CSF-Chugai, highly purified by depleting the inhibitor(s), was diluted to make 100 U/mL at a 10% concentration. These three CSA sources consistently induced NAP activity in a similar dose-dependent manner (Fig 6). The NAP score began to increase at a concentration of 1% and reached the upper limit of the normal range at a concentration of 10%, which is usually used in CFU-GM assay.

**Monocyte dependency of NAP activity induction in normal neutrophils.** The NAP scores of normal bone marrow LD cells and NP cells from the three control subjects were very low during preincubation. During the seven-day culture, the NAP score increased considerably in the LD cells (Fig 7A) and remained very low in the NP cells (Fig 7B).

**Neutrophil growth in liquid culture.** Both CML neutrophils and normal bone marrow neutrophils proliferated for seven days in all liquid cultures. In particular, CML neutrophils grew at a significantly higher rate in the presence of LD cells than with NP cells ($P < .01$) (Fig 8), even though there was little difference between their initial maturational composition in the neutrophil compartment (Fig 9). The mean ratio of monocytes-macrophages to neutrophils at days 0, 3, 5, and 7 were 0.015, 0.027, 0.035, and 0.046 in CML LD cells and 0.081, 0.115, 0.112, and 0.120 in normal LD cells, respectively. The mean ratios of monocytes-macrophages to neutrophils in NP cells from both CML and normal bone marrows did not exceed 0.015 until day 5. The ratio at day 7 was 0.024 in CML and 0.013 in normal subjects. The Ph1 chromosome was detected during the culture period (data not shown).

**DISCUSSION**

Because GM-colony forming cells from CML patients showed high NAP activity in the presence of a CSA source (GCT-CM), the factor responsible for the consistent elevation of NAP activity in liquid culture of CML LD cells was...
suspected to be colony-stimulating factor (CSF) released from monocytes. The NAP activity of NP cells with less than 0.2% monocytes was not significantly increased after seven days of liquid culture. During this period, NP cells consistently proliferated to produce mature neutrophils, although their growth rate was lower than that of LD cells. Furthermore, modification of monocyte number in CML cells by mixing the LD cells and NP cells at various ratios clearly demonstrated that NAP activity elevates proportionally to the number of monocytes present. NAP activity was also induced in NP cells cultured in the upper liquid with LD cells plated in the under agar layer. The degree of induction was again dependent on the number of monocytes in the underlayer. These observations indicate that NAP activity of CML neutrophils is induced in vitro, not by interaction between neutrophils and monocytes, but by soluble activity released by the monocytes.

The conditioned media of CML LD cells obtained from one of the three patients revealed simultaneous expression of both NAP-inducing activity and CSA, although CSA was relatively weak compared with the considerably high NAP-inducing activity. The three exogenous CSA sources showed similarly high NAP-inducing activity. The CSA–dose response curves of NAP activity were linear. These findings suggest a close relationship between the two activities but do not prove that these activities are derived from a single biological substance, ie, the CSF. Sato et al also demonstrated strong NAP-inducing activity in the cystic fluid of transplanted human squamous cell carcinoma, which is known to produce a large amount of CSF. According to Nicola et al, HPCM contains two types of CSF; CSFa may act as GM-CSF and EO-CSF, and CSFβ as G-CSF. Abboud et al also separated two types of CSF from GCT-CM. Even if NAP-inducing activity is the same as CSA, it is necessary to clarify what kind of CSF has the former activity as well. The possibility remains that NAP-inducing activity unrelated to CSF is released from CML monocytes and is present in the CSA sources.

The biochemical and functional aspects of neutrophils, such as phagocytosis and NAP activity, adhesiveness, and lactoferrin contents, have been found to be closely related. Because these characteristics appear to develop together and to become most marked in the segmented forms, NAP can be regarded as a marker enzyme for neutrophilic maturation. Lactoferrin, another marker of neutrophilic maturation, was reported to be induced in a dose-dependent manner by addition of exogenous CSA. Furthermore, there is an increasing number of reports that CSF can stimulate the functions of mature neutrophils. Weisbert et al showed that purified human GM-CSF enhances oxidative metabolism of mature neutrophils and demonstrated that the GM-CSF is identical to the neutrophil migration inhibition factor (NIF). In this context, it is interesting to clarify the relationship between NAP-inducing activity and CSFs.

In the liquid culture, monocyte depletion completely eliminated NAP induction in normal bone marrow neutrophils as well as in CML neutrophils. Thus both CML and normal neutrophils react similarly to the reduction of NAP-inducing factor derived from monocytes in the culture. In contrast to
CML neutrophils, LD cells from normal bone marrow showed no significantly higher proliferative activity than did NP cells. It is not clear at present why NP cells can proliferate at a similar rate with less endogenous CSA. The higher percentage of immature neutrophils capable of division in the NP cell sample seems to play a role in this phenomenon.

Judging from the results of previous reports and the present study, the low NAP activity of CML neutrophils does not seem to be an intrinsic defect due to their leukemic nature. However, it is still obscure why the NAP activity is uniformly and markedly low in vivo at the time of diagnosis. In theory, some inhibitory activity against NAP-inducing activity may exist in vivo. Patients' sera, plasma, conditioned media of mature neutrophils, and neutrophil debris were tested for their inhibitory effect on NAP-induction in autologous CML LD cell culture, but they were not inhibitory (data not shown). NAP-inducing activity and probably CSA may be low in vivo. Because, in this study, CML monocytes showed distinct NAP-inducing activity as well as CSA in vitro, and that CSA of the conditioned media of the bone marrow and the urine from CML patients are not low compared with normal controls, it is unlikely that these activities are absent in vivo.

There are also some clinical observations concerning the mechanism of low NAP activity in vivo. Kamada and Uchino observed during the preclinical phase of CML that NAP activity actually begins to decrease after peripheral leukocytosis is established. It is also well known that NAP activity increases after successful reduction of myeloid hyperplasia by chemotherapy, although the Ph clone persists. At the time of diagnosis, CML bone marrow usually consists of an extraordinarily large number of neutrophils. Subsequently, the ratio of monocytes to neutrophils becomes low compared with normal bone marrow. NAP induction in vivo may be closely related to the degree of myeloid hyperplasia. The amount of the NAP-inducing activity released by monocytes available to individual neutrophils may decrease after the hyperplasia is established. The amount of CSA in individual neutrophils may also be low. The consistent elevation of NAP activity in vitro can be explained by the higher ratio of monocytes in the LD cell sample than in vivo and the closed circumstance of 1 mL liquid culture where the neutrophils (target cells) and the monocytes/macrophages (effector cells) are kept in close contact. The low NAP activity of CML neutrophils seems to result from extreme myeloid proliferation, which might be induced by a more fundamental pathogenesis, such as recently postulated mechanism of altered c-abl protooncogene transcription due to the Ph translocation.

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