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

By Tatsuki Matsuo

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.

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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 100 mature
neutrophils (restricted to band and segmented forms) was consid-
ered 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 x 10⁶)
containing monocytes were suspended in 1 mL of a-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 centrifuga-
tion (3,000 rpm) and filtering with 0.45-μm Millipore filter
(Millipore Corp, Bedford, Mass).

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

Statistics. Student's t test used to assess the statistical signifi-
cance 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 signifi-
cant 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 culture to
avoid the effect of monocytes, which are known to produce
various biological substances, including CSF.¹⁴,¹⁵ 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 mono-
cytes 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

Fig 1. Neutrophil alkaline phosphatase (NAP) score in band
and segmented forms of neutrophils derived from granulocyte-
macrophage 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 cocul-
ture 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 layer 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-Chugai16 (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, i.e., 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 marrow35 and the urine36 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 Uchino37 observed during the preclinical phase of CML that NAP activity actually begins to decrease after peripheral leucocytosis is established. It is also well known that NAP activity increases after successful reduction of myeloid hyperplasia by chemotherapy, although the Ph1 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 Ph1 translocation.38

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T Matsuo