Types of Nuclear Endonuclease Activity Capable of Inducing Internucleosomal DNA Fragmentation Are Completely Different Between Human CD34⁺ Cells and Their Granulocytic Descendants

By Naoyuki Anzai, Hiroshi Kawabata, Toshiyasu Hirama, Hiroshi Masutani, Yasunori Ueda, Yatozo Yoshida, and Minoru Okuma

A hallmark of apoptosis is internucleosomal DNA fragmentation resulting from the activation of endonucleases. We characterized the endonuclease activity of human myeloid cell nuclei that cleaved their own nuclear chromatin to oligonucleosomal length fragments. Polymorphonuclear leukocytes (PMNs) of normal peripheral blood contained both Ca²⁺/Mg²⁺-dependent and DNase II-like acidic endonuclease activities in their nuclei. Immature myeloid cells of normal bone marrow at various stages of granulocytic maturation had similar nuclease activities. In contrast, a clear difference was shown in the circulating CD34⁺ cells, in that only Mg²⁺-dependent, Ca²⁺-independent endonuclease activity was detected. Consistent with these findings is the emergence of the Ca²⁺+/Mg²⁺-dependent and acidic endonuclease concomitantly with the disappearance of the Mg²⁺-dependent endonuclease when CD34⁺ cells were induced to differentiate in vitro toward granulocytes. Leukemic cell lines of all lineages also had Mg²⁺-dependent nuclease activity. Our results suggest an association of the Mg²⁺-dependent endonuclease with hematopoietic progenitor cells and that the relative activities of the nuclear nuclease in human myeloid cells change substantially during granulocytic differentiation.

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CD11b, CD14, CD16, CD19, CD56, CD57 (Leu-7; Becton Dickinson), and glycophorin A. Negative selection was performed using IgG-coated dishes and immunomagnetic beads. Finally, the cells were incubated with CD34-coated immunomagnetic beads (Dynabeads M-450 CD34; Dynal) for 1 hour at 4°C. To evaluate the percentage of CD34+ cells, portions of microsphere-resetting cells were treated with 100 U/mL chymopapain (Sigma Chemical Co, St Louis, MO) at 37°C for 30 minutes. Then, the microsphere-free cells were labeled with CD34 (Tük-3; Dako A/S) MoAb and with fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragment of rabbit antirat mouse IgG (Dako A/S). The labeled cells were analyzed by FACSscan (Becton Dickinson).

Culture of CD34+ Cells

Isolated CD34+ cells were cultured in Iscove’s modified Dulbecco’s medium (Gibco, Grand Island, NY) supplemented with 30% FCS at 37°C in a humidified 5% CO2 atmosphere. At initiation and every 48 hours thereafter, cultures received 100 U/mL interleukin (IL)-3 (Kirim Arogen, Tokyo, Japan), 50 ng/mL granulocyte-stimulating factor (G-CSF; Chugai, Tokyo, Japan), and 50 ng/mL stem cell factor (Kirim Arogen). The differentiation of CD34+ cells was assayed by FACSscan for the expression of the following surface markers: CD13 (MCS-2; Nichirei, Tokyo, Japan), CD14, CD15 (Leu-M-1; Becton Dickinson), CD33 (VM-54; Dakopatts), and CD34.

Cell Lines

HL-60, a promyelocytic leukemia cell line; KG-1, a myeloblastic leukemia cell line; K562, a chronic myelogenous leukemia cell line; THP-1 and U937, monocytoid cell lines; HEL, an erythroleukemia cell line; P39, a myelomonocytoid cell line; Raji, a Burkitt's lymphoma cell line; RPMI8226, a myeloma cell line; LC4-1, a B-cell lymphoblastoid cell line; and Jurkat and Molt-4, T-lymphoblastoid cell lines; PHA-L and U937, monocytoid cell lines; HEL, an erythroleukemia cell line; RPMI8226, a myeloma cell line; LC4-1, a B-cell lymphoblastoid cell line; and Jurkat and Molt-4, T-lymphoblastoid cell lines, were obtained from Japanese Cancer Research Resources Center (JCRRC). CD34+ blasts, were kindly provided by T. Kitamura (DNAX, Palo Alto, CA), and K. Tohyama (Fukui Medical School, Fukui, Japan), respectively. The cells were suspended in 1.5 mmol/L MgCl2. Then the nuclei were washed twice with 1.5 mmol/L MgCl2 containing 0.2% NP-40 to rupture the cytoplasmic membrane. Then the nuclei were incubated in 10 mmol/L TRIS-HCl pH 7.5, 25 mmol/L KCl, 5 mmol/L MgCl2 buffer with 0.25 mol/L sucrose in a Dounce homogenizer, and then 2 vol of TKM buffer with 2.3 mol/L sucrose was added. The cell lysates were layered onto TKM buffer with 2.3 mol/L sucrose and centrifuged for 1 hour at 100,000g. The nuclear pellets were washed once with 1.5 mmol/L MgCl2.

Acid Phosphatase Measurement

The nuclear pellets were incubated in 10 mmol/L TRIS-HCl pH 7.5, 0.6 mol/L NaCl, and 0.2% NP-40 for 1 hour on a rotating platform and centrifuged at 183,000g for 1 hour at 4°C. The acid phosphatase activity of the supernatants was measured using a commercially available kit (Wako, Osaka, Japan), based on the Bessey-Lowry method using p-nitrophenyl phosphate as a substrate.

Autodigestion

The isolated nuclei were incubated in digestion buffer at 37°C for 16 hours. The incubation buffers for determining the activities of Ca2+/Mg2+-dependent, Mg2+-dependent, and acidic endonucleases, respectively, were as follows: buffer 1: 10 mmol/L TRIS-HCl pH 7.6, 10 mmol/L CaCl2, 5 mmol/L MgCl2, 1 mmol/L dithiothreitol (DTT); buffer 2: 10 mmol/L TRIS-HCl pH 7.6, 1 mmol/L MgCl2, 2 mmol/L EGTA, 1 mmol/L DTT (this condition was determined previously using P39 cells17); and buffer 3: 50 mmol/L bis-TRIS-HCl pH 6.0, 5 mmol/L EDTA, 1 mmol/L DTT. Buffer conditions 1 and 3 were determined primarily using PMNs (see Results). The reaction volume was increased to 100 μL, and the reaction was terminated by adding 5 μL of 0.5 mol/L EDTA pH 8.0, 2 μL of 10% sodium dodecyl sulfate, and 10 μL of 20 mg/mL proteinase K. After incubation of the mixture at 50°C for 1 hour, the samples were precipitated with an equal volume of 2 mol/L sodium iodide and 2 vol of 2-propanol. After centrifugation at 14,000g for 15 minutes and rinsing with 70% ethanol, the pellets were dissolved in 10 mmol/L TRIS-HCl pH 8.0, 1 mmol/L EDTA containing 0.1 μg/mL of RNase A. The samples were incubated at 37°C for 1 hour, and the DNA concentration was estimated by measuring the optical density (OD) at 260 nm. DNA (2 μg per lane) was electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining, and the gels were photographed under UV illumination.

Quantitative Analysis of Nucleosome Activities

The method for quantitative analysis of the degree of nucleosomal-size DNA fragmentation has been described elsewhere. Briefly, each lane of the electrophoretic image was divided into three areas: the large-molecular-size area (area L) containing DNA larger than 10 kbp, the middle-molecular-size area (area M) containing DNA between the sizes of 10 kbp and 300 bp, and the single-nucleosomal-size area (area S) containing DNA smaller than 300 bp. We then calculated the integration of the intensity of each area (IL, IM, and IS, respectively). Next, the integration of the whole lane area on the gel (IA) was given by the sum of IL, IM, and IS.

Nuclear Isolation

The cells were washed three times with PBS. All subsequent steps were performed at 4°C.

Method 1. The cells were suspended in 1.5 mmol/L MgCl2. After centrifugation, the pellets were resuspended and gently shaken in 1.5 mmol/L MgCl2 containing 0.2% NP-40 to rupture with the cytoplasmic membrane. Then the nuclei were washed twice with 1.5 mmol/L MgCl2. Nuclei were prepared by this method in all experiments unless otherwise indicated.

Method 2. To obtain nuclei with minimal cytoplasmic contamination, ultracentrifugation through 2.3 mol/L sucrose was used. The cells were homogenized in TKM (50 mmol/L TRIS-HCl pH 7.5, 25 mmol/L KCl, 5 mmol/L MgCl2) buffer with 0.25 mol/L sucrose in a Dounce homogenizer, and then 2 vol of TKM buffer with 2.3 mol/L sucrose was added. The cell lysates were layered onto TKM buffer with 2.3 mol/L sucrose and centrifuged for 1 hour at 100,000g. The nuclear pellets were washed once with 1.5 mmol/L MgCl2.

Characterization of Nuclear Endonuclease of PMNs

We examined the characteristics of endonuclease activity of PMNs. Figure 1 shows that the activity at alkaline pH was dependent on Ca2+ and Mg2+, and the optimal concentra-
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Fig 1. Effects of concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) on autodigestion of PMN nuclei at pH 7.6. (A) PMN nuclei were incubated with 1 mmol/L EGTA or various concentrations of CaCl\(_2\) in buffer containing 10 mmol/L TRIS-HCl pH 7.6, 5 mmol/L MgCl\(_2\), and 1 mmol/L DTT for 16 hours at 37°C. (B) PMN nuclei were incubated with various concentrations of MgCl\(_2\) in buffer containing 10 mmol/L TRIS-HCl pH 7.6, 5 mmol/L CaCl\(_2\), and 1 mmol/L DTT for 16 hours at 37°C. DNA extracted from nuclei before incubation was used as a control. DNA molecular weight markers expressed in base pairs are indicated on the left side of these figures.

Fig 2. Effects of pH on the endonuclease activities of PMN nuclei. PMN nuclei were incubated in 10 mmol/L buffer with either 5 mmol/L MgCl\(_2\), 5 mmol/L CaCl\(_2\) (○) or 1 mmol/L EDTA (●) for 16 hours at 37°C. The buffers used were sodium acetate, pH 4.0 to 5.5; bis-TRIS-HCl, pH 6.0 to 7.0; and TRIS-HCl, pH 7.2 to 9.0. The activities were expressed as micrococcal nuclease (MN) equivalent of DNA fragmentation after autodigestion of PMNs. Activity less than 0.1 mU MN equivalent (indicated by horizontal line) was estimated as negative. Results are means of triplicate samples.
Table 1. Effects of Several Compounds on Activities of Ca\(^{2+}/Mg\(^{2+}\)-Dependent and Acidic Endonucleases

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mmol/L)</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca(^{2+}/Mg(^{2+})-Dependent</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Aurintricarboxylic acid</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>NaCl</td>
<td>100</td>
<td>19</td>
</tr>
<tr>
<td>KCl</td>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>2</td>
<td>55</td>
</tr>
</tbody>
</table>

PMN nuclei were incubated in buffer 1 (for Ca\(^{2+}/Mg\(^{2+}\)-dependent endonuclease) or buffer 3 (for acidic endonuclease) with or without inhibitors for 16 hours at 37°C. Relative activity is expressed in terms of percent MN equivalent of DNA fragmentation (%FR) compared with that without inhibitor as 100%.

Table 2. Comparison of Acid Phosphatase Activity and Endonuclease Activities in Nuclei Prepared by Two Methods

<table>
<thead>
<tr>
<th></th>
<th>Method 1</th>
<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase activity/DNA (IU/g)</td>
<td>358 ± 188</td>
<td>23 ± 14</td>
</tr>
<tr>
<td>Ca(^{2+}/Mg(^{2+})-dependent endonuclease activity (MN equivalent mU)</td>
<td>1.06 ± 0.19</td>
<td>0.36 ± 0.15</td>
</tr>
<tr>
<td>Acidic endonuclease activity (MN equivalent mU)</td>
<td>7.43 ± 3.52</td>
<td>0.91 ± 0.52</td>
</tr>
</tbody>
</table>

Acid phosphatase activity of nuclear extracts was measured as described in Materials and Methods. PMN nuclei were incubated in buffer 1 (for Ca\(^{2+}/Mg\(^{2+}\)-dependent endonuclease) or buffer 3 (for acidic endonuclease) for 16 hours at 37°C. The activities were expressed as MN equivalent of DNA fragmentation after autodigestion of PMNs. Results are means ± SD of four experiments.

Compared with that by method 1 in the experiment using P39 (data not shown).

Features of Nuclear Endonuclease of Immature Myeloid Cells and Hematopoietic Progenitor Cells

Two types (the Ca\(^{2+}/Mg\(^{2+}\)-dependent and the acidic) of activity were observed with no detectable level of the Mg\(^{2+}\)-dependent endonuclease activity in nuclei of immature bone marrow myeloid cells. Thus, the pattern of nuclease activity in these cells was similar to that in PMNs (Fig 4), although DNA fragmentation tended to form a smear rather than a ladder on electrophoresis.

In contrast, the Mg\(^{2+}\)-dependent endonuclease activity, with no apparent activity of the Ca\(^{2+}/Mg\(^{2+}\)-dependent or the acidic endonuclease found in PMNs, was detected unequivocally in nuclei of CD34\(^+$\) cells (Fig 3B). In the presence of EGTA but without Mg\(^{2+}\) ions, no DNA fragmentation was observed in CD34\(^+$\) cells (data not shown), thus confirming

![Fig 3. Autodigestion of PMN nuclei (A) and CD34\(^+$\) cells (B). DNA extracted from nuclei before incubation was used as a control. Buffer conditions (1, Ca\(^{2+}/Mg\(^{2+}\)-dependent endonuclease; 2, Mg\(^{2+}\)-dependent endonuclease; and 3, acidic endonuclease) were as described in Materials and Methods. DNA molecular weight markers are indicated in base pairs and are the same as in Fig 1.](image-url)
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Fig 4. MN equivalent of DNA fragmentation after autodigestion of PMNs, immature myeloid cells, and CD34+ cell nuclei. Activity less than 0.1 mU MN equivalent (indicated by horizontal line) was estimated as negative. MN equivalent of %FR of DNA without incubation was used as a control. Buffer conditions 1 to 3 were the same as in Fig 3. Results are means ± SD of triplicate samples. C, control.

Fig 5. Time courses of changes in endonuclease activities of differentiating CD34+ cell nuclei. Isolated CD34+ cells were incubated in suspension culture containing IL-3, stem cell factor, and G-CSF. Nuclei of floating cells were isolated and subjected to autodigestion at the indicated times. DNA extracted from nuclei before autodigestion was used as a control. Buffer conditions 1 to 3 are the same as in Fig 3. DNA molecular weight markers are indicated in base pairs and are the same as in Fig 1.

DISCUSSION

The present study showed that three types of endonucleases exist in myeloid cell nuclei, and the pattern of nuclease activity expression is strikingly different between CD34+ cells and mature neutrophils. The three types of endonucleases (Ca2+/Mg2+-dependent, Mg2+-dependent, and acidic) could be distinguished from each other with regard to their cation requirement, optimal pH, and response to inhibitors.

Several candidate endonucleases involved in apoptosis have been proposed. These include (1) a Ca2+/Mg2+-dependent endonuclease, which has been investigated most intensively, such as NUC18 and DNase I; (2) a Ca2+-independen-
dent form\textsuperscript{17,26,27}, and (3) a cation-independent acidic form such as DNase II.\textsuperscript{14,28} However, there have been few reports on the endonucleases of PMNs. Only one report suggested the presence of an ion-insensitive endonuclease in PMN nuclei, but no detailed characterization was reported.\textsuperscript{29} In this study, we determined the characteristics of the activities of the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent and the acidic cation-independent endonuclease of PMN nuclei. Similar enzyme activities with regard to ion requirement, pH range, and inhibitors to each have been reported previously in other tissues.\textsuperscript{12-14} Further characterization and isolation of these nucleases will be necessary to determine their identities.

Some investigators have not detected endonuclease activity in leukemia cell nuclei.\textsuperscript{15,27} This discrepancy from our results may be attributed to different experimental conditions for examining nuclease activity, as well as the possible uneven intracellular distribution of the nucleases. It has been reported that three types of endonuclease activities (Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent, Mg\textsuperscript{2+}-dependent, and acidic) were recovered mainly from cytoplasmic and nuclear sap fractions in rat thymocytes.\textsuperscript{30} Our observation that nuclease activities were reduced to a great extent with the strict nuclear isolation method (method 2) indicates that the endonuclease activities in PMNs are distributed mainly in the cytoplasmic fraction. Therefore, our assay system (method 1) may overestimate nuclear endonuclease activities. The acidic nuclease of PMNs, like DNase II, may be primarily of lysosomal origin. The precise subcellular localization of apoptotic endonucleases remains controversial. However, the present results strongly suggest that at least a certain proportion of these nucleases exist in PMN nuclei. In contrast, substantial amounts of the Mg\textsuperscript{2+}-dependent nuclease may be present in nuclei of myeloid cell lines. In PMNs, the Mg\textsuperscript{2+}-dependent nuclease activity was identified in neither nuclear nor cytoplasmic fractions (data not shown).

Examination of three types of endonuclease activities revealed a clear-cut difference between CD34\textsuperscript{+} cells and immature myeloid cells or PMNs. Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent and acidic, but not Mg\textsuperscript{2+}-dependent, endonuclease activities were found in immature myeloid cell and PMN nuclei. In contrast, only Mg\textsuperscript{2+}-dependent nuclear endonuclease activity could be detected in CD34\textsuperscript{+} cells. Furthermore, leukemic cell lines of all lineages have been found to contain the same Mg\textsuperscript{2+}-dependent activity as that of CD34\textsuperscript{+} cells. These results suggest that hematopoietic progenitor cells contain the Mg\textsuperscript{2+}-dependent endonuclease activity in their nuclei. CD34\textsuperscript{+} cells may lose the Mg\textsuperscript{2+}-dependent endonuclease activity and acquire new activities along the differentiation pathway to granulocytes. This hypothesis was confirmed by the differentiation experiments of CD34\textsuperscript{+} cells. A few possibilities may be raised concerning the significance of these new findings. It is possible that the endonuclease involved in apoptosis differs between progenitor cells and granulocytes. Alternatively, de novo synthesis of the enzyme, or modification of activity of endonuclease such as degradation of inhibitors or access of the enzyme to the nucleus from the cytoplasm, may be necessary for apoptosis.

Ca\textsuperscript{2+} has been postulated to trigger endonuclease activation and apoptosis in some systems.\textsuperscript{31,32} In contrast, Ca\textsuperscript{2+} appears to inhibit apoptosis of the progenitor cell line caused by factor withdrawal.\textsuperscript{3} CD34\textsuperscript{+} cells require growth factor(s) for their survival, as supported by the results of a recent study in which IL-3 and c-kit ligand suppressed apoptosis of CD34\textsuperscript{+} cells depending on their differentiation stage.\textsuperscript{33} Collectively, our observations favor the hypothesis that the Mg\textsuperscript{2+}-dependent endonuclease is activated in apoptosis arising from factor deprivation.

There are two possible explanations for why the Mg\textsuperscript{2+}-dependent endonuclease activity is not detected in PMN nuclei. One possibility is that the protein itself is lost, or its amount is markedly diminished. Alternatively, the activity may be inhibited by other nuclear components. Indeed, we have confirmed the presence of inhibitors of the Mg\textsuperscript{2+}-dependent endonuclease in nuclear extracts of PMNs. We are currently attempting to isolate the nuclease and its inhibitor(s), with the hope of validating one or other of the above possibilities.

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