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Detection of myeloperoxidase activity in primary leukemic cells by an enhanced chemiluminescent assay for differentiation between acute lymphoblastic and non-lymphoblastic leukemia

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ABSTRACT

Background: Myeloperoxidase (MPO) plays a crucial role in the differentiation of acute lymphoblastic leukemia (ALL) and acute non-lymphoblastic leukemia (ANLL). In this report, we proposed the application of the enhanced chemiluminescent (ECL) technique to the determination of MPO activity in blasts of acute leukemia (AL).
Methods: Bone-marrow samples were obtained from 23 patients with AL (ALL, 5 cases; ANLL, 13 cases; AUL, 1 cases; mixed-lineage AL, 4 cases). Cells were incubated with a standard reaction mixture and chemiluminescence was measured. The mean peak light emission (PLE) was assessed.
Results: When the cut-off point of PLE was settled at 2483, which was set for the discrimination between ANLL and ALL (mean + 3 × SD of ALL samples, $n = 5$), all cases of ALL were MPO-negative, and ten of the thirteen ANLL patients were MPO-positive, which was concordant with cytochemical staining. In addition, this technique was able to demonstrate MPO activity in 4 mixed-lineage AL cases which did not stain for MPO in cytochemistry preparations.
Conclusions: Our ECL technique is simple, inexpensive, and easier to perform compared to other procedures used to measure MPO activity in AL.

1. Introduction

AL is a heterogeneous hematologic malignant disease. According to the French–American–British (FAB) criteria, AL is classified into ALL and ANLL. The latter must be distinguished from ALL at diagnosis because the therapeutic strategies and prognosis vary considerably between ALL and ANLL. In the FAB classification, MPO is considered to be one of the main markers of myeloid lineage, and plays an important role in the diagnosis and differentiation of ALL and ANLL.

Cytochemical staining, electron microscope cytochemistry, immunocytochemical analysis, flow cytometry, Northern blotting and reverse transcription-polymerase chain reaction have been used to assay the activity or expression of MPO [1–5], but each method has its own shortcomings. The conventional cytochemical method for MPO is observation under light microscopy. But it requires expertise in cell-morphology recognition, and cannot discriminate minimally differentiated AL because the cytochemical reaction for MPO is negative [6]. Immunocytochemical analysis using a monoclonal antibody against MPO is highly specific but requires a highly specific antibody. Electron microscope cytochemistry is the most sensitive method for MPO analysis but requires special instrumentation and is labor-intensive, making it unsuitable for routine clinical use. Chemiluminescence techniques are used in biomedical science and clinical medicine because they: (i) do not produce radioactive waste; (ii) are relatively simple; (iii) require inexpensive instrumentation; and (iv) have ultra-sensitive detection limits [7,8]. Two studies using a chemiluminescence assay have been conducted to determinate MPO activity [6,9]. Fonseca et al. demonstrated the use of the enol ether of isobutyraldehyde to differentiate ALL and ANLL [9], but prompt clinical application as a complementary test was hindered by the lack of a commercial source of this substrate. The same research group subsequently proposed a new highly sensitive ECL technique based on the separated commercially available substrate of p-iodophenol and luminol for the determination of MPO activity in blasts of minimally differentiated ANLL-M0 and ANLL-M7 [6]. However, their ECL technique was complicated and difficult to standardize. To facilitate the chemiluminescent method used for the detection of MPO activity, we employed a

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simplified ECL assay with a commercially available kit. We report a new application with the commercially available kit to detect MPO activity in leukemic cells from AL patients.

2. Materials and methods

2.1. Cell culture

Cells (HL-60, Jurkat, Raji) were provided by the Cell Culture Central of Xiangya Medical School of Central South University (Changsha, Hunan, China). Cells were maintained at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 (HyClone, China) containing 10% newborn bovine calf serum (Sijiqin, China).

2.2. Morphologic analysis

Bone-marrow smears were made from patients and examined under light microscopy. Patients were diagnosed and classified according to FAB criteria [10]. All of the patients provided written consent, and Ethics permission was obtained for the use of all samples.

2.3. MPO cytochemical staining

MPO solution-A (benzidine 0.3 g, 99 ml of 95% ethanol, 1 ml of a saturated solution of nitroferrocyanic sodium) was added on the slide to cover the fresh bone-marrow smear. This was allowed to stand for 2 min at room temperature (RT). An equal volume of MPO solution-B (0.3 ml of 30% H_2O_2, 25 ml of distilled water) was added, incubated for 5 min at RT, followed by washing and re-staining with Wright's staining solution for 15 min. After washing, the slide was observed under light microscopy. MPO activity was considered positive if the percentage of MPO-staining cells was ≥3.

2.4. Immunophenotyping

Immunophenotyping was performed on fresh cell with a panel of monoclonal antibodies by direct immunofluorescence, which included: CD3, CD13, CD14, CD19, CD20, CD33, CD34 and HLA-DR, and analyzed by flow cytometry in a FACSCalibur machine (Becton Dickinson Bioscience). All antibodies were from Becton Dickinson Bioscience (USA). In brief, bone marrow containing about 2×10^5 white blood cells was added to each tube, followed by incubation with 20 µl of monoclonal antibody (CD3, CD13, CD14, CD19, CD20, CD33, CD34 and HLA-DR) for 30 min at RT in a darkened environment. This was followed by the addition of 2 ml of red blood cell lysing solution and incubation at RT for 15 min in the dark. The suspension was removed after centrifugation at 1500 rpm for 5 min, and the pellet washed and resuspended. Blasts were gated based on their CD45 expression and side scatter, and analyzed. Negative controls included a mouse isotype-matched non-relevant immunoglobulin. CD expression was considered positive (+) if the number of CD-expressed cells was 20–40%; it was (++) if it was 40–70%; it was (+++) if it was >70%; and was negative (−) if it was <20%.

Table 1
Patient characteristics.

No	Sex	Age	Blast	CD expression							
			(%)	CD3	CD13	CD14	CD19	CD20	CD33	CD34	HLA-DR
ALL											
1	M	34	73	−	−	−	+++	−	−	+	+++
2	M	53	80	−	−	−	+++	−	−	+++	+++
3	M	1	65	−	−	−	+++	−	−	+++	+++
4	M	8	90	−	−	−	+++	−	−	+++	+++
5	F	26	85	−	−	−	+++	+++	−	+++	+++
ANLL											
6	M	64	91	−	+++	−	−	−	+++	+++	+++
7	M	46	72.4	−	+++	−	−	−	+++	−	+++
8	M	42	62	−	−	−	−	−	+++	+++	+++
9	F	26	84	−	+	ND[a]	−	−	+++	+	+++
10	M	21	58	−	−	−	−	−	+++	+++	+++
11	M	58	67.5	−	++	−	−	−	+++	+++	+++
12	F	7	56.7	−	+++	+	−	−	++	++	+++
13	M	12	82	−	++	−	−	−	+++	+	−
14	F	19	69	−	+++	+	−	−	+++	+	+++
15	M	35	77	−	+++	−	−	−	+++	−	−
16	M	57	88.6	−	+++	+	−	−	+++	++	+++
17	M	10	89.3	−	−	−	−	−	+	+++	+++
18	M	19	80.5	−	++	++	−	−	+++	+	+++
MLL[b]											
19	M	18	84	−	+++	−	++	−	++	+++	+++
20	M	16	60	−	+	+	+	−	+	+++	+++
21	F	77	65	−	+	−	++	ND	+	+++	+++
22	F	9	87	−	+++	−	++	−	+	+++	+++
AUL											
23	M	9	79	−	−	−	−	−	−	++	+++

[a] ND: Not detected.
[b] Number 19: 42% myeloblast + 42% (lymphoblast + prolymphocyte). Number 20: 25% prolymphocyte + 35% (monoblast + promonocyte). Number 21: 45% myeloblast + 20% prolymphocyte. Number 22: 45% myeloblast + 42% prolymphocyte.

Table 2
Comparison of MPO activity analyzed by 2 assays.

Patient no.	ECL	Cytochemical staining	Patient no.	ECL	Cytochemical staining
1	−	−	13	+	+
2	−	−	14	−	−
3	−	−	15	+	+
4	−	−	16	+	+
5	−	−	17	−	−
6	+	+	18	−	−
7	+	+	19	+	−
8	+	+	20	+	−
9	+	+	21	+	−
10	+	+	22	+	−
11	+	+	23	−	−
12	+	+			

−: MPO-negative; +: MPO-positive.

2.5. Isolation of bone marrow mononuclear cells (BMMNCs)

BMMNCs were isolated by density gradient centrifugation. Briefly, 3–4 ml of bone marrow was centrifuged at 2000 rpm for 5 min. After removing plasma, the remaining bone marrow was diluted with an equal volume of phosphate-buffered saline (PBS), and slowly added to a tube containing a half-volume of cell isolation solution (Solarbio, China). BMMNCs were obtained by centrifugation at 2000 rpm for 20 min, washed and counted.

2.6. Determination of MPO activity

MPO activity in leukemic cells was determined by an ECL assay. In brief, 1×10^5 of BMMNCs was placed in a tube and mixed with PBS solution to produce a final volume of 50 µl. Two-hundred microliters of 0.2 mol/L Tris–HCL (pH 8.7) was added. The reaction was allowed to proceed for 30 min at 4 °C followed by incubation with ECL substrate solution (Beyotime Biotech, China). Light emission was immediately measured with a fluorescence spectrophotometer (Jobin Yvon Fluorolog3, France). The cutoff point set for discrimination between ALL and ANLL was defined as the peak light emission (PLE) of mean + 3 × standard deviation (mean + 3 × SD) from ALL samples.

2.7. Specificity analysis

To analyze the specificity of the ECL assay, 1×10^5 of cells (HL-60, Jurkat and Raji) was used to determine MPO activity by the procedure described above.

3. Results

3.1. Sample characteristics

Patient characteristics are shown in Table 1; samples were collected from patients with AL diagnosed at the Xiangya Hospital of Central South University (Changsha, Hunan, China). Most samples were obtained at diagnosis and before initiation of therapy. Multiple forms of AL were observed, including five cases of ALL, 13 cases of ANLL, four patients with mixed-lineage AL, and one patient with acute undifferentiated leukemia (AUL). Mixed-lineage AL was diagnosed if the blasts had the myeloid markers CD13 or CD33 and one of the T- or B-lineage markers [2]; and AUL was defined if immunologic markers disclosed one of the earlier molecules such as HLA-DR, CD34, or CD7, together with the lack of myeloid markers as CD13, CD33, and T- and B-lineage markers such as CD10, CD19, CD22, CD24, CD3, and CD5[2]. Cytochemical staining showed that all five patients with ALL, four patients with mixed-lineage AL, and one patient with AUL were MPO-negative (Table 2). In 13 ANLL patients, 10 cases were MPO-positive, and the remaining three were MPO-negative (Table 2).

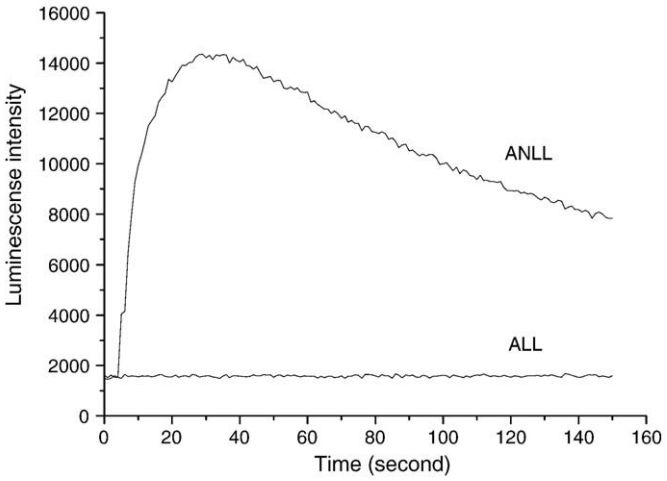

Fig. 1. Light emission following ECL substrate solution addition to 1×10^5/250 μl blast cells from ALL or ANLL.

3.2. MPO activity

Using the ECL assay with a commercially available ECL kit, we initially measured MPO activity in 23 AL patients. Fig. 1 shows the typical light-emission pattern of measurement of MPO activity in primary leukemic cells from one ANLL patient and one ALL patient. The mean PLE for blank control (PBS), 1×10^5 of BMMNCs from ALL or ANLL patients was 1644, 1822 and 13008, respectively. Mean PLE for MPO activity in leukemic cells from ANLL patients was notably higher than that from ALL patients. The cutoff point set for discrimination between ALL and ANLL (calculated according to the formula of PLE of mean $+3\times$SD from ALL samples) was 2432. Based on this cutoff point, all 5 ALL patients, 3 of 13 ANLL patients, and 1 with AUL were MPO-negative; the remaining 10 ANLL patients, and 4 mixed-lineage AL patients were MPO-positive (Table 2). In 13 ANLL cases, 2 patients with a M3 subtype had a highest mean PLE. Furthermore, the specificity of the ECL assay was evaluated. MPO is an enzyme present only in myeloid cells. The results showed that MPO activity in myeloid-lineage cell of HL-60 was positive, but it was negative in T-lymphoid-lineage cell of Jurkat and B-lymphocyte Burkitt's lymphoma cell of Raji. These findings suggest it would be useful for specific detection of active MPO in myeloid-lineage cell.

4. Discussion

Because the therapeutic strategies vary considerably between ALL and ANLL, ALL must be distinguished from ANLL at diagnosis. MPO is considered highly specific for myeloid lineage, and two reports have even described it as being more useful than CD13 or CD33 for the detection of myeloid leukemia [2]. The usefulness of cytometry is unquestionable. However, an enhanced chemiluminescent assay is useful if the nature of leukemic cell cannot be determined exactly by cytometry. In this study, using an ECL assay with a commercially available kit, we initially measured MPO activity in 23 patients with AL. Compared with routine cytochemical staining, MPO activities determined by ECL assay from all 5 ALL patients (100%), thirteen of thirteen ANLL patients (100%) and 1 AUL patient (100%) were consistent. The ECL assay used in this study could therefore be used in the clinic to detect MPO activity in primary leukemic cells for differentiation between ALL and ANLL. Three of 13 patients with ANLL were MPO-negative. One patient (number 14) had 69% immature cells in the bone marrow, expressed CD13 (87.5%), CD14 (38.1%), CD33 (94%), CD34 (40.2%) and HLA-DR (94.6%); patient number 17 had 89.3% blasts, expressed CD33 (25.9%), CD34 (98.8%) and HLA-DR (99.4%); and patient number 18 had 80.5% blasts, expressed CD13 (67%), CD14 (59.6%), CD33 (93.1%), CD34 (25.3%) and HLA-DR (90.9%). Patient number 14 and patient number 18 of the 3 patients had the M4 subtype of ANLL and a higher expression of CD33. Patient number 17 expressed more CD34 antigen, but less CD33 antigen, which could be classified into minimally differentiated ANLL of the M0 subtype according to the diagnostic criteria of CD13- or CD33-, CD34- and HLA-DR-positive leukemia without T- or B-lineage markers [2]. For the two ANLL patients with the M4 subtype, most blast cells in their bone marrow were myeloblasts and monoblasts, which are often MPO-negative. Most M0 subtypes of ANLL patients were MPO-positive according to electron microscopy, but it was reported that about 19% of ANLL patients with the M0 subtype were MPO-negative [2]. Eguchi et al. and Costa et al. demonstrated by ultrastructural studies and ECL studies that MPO activity was negative in one-fifth or two out of seven ANLL-M0 case [2,6]. Patient number 17 was therefore similar with the AUL patient (number 24) who expressed only CD34 antigen (64.5%), and may have very weak MPO activity due to very immature blast cells. In the 4 mixed-lineage AL patients, 3 cases were mixed-AL of myeloid and lymphoid lineage, the other one was of lymphoid and monocytic lineage. All four cases were MPO-positive, but were as negative detected by cytochemical staining. This result may be associated with a difficulty in cell-morphology recognition because mixed-lineage AL often contains at least two different-lineage blasts, whereas MPO cytochemical staining is observed under light microscopy based on knowledge of cell morphology. The morphology of immature cells (particularly mixed-lineage AL) is very heterogeneous, making it difficult to distinguish. The ECL method described in this study does not require expertise in cell-morphology recognition; it is rapid, simple, objective, specific, and can be done with inexpensive commercial chemicals. Together with ultra-sensitive detection limits, using our ECL assay to detect MPO activity is superior to light microscopy.

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