Deficient Histone Acetylation in Acute Leukemia and the Correction by an Isothiocyanate

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Key Words

Acetylation • Acute leukemia • Histone • Histone deacetylases • Leukemia • Pathological markers

Abstract

Objective: Since histone hypoacetylation due to excess histone deacetylases (HDACs) has been associated with transcriptional repression in leukemia, we aimed to determine deficient histone acetylation in patients with acute leukemia and the effect of its correction by an isothiocyanate. **Methods:** The acetylation status of histones H3 and H4 in cells from patients with untreated acute leukemia was determined by Western blot. Deficient histone acetylation was analyzed in relation to the disease state. Bone marrow cells from 10 patients with acute myeloid leukemia (AML) were cultured in phenylhexyl isothiocyanate (PHI) to evaluate correction of the deficiency. **Results:** Acetylation of histones H3 and H4 was virtually undetectable or significantly lower in acute leukemia. This deficiency was consistent among all the patients examined. Histone acetylation was up-regulated in the presence of PHI, revealing an excess of deacetylation activity in AML. PHI treatment induced apoptosis, indicating HDAC inhibition was able to correct the deficiency. **Conclu-**

sions: Deficient histone acetylation may represent an aberration at the epigenetic level in acute leukemia. PHI might represent a target for correcting deficient acetylation, and potential epigenetic regulators for preventing the progression of leukemia.

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Introduction

The N-terminal tails of core histones are accessible on the surface of nucleosomes, subjecting them to a variety of interactions with regulatory proteins and enzymes. Among the many post-translational modifications of histones is histone acetylation, which plays a key role in the regulation of transcription by modulating chromatin structure [1–4]. Acetylated lysines of histones provide interaction with specific effector modules such as bromodomain complexes which may mediate transcriptional regulation [5]. The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila* [6]. Acetylation may also neutralize the charge interactions between histones and DNA, which allows for chromatin remodeling and unfolding [7]. In

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Table 1. Clinical features of leukemia patients enrolled in the study

Patient	Age, years	Sex	Sample	Diagnosis	Blast, %	Treatment	Response	Culture with PHI
P1	50	M	BM	AML	93	ATRA	CR	no
P2	41	M	BM	AML	69	BMT	CR	no
P3	10	F	BM	ALL	75	–	n/a	no
P4	11	F	BM	AML	86.5	ATRA	CR	no
P5	26	F	BM	AML	91.5	MA	PR	no
P6	9	M	BM	ALL	89	–	n/a	no
P7	16	F	BM	ALL	55.5	VMP	CR	no
P8	13	M	BM	AML	82.5	–	n/a	no
P9	12	M	BM	ALL	90	–	n/a	no
P10	3	M	BM	ALL	74	–	n/a	no
P11	2/12	F	BM	ALL	86.5	–	n/a	no
P12	48	M	BM	AML	92.5	–	n/a	no
P13	19	F	BM	AML	74	–	n/a	no
P14	62	F	BM	AML	56.5	–	n/a	no
P15	2/12	M	BM	ALL	87	–	n/a	no
P16	39	M	BM	AML	66.5	–	n/a	yes
P17	11	M	BM	AML	77.5	ATRA	CR	yes
P18	12	F	BM	AML	78.5	–	n/a	yes
P19	48	M	BM	AML	88.5	DA	CR	yes
P20	38	M	BM	AML	69	DA	PR	yes
P21	43	M	BM	AML	93	MA	CR	yes
P22	55	F	BM	AML	88	MA	CR	yes
P23	69	M	BM	AML	90	DA	PR	yes
P24	10	M	BM	AML	65	–	n/a	yes
P25	26	F	BM	AML	91.5	DA	PR	yes

M = Male; BM = bone marrow; ATRA = all-trans-retinoic acid; CR = complete remission; BMT = bone marrow transplantation; F = female; n/a = non-applicable; MA = mitoxantrone-cytarabine; VMP = velcade-melphalan-prednisone; DA = daunorubicin-cytarabine; PR = partial remission.

general, histone acetylation provides transcriptional regulators greater access to genes and is associated with transcriptional activation. On the other hand, histone deacetylation is associated with transcriptional repression. The enzymes regulating the level of histone acetylation, i.e. the acetyltransferases, and the level of histone deacetylation, i.e. the histone deacetylases (HDACs), are important in the regulation of transcription, cell growth and differentiation [8, 9]. Aberrant activities of these enzymes such as excessive recruitment of HDACs, or mutations in acetyltransferases, have been implicated in the carcinogenesis of leukemia and a number of other cancers [8–10]. Inhibitors of HDACs have been considered to be promising drugs for leukemia treatment.

The nature of the defect in acute leukemias, with regard to the status and level of histone acetylation, has not previously been clearly presented. There are a limited number of reports in this area, especially about cells from

patients with acute leukemia. We have focused our analysis on histones H3 and H4, since the majority of acetylation occurs on these histones [11]. Additionally, our previous analyses with leukemia cell lines have provided indications of potential defects in acetylation of histones H3 and H4 [12, 13]. Furthermore, we experimented with phenylhexyl isothiocyanate (PHI) to enhance and correct the deficiency in histone acetylation. PHI is known to be a potent chemopreventive agent and was the first isothiocyanate demonstrated to inhibit a spontaneous leukemia in mice [14]. PHI was the first isothiocyanate, as compared with others, shown to be an inhibitor of HDACs in leukemia cells and displayed significant effects in modifying histone acetylation [12]. As a result, we have found that PHI mediates growth arrest and apoptosis in leukemic T cells [13] and myeloid cells in cultures [12], as well as in xenografts [14]. PHI-mediated apoptosis was more selective for replicating tumor cells than for non-replicat-

ing normal cells [14], indicating that it warrants to be further investigated as a potential chemopreventive and therapeutic agent. Other isothiocyanates, including sulforaphane and phenethyl isothiocyanates, have demonstrated the ability to induce growth arrest and apoptosis in cancers of multiple organs. Together, they indicate that the cruciferous vegetables, such as broccoli, cabbages, watercress, Brussel's sprouts and others, which contain natural isothiocyanates, could be a commonly accessible source for chemoprevention. The major mechanisms of the isothiocyanate-mediated anti-cancer effects include cytoprotection to protect cells from the damage caused by carcinogens [15, 16], and the induction of growth arrest and apoptosis in tumor cells [17–19]. In this report, we demonstrated that in contrast to normal cells, a deficiency in global histone acetylation is present in acute leukemias of both myeloid and lymphoid origins. Additionally, we have described that exposure to PHI has significantly enhanced the level of histone acetylation and apoptosis induction in leukemia cells.

Materials and Methods

Study Subjects

Approval of the clinical study for using discarded bone marrows and peripheral blood specimens after diagnosis was obtained from the human committee of Zhangzhou Affiliated Hospital of Fujian Medical University, China. Each individual provided informed consent prior to participation in the study. This study included 25 in-patients with primary untreated acute leukemia, classified by the French-American-British Cooperative Group with morphology, immunology, cytogenetics and molecular biology criteria. Twenty-five patients were enrolled, 7 with acute lymphoid leukemia and 18 with acute myelogenous leukemia (AML) (table 1). The median age was 16 years (range 2.5–69). Individuals without leukemia, including 1 patient with anemia, 1 with infectious disease, 2 with idiopathic thrombocytopenic purpura, as well as 1 healthy volunteer, were enrolled in the study (table 1).

Evaluation of Histone Acetylation

Mononuclear cells from leukemia patients were isolated by Ficoll-Hypaque density gradient centrifugation [14], and the preparations containing 90% or more myeloid blasts by morphology were used in the study. Bone marrow mononuclear cells from 4 non-leukemia patients and the peripheral blood mononuclear cells from a healthy donor were similarly isolated. Total proteins of the mononuclear cells were prepared with a Pierce lysis buffer (Pierce, Rockford, Ill., USA). Western blotting was performed according to previously described methods [12]. Each lysate was used at 15 µg or more, and immunoblotting was performed with specific antibodies against acetylated histone H3 or H4 (Upstate Biotechnology, Lake Placid, N.Y., USA). An antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used as a loading control.

Cell Cultures and Apoptosis

PHI, >98% pure, was purchased from LKT Lab (St. Paul, Minn., USA). Bone marrow mononuclear cells were isolated from 10 patients with AML (P16–P25; table 1) by Ficoll-Hypaque centrifugation. Preparations containing >90% blasts were seeded at 5×10^5 cells/ml of RPMI-1640 medium with 15% heat-inactivated fetal calf serum, 100 IU/ml of penicillin and streptomycin, and maintained at 37°C in a 5% CO₂ atmosphere. Some of these cultures were exposed to various concentrations of PHI, using a stock solution in 75% methanol [12]. Cultures supplemented with the methanol medium were used as a vehicle control. Apoptosis was evaluated by the annexin V staining procedure (BD Biosciences, San Jose, Calif., USA), and the apoptotic cell proportion among the total cells (apoptosis rate) was quantified with a FAC-Scan flow cytometer according to the manufacturer's instructions. Statistical analysis was performed with a software SPSS 11.5, and $p < 0.05$ was considered to be statistically significant.

Results

Deficient Histone Acetylation in Acute Leukemia

The status of acetylation of histone H3 or H4 of mononuclear cells from patients with acute leukemia, and from individuals without leukemia, was compared. Fifteen patients with acute leukemia of myeloid or lymphoid lineages were enrolled in the study (P1–P15; table 1). Their bone marrow mononuclear cell preparations isolated by Ficoll-Hypaque density gradient centrifugation that had >90% blasts were examined for the expression of acetylated histones H3 and H4, with Western blotting. Figure 1a shows the expression level of acetylated histones from 6 leukemia patients (P1–P6) and the bone marrow mononuclear cell preparations from 4 non-leukemia individuals that included 2 patients with idiopathic thrombocytopenic purpura, 1 with anemia and 1 with infectious disease. The expression of acetylated histones H3 and H4 was either absent or barely visible in all the leukemia specimens, and was significantly lower than the expression of the non-leukemic cell preparations. Bone marrow mononuclear cells from another 9 patients with acute leukemia (P7–P15) were also compared with that of a healthy donor (fig. 1b). As demonstrated, all 9 patients had a significantly lower expression of acetylated histones H3 and H4 than that of the healthy donor. Among them, 2 preparations from patients P9 and P11 had no visible acetylated histone H4. These results clearly demonstrated that the cells from the acute leukemia patients of all age groups (from age 2.5 to 69) that were examined were deficient in acetylation of histones H3 and H4.

Fig. 1. Deficient histone acetylation of the cell preparations from patients with acute leukemias as compared with the mononuclear cells from individuals without leukemia. **a** Western blots show the level of acetylated histone H3 or H4 of bone marrow mononuclear cells isolated from 6 acute leukemia cases (P1–P6) and the bone marrow mononuclear cell preparations from 4 individuals without leukemia (N1–N4). N1, an individual with anemia; N2, a person with infectious disease; N3 and N4 had idiopathic thrombocytopenic purpura. **b** Status of histone acetylation of cells from patients with acute leukemias (P7–P15) and the mononuclear cells from a healthy donor N5. Antibody against β -actin was used as a loading control.

Enhancing Histone Acetylation by PHI

Previously, we demonstrated that exposure of leukemic cell lines to PHI, an inhibitor of the activity and expression of HDACs, enhanced acetylation of histones H3 and H4 [12, 13]. The next experiments were performed to examine whether PHI could alter the status of acetylation of core histones of the cells from AML. The bone marrow mononuclear cells that had >90% blasts from 10 patients (P16–P25; table 1) were isolated and cultured without or with PHI at various concentrations. The status of histone acetylation was evaluated during a short culture period of 3–7 h. In the presence of PHI, the accumulation of acetylated histone H3 or H4 was significantly enhanced with all 10 patients, as compared with control cultures without PHI that had barely visible levels. Figure 2a illustrates the typical results obtained from 3 such cases (P17, P20 and P22). Significant enhancement of acetylation of both histones H3 and H4 was observed with PHI used at $\geq 10 \mu\text{M}$. The enhancement was also time related, with a larger magnitude of increase seen after 7 h of exposure, as compared with that after 3 h (fig. 2a).

Apoptosis Induction by PHI

After exposure to PHI, the presence of apoptotic cells among the patient cells was determined by the annexin V staining. Figure 2b depicts the experimental results with 4 cases (P16, P17, P20 and P22). Significant concentration- and time-related increase in apoptotic cells is statistically significant as compared with the control cells without PHI ($p < 0.05$). PHI used at $10 \mu\text{M}$ was found to be effective in inducing apoptosis after 3 h. For example, patient P16 shows that the apoptotic cells, 21% after 3 h with $10 \mu\text{M}$ PHI, was increased to approximately 42% after 7 h.

Discussion

In this study, evidence is presented that histone acetylation is significantly deficient in the patient cells from acute leukemia patients, as compared with cells from individuals without leukemia. This deficiency was revealed in all the acute leukemia cases investigated in all age groups from 2.5 to 69 years, including both myeloid and lymphoid lineages. The levels of acetylated H3 and H4 were examined in the patients with or without complete

Fig. 2. Enhancement of acetylation of histones H3 and H4 and apoptosis induction in the mononuclear cells from AML patients by exposure to PHI. **a** The bone marrow mononuclear cell preparations from 3 AML patients (P17, P20 and P22) were isolated and cultured without or with the exposure to PHI. Western blot figures show enhanced histone acetylation in the presence of PHI. The upper part of the blots is the status of acetylation of histone H3, and the lower part is the status of histone H4. Antibody against β -actin was used as a loading control. **b** Graphs show the increase in apoptotic cells among the cultured cell preparations from patients P17, P20 and P22 after PHI exposure. The left graph denotes the apoptosis rate after 3 h exposure to PHI in culture and the right graph the apoptosis rate after 7 h.

remission response. The patient cell preparations used for the analyses were enriched with primary acute myelogenous blasts, while the normal cells were similarly prepared from the bone marrows of individuals without leukemia, indicating that the comparison of these cell types was appropriate. Our findings have revealed, for the first time, a deficiency in histone acetylation as an abnormality at the epigenetic level in acute leukemias. Since most acetylation occurs on histone H3 [11], our findings represent the majority of the histone acetylation in acute leukemias. Our findings present an aberration in histone acetylation. This aberration in the structure of chromatin and nucleosomes could potentially be a new diagnostic and pathological marker of the disease.

A deficiency in histone acetylation in acute leukemia would imply excessive deacetylation, which could be due to an excessive deacetylase activity, a deficient acetyltransferase activity, or a combination of the two. Here, we demonstrated that deficient histone acetylation in AML

could be consistently upregulated with PHI, which is a known HDAC inhibitor [12], thus supporting the presence of an excessive deacetylation in AML. This interpretation is in line with reports that describe excessive recruitment of HDACs in leukemia [8, 9]. PHI was previously shown to augment the level of P300, one of the acetyltransferases, in addition to inhibiting HDACs, in leukemic HL-60 promyelocytes [12]. It is most likely that the enhancement of histone acetylation in the patient cell preparations was achieved by this effect to downregulate deacetylation. The experimental results showed that the acetylation of histones H3 and H4 was accumulated along with a significant development of apoptosis. Apoptosis induction by PHI has been described with leukemic cell lines [12, 13]. The apoptosis was shown to be mediated by the intrinsic pathway, similar to that induced by HDAC inhibitor drugs like suberoylanilide hydroxamic acid, oxamflatin and depsipeptide [20–22].

Histone hypoacetylation is associated with heterochromatin and transcriptional repression. HDACs have been suggested to be targets of myeloid leukemia treatment since HDACs are present in the complexes of transcriptional coregulators such as mSin3A and N-CoR, which are required for repressing target genes [23, 24]. The use of HDAC inhibitors could de-repress the genes that are silenced due to hypoacetylation. Reactivation of the cell cycle regulator p21 in leukemia cells by HDAC inhibitor drugs [25], and also by PHI in our experiments [12], are examples. The leukemia cells are thus restored with their previously lost biological functions.

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