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SENSITIVITY OF NORMAL AND MALIGNANT HUMAN LYMPHOCYTES TO 5-AMINOLEVULINIC ACID-MEDIATED PHOTODYNAMIC DAMAGE

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Aim: **To compare the sensitivity of normal and malignant human lymphocytes to 5-aminolevulinic acid (ALA) — mediated photodynamic damage.** ***Methods:*** **Blood lymphocytes isolated by Ficoll-sodium metrizoate density gradient from healthy donors (6) and hematologic patients (20) with different forms of lympholeukemia, and also transformed lymphocytes of human B-cell (Raji, Namalwa) and T-cell (MT-4, HUT-78) lines were inestigated. Diagnoses of chronic lymphoproliferative disorders were made on the grounds of morphological, cytochemical and immunocytochemical studies of peripheral blood and bone marrow cells, with immunophenotype determination by monoclonal antibodies to differentiation antigens of T, B lymphocytes and NK cells and immunocytochemical ABC-AP method. Cells of leukemic B- and T-cell lines were cultured in standard RPMI-1640 medium. For photodynamic treatment, the cells were incubated with ALA and then irradiated by a helium-neon laser (wavelength of 633 nm). The number of dead cells was determined in 20 h with trypan blue dye exclusion test.** ***Results:*** **The striking difference in responsiveness to ALA-mediated photodynamic treatment (ALA-PDT) between normal lymphocytes and cells isolated from lymphatic leukemia patients was established. A bulk of leukemic cells (mean for 10 patients with B-CLL — 62.06 ± 4.03%) were destroyed under the lowest ALA-PDT doses tested: 1 mM ALA, irradiation dose of 25 J/cm^2. However, it was virtually impossible to attain any appreciable damage of lymphocytes from healthy donors even with the highest treatment doses (5 mM ALA, 150 J/cm^2). High sensitivity to ALA-PDT of malignant lymphocytes was confirmed in experiments with human T- and B-cell leukemic cell lines, and in these experiments, an anomalous reaction to the treatment of Raji cells was also detected. The mechanisms of the difference between normal and malignant lymphocytes are discussed in terms of altered heme-synthesis processes in malignant cells.** ***Conclusions:*** **1) It is shown for the first time that blood lymphocytes from lymphatic leukemia patients are highly sensitive to the damage with ALA-PDT while lymphocytes of normal donors are practically not damaged. 2) Transformed lymphocytes of human T-cell lines are more sensitive than lymphocytes of B-cell lines. 3) Lymphocytes of the Raji line display anomalous dose-effect dependence with ALA-PDT. 4) It is proposed to evaluate the drastic difference in ALA-PDT responsiveness of normal and malignant lymphocytes as a possible simple and low-traumatic test for B-CLL screening among the elderly people.**

Key Words: **blood lymphocytes, donors, leukemic patients, T- and B-cell lines, photodynamic treatment, 5-aminolevulinic acid, B-CLL screening.**

Among the photosensitizing substances, utilized for photodynamic therapy of tumors (PDT), 5-aminolevulinic acid (ALA) is the only one that is not a photosensitizer proper but a metabolic precursor of an active product protoporphyrin IX (Pp IX) in which it converts upon introduction into organism [1, 2]. Therefore, levels of the Pp IX accumulation in different tissues may vary depending on tissue metabolism. It was shown that in tumors the Pp IX formation from exogenic ALA was more intensive than in normal tissues [3, 4] presumably because malignant cells have higher activity of rate-limiting enzyme porphobilinogen deaminase [5] and lower activity of another enzyme ferrochelatase [6] controlling under normal conditions the Pp IX overproduction by its transformation to heme [7].

Taking into account that photodynamic elimination of malignant lymphocytes may potentially have some important practical applications (e. g., graft purging from residual leukemic cells in the course of autologous bone marrow transplantation; PDT of cutaneous T-cell lymphomas), we set the task of studying antileukemic efficiency of ALA-mediated PDT using as model targets both human leukemia cell lines and neoplastic lymphocytes isolated from patients with different forms of leukemia. The results obtained demonstrate pronounced difference in resistance to ALA-PDT of lymphocytes from leukemic patients compared to the cells from normal subjects suggesting the possibility to employ this difference as an additional marker of lymphoid malignancy.

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Abbreviations used: ALA – 5-aminolevulinic acid; B-CLL – B-cell chronic lymphocytic leukemia; PDT – photodynamic therapy; Pp IX – protoporphyrin IX; T-LGLL – large granular lymphocytic leukemia, T-cell variant.

MATERIALS AND METHODS

Mononuclear cells, hereafter referred to as lymphocytes, were isolated from freshly drawn heparinized peripheral blood of 20 haematologic patients, which were treated in different Kiev clinics, and 6 healthy donors (3 men and 3 women, median age 31 years, range 22–40) by a density-gradient technique (Ficoll-sodium metrizoate d = 1.077 g/cm^3) [8]. Preliminary clinical diagnoses of chronic lymphoproliferative disorders were verified by authors on the grounds of morphological, cytochemical and immunocytochemical investigations of peripheral blood and bone marrow cells [9]. Immunophenotype determination was carried out by means of a panel of monoclonal antibodies (DakoCytomation, Denmark) to differentiation antigens of T lymphocytes (CD2, CD3, CD4, CD5, CD7, CD8), B lymphocytes (CD19, CD20, CD23, CD43), and NK cells (CD16, CD57), and by immunocytochemical ABC-AP method [9]. From each patient an informed consent to participate in the investigation was obtained.

B-cell lines Raji and Namalwa (human Burkitt lymphoma) [10, 11] and T-cell lines MT-4 (human T-cell leukemia) [12] and HUT-78 (human cutaneous T-cell lymphoma) [13] were obtained from culture bank of R.E. Kavetsky Institute for Experimental Pathology, Oncology and Radiobiology and were cultured in standard RPMI-1640 medium supplied with 10% fetal bovine serum. For photodynamic treatment, cell suspensions in Hanks' balanced salt solution (pH = 7.2) were prepared from cultures of leukemic cell lines in log phase of growth or lymphocytes freshly isolated from heparinized blood. Solutions of ALA (Synbias, Ukraine) were prepared immediately before experiments in the buffered salt solution, pH = 7.2. A cell suspension ($2 \cdot 10^6$/ml) was incubated for 4 h with 1.0–5.0 mM ALA under 37 °C and then irradiated by helium-neon laser (wavelength of 633 nm). Irradiation doses varied from 10 J/cm^2 to 150 J/cm^2 depending on cell resistance. After the light treatment, cells were incubated (37 °C) in RPMI-1640 medium with fetal serum for additional 20 h for photodynamic alterations to fully develop. The number of dead cells was determined by trypan blue dye exclusion test.

RESULTS

Lymphocytes isolated from healthy donors turned out quite resistant to the photodynamic treatment with ALA. As it is shown in Table 1, application of the entire range of tested doses up to 150 J/cm^2, that was 6 times more than the dose served usually as an effective standard in experiments with malignant lymphocytes (25 J/cm^2), could not induce any significant death of treated cells. Equally unsuccessful were attempts to boost photodynamic alteration of the cells raising concentration of ALA from 1 mM (used for evaluation of lymphocytes killing in studies with leukemia patients) to 2–5 mM.

On the contrary, lymphocytes obtained from patients with lymphatic leukemia, yielded in general easily to photodynamic damage (Table 2). In 9 from 16 such patients (№ 1–7, 10, 13), more than 50% of cells died under chosen standard regimen of PDT treatment (1 mM ALA, 25 J/cm^2). Most (7) of these 9 leukemia cases were diagnosed by cell cytomorphology and immunophenotype as B-cell chronic lymphocytic leukemia (B-CLL). Among other 7 cases, in 3 patients with B-CLL (№ 8, 9, 12) the number of dead cells also approached 50% (48, 45, 41%). And only in remainder 4 patients (№ 11 — hairy cell leukemia and № 14–16 — T-cell large granular lymphocyte leukemia) the number of dead cells was low: 24.4; 3.7; 4% and 16%, respectively.

For the sake of comparison, in the Table 2 three cases (№ 18–20) are also given in which a preliminary clinical diagnosis of leukemia was not confirmed by laboratory investigations, and the cell response to photodynamic treatment proved to be accordingly insignificant: 5.7; 6.6% and 17.2% of dead cells, respectively.

In parallels with lymphocytes isolated from blood of leukemia patients, resistance to ALA-PDT of leukemic B-cell lines (Raji and Namalwa) and T-cell lines (MT-4 and HUT-78) was studied . Photodynamic vulnerability of these cultured neoplastic lymphocytes was also found to be by far exceeding such of normal blood lymphocytes (see Table 3). And as it is seen from the Table 3, among 4 cultures tested, Raji stood out for its comparatively high resistance to ALA-PDT while HUT-78 seemed to be the most responsive.

Table 1. ALA-PDT action on lymphocytes of healthy donors

Donors	ALA concentration (mM)	Laser radiation doses (J/cm^2)	Number of dead cells (%)
№ 1	1.0	10.0	1.0
	1.0	18.0	1.0
	1.0	25.0	1.0
№ 2	2.0	10.0	0.0
	2.0	18.0	0.2
	2.0	25.0	0.5
№ 3	3.0	10.0	1.0
	3.0	18.0	1.0
	3.0	25.0	1.0
№ 4	4.0	10.0	0.0
	4.0	18.0	0.0
	4.0	25.0	0.1
№ 5	2.0	50.0	0.5
№ 6	5.0	100.0	1.5
	5.0	150.0	1.5
Control (untreated cells)			0.5

DISCUSSION

In addition to selective low-invasive destruction of tumors, PDT may be instrumental in elimination of malignant lymphoid cells, as it is illustrated by bone marrow transplants purging from residual leukemic cells with merocyanine 540 assisted PDT [14], a procedure proposed to improve efficiency of leukemia patients' therapy. In that case, selectivity of malignant lymphocytes killing, as opposed to normal lymphocytes and bone marrow progenitors, acquires particular importance.

The data presented here show that there is a striking difference in responsiveness to ALA mediated PDT of blood lymphocytes obtained from healthy subjects and leukemia patients. Whereas in most cases, a bulk of leukemic cells were quite easily destroyed by ALA-PDT without necessity to resort to higher photosensitizer concentrations or light radiation doses (see Table 2, № 1–10, 12, 13), it was virtually impossible to attain any appreciable damage of normal lymphocytes even with the largest ALA-PDT doses tested. The contrasting reactions observed may be explained by peculiarities of heme metabolism in malignant cells. Heme synthesis from glycine and succinyl CoA under normal conditions is a subject of the negative feedback regulation [7]: an increase in the heme formation inhibits activity of a rate limiting enzyme aminolevulinat- synthase and decreases ALA concentration. If exogenic ALA is introduced in organism the limiting link is passed round, and the process proceeds down the chain to synthesis of Pp IX. In normal lymphocytes, the Pp IX by means of ferrochelatase joins Fe^{2+} being transformed to heme that unlike PpIX has no photosensitizing properties. But in malignant cells, ferrochelatase activity is low [6]. What is more, in the cells, intracellular iron stores are spent for DNA and cytochrome synthesis [15]. As

Table 2. ALA-PDT action on lymphocytes of patients with hematological diseases

Patients	Diagnoses	Phenotype (positive cells, %)												Number of dead cells (%)
		CD19	CD20	CD23	CD43	CD2	CD3	CD4	CD5	CD7	CD8	CD16	CD57	
1	B-cell chronic lymphatic leukemia (B-CLL)	80	85	65			7	7	55	7	7			73.5 ± 2.36
2	B-CLL	90	90	90			5	Single cells	95	Single cells	Single cells			61.8 ± 5.48
3	B-CLL	80	80	70			12	7	90	7	7			53.0 ± 2.1
4	B-CLL/non-Hodgkin`s lymphoma	50	50	54	80		37		82					60.3 ± 7.74
5	B-CLL	90	90	80			5	Single cells	90	Single cells	Single cells			85.4 ± 4.09
6	B-CLL	75	70	60	80		15							67.2 ± 4.42
7	B-CLL	85	75	65			20		50					72.8 ± 4.66
8	B-CLL	70	64	50			24		57					48.0 ± 2.03
9	B-CLL	70	75	26			24	13	52	12	12			45.0 ± 2.16
10	Non-Hodgkin`s lymphoma (early leukemization phase)	65	70				30	18		15	15			53.6 ± 2.4
11	Hairy cell leukemia	50	53	-/+			43	43	42	43	43			24.4 ± 2.68
12	B-CLL	55	60	50			37	20	55	18	17			41.0 ± 2.7
13	T-cell chronic lymphatic leukemia				95		90	88	80		0			90.0 ± 3.5
14	Large granular lymphocytic leukemia, T-cell variant (T-LGLL)				99		90				90	68	36	3.7 ± 1.03
15	T-LGLL					85	90	90	90	25	10	8		4.0 ± 0.5
16	T-LGLL					93	95	10	25		77	63	79	16.0 ± 1.76
17	Langerhan`s cell histiocytosis	11	11				73	30		30	34			20.5 ± 1.84
18	Polyclonal B-lymphocyte proliferation	33	28				60							5.7 ± 1.2
19	Polyclonal B-lymphocyte proliferation	21	17	84			78							6.6 ± 0.24
20	Polyclonal B-lymphocyte proliferation													17.2 ± 1.96

a result, in malignant cells Pp IX is accumulated giving rise to the cell photosensitivity.

It is of note that most of the cases analyzed here, that showed considerable lymphocyte responsiveness to ALA-PDT, were constituted by B-cell leukemia patients, including 10 patients with B-CLL. Other four cases were a patient with T-cell chronic lymphatic leukemia (see Table 2, № 13) and three patients with T-cell large granular lymphocyte leukemia (see Table 2, № 14–16). Curiously enough, these four cases occupied extreme (but opposite) positions on the general (for studied leukemia patients) scale of ALA-PDT lymphocyte responsiveness: the first one with the largest portion of cells killed (90%) and the other three — with the smallest (3.7; 4; 16%). It remains to wait until more patients with these leukemia types are analysed to decide whether such reactivity levels are typical of them. As to B-CLL patients, it seems that more than 40% killing of lymphocytes (mean for 10 patients is 62,06% ± 4,03%) under standard conditions chosen is quite characteristic. Given relatively high incidence of B-CLL among the elderly population [16], drastic distinctions in lymphocytes ALA-PDT responsiveness between the patients and healthy people should be evaluated as a possible simple and low-traumatic test for B-CLL screening, especially if it is established that there is no essential overlap in the cell reactivity with reactive lymphocytosis (the work in progress). In this connection, three cases with negative diagnoses, deliberately included in Table 2 (№ 18–20), are demonstrative. As it happened, ALA-PDT responsiveness tests with the patients were carried out before immunocytological investigation of the material (responsibility of another laboratory) was accomplished, and strong doubts were cast on the preliminary leukemia diagnosis forestalling a formal diagnostic conclusion.

It was interesting to find out whether leukemic lymphocytes, cultured as established cell lines, retain their hightened ALA-PDT responsiveness in comparison to normal cells. The data obtained with two B-cell lines (Namalwa and Raji) and two T-cell lines (MT-4 and HUT-78) witness that it is the case (Table 3) and corroborate the findings of other authors [17–23], though all of them worked with some other leukemic cell lines. From Table 3 it also appears that among four cell lines studied, the Raji is distinctly distinguished by its relatively low responsiveness, surprisingly independent of ALA-PDT doses applied. Considering the leading role apoptotic mechanisms play in photodynamic damage of tumors [24],the anomalous reaction of Raji cells is presumably connected with altered apoptosis pathways discovered in the cells [25]. Taken as a whole, two T-cell lines tested are more ALA-PDT responsive than B cell lines (could that be correlated to extremely high cell responsiveness of the only T-cell leukemia patient analysed (see Table 2, № 13)?).

To the best of our knowledge, there are no published data on the ALA-PDT responsiveness of lymphocytes from patients with lymphatic leukemia. In a paper [26], preferential ALA-PDT killing of malignant lymphocytes from a patient with cutaneous T-cell lymphoma compared to normal peripheral blood lymphocytes was described. Hrkal`s group reported [27] that in 8 out of 10 patients with acute myeloid leukemia, ALA-PDT treatment of mononuclear cell preparations resulted in substantial reduction of blast cells number whereas the viability of normal lymphocytes was little affected.

Table 3. ALA-PDT action on leukemia cell lines

Cell lines	ALA concentration (mM)	Laser radiation doses (J/cm^2)	Number of dead cells (%)
B-cell lines			
Raji	1.0	25	18.25 ± 1.7
	2.5	50	15.3 ± 2.3
	4.0	100	17.0 ± 2.0
Namalwa	1.0	25	36.0 ± 5.7
	2.5	50	63.5 ± 2.5
	3.0	75	97.6 ± 3.1
T-cell lines			
MT-4	1.0	25	54.8 ± 3.4
	2.5	50	96.3 ± 4.8
HUT-78	1.0	10	74.3 ± 2.3
	1.0	25	95.7 ± 0.8

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ЧУВСТВИТЕЛЬНОСТЬ НОРМАЛЬНЫХ И МАЛИГНИЗИРОВАННЫХ ЛИМФОЦИТОВ ЧЕЛОВЕКА К ФОТОДИНАМИЧЕСКОМУ ВОЗДЕЙСТВИЮ, ОПОСРЕДОВАННОМУ 5-АМИНОЛЕВУЛИНОВОЙ КИСЛОТОЙ

Цель: изучить чувствительность к фотодинамическому воздействию, опосредованному 5-аминолевулиновой кислотой (АЛК), нормальных и малигнизированных лимфоцитов человека. _Методы:_ объектом исследования служили лимфоциты, выделенные с помощью градиента плотности фиколл-верографина из крови здоровых доноров (6) и гематологических больных (20) с различными формами лимфолейкозов, а также трансформированные лимфоциты В-клеточных (Raji, Namalwa) и Т-клеточных (МТ-4, HUT-78) линий человека. Диагноз хронического лимфопролиферативного заболевания ставили на основании морфологического, цитохимического и иммуноцитохимического исследования периферической крови и клеток костного мозга; иммунофенотипирование проводили с использованием панели моноклональных антител к дифференцировочным антигенам Т-, В-лимфоцитов, NK-клеток и АВС-АР-метода. Трансформированные лимфоциты клеточных линий культивировали в стандартной RPMI-1640 среде с 10% эмбриональной телячьей сывороткой. Для фотодинамического воздействия клетки инкубировали с 1,0–5,0 мМ АЛК в течение 4 ч и облучали гелий-неоновым лазером (длина волны – 633 нм), варьируя дозу от 10 до 150 Дж/см^2 . Количество погибших клеток после дополнительной 20-часовой инкубации определяли с помощью теста с трипановым синим. _Результаты:_ показано, что нормальные и малигнизированные лимфоциты человека резко отличаются по чувствительности к АЛК-опосредованному фотодинамическому действию (АЛК-ФД). Значительная часть лимфоцитов крови больных лейкозом (в среднем 62,06 ± 4,03% у 10 пациентов с В-клеточным хроническим лимфолейкозом, В-ХЛЛ) погибала при минимальных параметрах воздействия (1 мМ АЛК, доза облучения 25 Дж/см^2), тогда как даже наивысшая доза (5 мМ АЛК, 150 Дж/см^2) не влияла на жизнеспособность нормальных лимфоцитов. В опытах _in vitro_ в Т-клеточных линиях отмечали больший процент гибели клеток, чем в В-клеточных. При этом линия Raji отличается парадоксальным отсутствием зависимости количества погибших клеток от дозы воздействия, что, по-видимому, связано с известным для этой линии дефектом сигнальных путей апоптоза. Обсуждаются вероятные механизмы установленных отличий между нормальными и малигнизированными лимфоцитами в контексте альтерации процессов синтеза клетками гема при малигнизации. _Выводы:_ 1) впервые показано, что малигнизированные лимфоциты крови больных лимфолейкозом проявляют высокую чувствительность к АЛК-ФД при фактическом отсутствии такой чувствительности у лимфоцитов здоровых доноров; 2) озлокачествленные лимфоциты Т-клеточных линий более чувствительны к АЛК-ФД по сравнению с В-клеточными; 3) для клеток линии Raji установлена аномальная зависимость доза/эффект; 4) на основе полученных результатов предложен простой и малотравматичный тест для скрининга пожилых людей на наличие В-ХЛЛ.

Ключевые слова: лимфоциты крови, здоровые доноры, больные лейкозом, клеточные линии, фотодинамическое воздействие, 5-аминолевулиновая кислота, скрининг В-ХЛЛ.

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