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Blood cells in thyroid cancer patients: a possible influence of apoptosis

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Abstract: The side effects of radioactive iodine (131-I) treatment of differentiated thyroid cancer (DTC) patients include reduction of peripheral blood cell counts. The aim of this study was to analyze some potential changes in blood cell counts of DTC patients after 131-I therapy, especially CD3-positive, CD19-positive, and CD56-positive peripheral blood lymphocytes (PBL), as well as the possible role of apoptosis in selected lymphocyte populations. The study group included 24 thyroid cancer patients and 24 control subjects. Peripheral blood samples from patients and controls were analyzed using 5-color flow cytometry. Apoptotic cells were detected using an Annexin V-FITC/7-AAD kit. There was a statistically significant decrease of all blood cells after the 131-I therapy. The CD19+ B lymphocyte population was the most affected (5.82 ± 3.21% before therapy vs. 3.93 ± 2.60% after therapy, p = 0.008). This decrease was correlated with the degree of apoptosis of peripheral blood lymphocytes (Spearman's r = 0.563, p = 0.013). We concluded that 131-I therapy of DTC patients led to a decrease of all peripheral blood cells, especially CD19+ B lymphocytes. This directly correlated with apoptosis of PBLs, indicating that radiation damage to B cells leads to subsequent elimination by apoptosis.

Keywords: Differentiated thyroid cancer, peripheral blood cells, apoptosis

1 Introduction

Thyroid cancer is the most common malignancy of the endocrine system; its incidence has been increasing over the past 20 years [1]. Differentiated thyroid cancer (DTC) accounts for more than 90% of all thyroid cancers and includes papillary, follicular, and poorly differentiated histological types [2]. Following surgery, treatment of DTC patients with radioactive iodine (131-I) is a standard procedure for the ablation of remnant thyroid tissue and for the treatment of iodine-avid metastases [3, 4]. According to the recommendations of American Thyroid Association (ATA), 131-I administration is indicated in patients with a moderate to high risk of recurrence, based on age, tumor size, lymph node status, and extrathyroidal extension as well as on histological type of the thyroid tumor [2,3]. Side effects of 131-I treatment may occur in many organ systems, including the lacrimal glands, bone marrow, lungs, and reproductive organs [5,6]. Although transient anemia and thrombocytopenia following 131-I treatment have been described [6-9], the most pronounced effect of ionizing radiation is on peripheral blood leukocytes [7, 10]. Most published findings indicate that the effect of 131-I on peripheral blood lymphocytes depends on their phenotype and the time elapsed since the application of 131-I. However, the rapid fall in peripheral blood lymphocyte counts after 131-I treatment has not been clarified to date. Because we have recently shown increased apoptosis of peripheral blood lymphocytes in DTC patients treated with 131-I [11], the objective of this study was to investigate whether that treatment had an impact on CD3-positive, CD19-positive, and CD56-positive lymphocytes counts in peripheral blood of DTC patients after 131-I therapy.

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2 Material and methods

2.1 Study population

The study was approved by the Ethical Committee of the Clinical Center Kragujevac. All patients and control subjects gave their written informed consent according to the Declaration of Helsinki.

The study population included 24 well-differentiated thyroid cancer patients (17 females and 7 males), mean age 53.46 ± 14.33 years (range 21 to 78 years). Among the 24 DTC patients, 17 (70.83%) had papillary carcinoma and 7 (29.17%) had the follicular variant of papillary carcinoma. Sixteen patients had no clinical evidence of metastasis, whereas eight patients had metastasis in the lymph nodes, bone, or lungs. Four to 6 weeks after surgical total thyroidectomy, and 10 days after a low-iodine diet, patients were treated at the Nuclear Medicine Department of the Clinical Center Kragujevac, according to guidelines of European Association of Nuclear Medicine (EANM) [3], with fixed nominal activities of 3.7 GBq (100 mCi) (13 patients) or 5.5 GBq (150 mCi) (11 patients) of orally administered 131-I. At the time of 131-I administration, all 24 patients were hypothyroid after thyroid hormone withdrawal (TSH > 30 mIU/L).

The control group consisted of 24 healthy subjects, 19 (79.16%) females and 5 (20.84%) males, mean age of 47.50 ± 12.62 years (range 31–80 years). They were colleagues and relatives who were willing to engage in the study.

2.2 Isolation of peripheral blood mononuclear cells

Blood samples were collected in the morning in polystyrene tubes. Heparinized peripheral blood (10 ml) was centrifuged at 400xg for 10 min to separate plasma and cells. Peripheral blood mononuclear and polymorphonuclear cells were fractionated by single step continuous density-gradient centrifugation with Lymphoprep (Lymphoprep 1.077, Nicomed Pharma AS, Oslo, Norway). The separated mononuclear cells were washed three times with isotonic phosphate buffered saline and diluted with the same solution to a final concentration of 1 million cells/ml.

2.3 Staining for Flow Cytometry

Peripheral blood mononuclear cells of patients in each group were analyzed using 5-color flow cytometry. A 100 µl aliquot of the final solution of peripheral blood mononuclear cells was incubated for 15 min at room temperature with 10 µl of each monoclonal antibody (anti-CD3 labeled with ECD, anti-CD19 labeled with FITC, anti-CD56 labeled with PE, anti-CD45 labeled with PC5, and pairing isotype controls IgG1). After incubation, the samples were washed twice in PBS and fixed with 2% (w/v) paraformaldehyde in PBS for 10 min at room temperature prior to flow cytometry.

2.4 Detection of apoptosis

Apoptotic cells were detected using an Annexin V-FITC/7-AAD kit (Beckman Coulter IM3614). Namely, in the early phase of apoptosis, cell membrane integrity is maintained, but the cells lose membrane phospholipid asymmetry. Phosphatidylserine (PS), a negatively charged phospholipid located in the inner leaflet of the plasma membrane, is then exposed at the cell surface. The calcium and phospholipid binding protein, Annexin V, binds preferentially to PS. In late apoptosis the cell membrane loses integrity and exposes DNA to viable dyes [12]. It is considered that Annexin V negative and 7-AAD negative cells are viable, Annexin V positive and 7-AAD negative cells are in the early stages of apoptosis, whereas Annexin V positive and 7-AAD positive cells are in late apoptosis. After isolation, cells were washed in PBS (p5493 Sigma-Aldrich) and re-suspended in ice cold binding buffer to a final concentration of 1 million cells/ml. Samples for analysis were prepared using 100 µl final solution incubated in the dark for 15 min with Annexin V-FITC (10 µl) and 7-AAD (20 µl) and re-suspended in 400 µl ice-cold binding buffer. Finally, cells were analyzed on an FC500 Beckman Coulter flow cytometer to the number of 20,000 events, gating lymphocytes on the FS/SS diagram. The percentages of early and late apoptotic cells were determined using CXP Cytometer software.

2.5 Statistical analysis

All values were expressed as mean ± standard deviation (SD). The commercial SPSS version 10.0 for Windows was used for statistical analysis. Student's *t*-test was employed for comparison of paired samples. For nonparametric variables, differences between two independent groups were determined by the Mann-Whitney U-test, whereas the Wilcoxon test was used for dependent groups. The observed variables were compared by the bivariate correlation test and Spearman's r coefficient. p values less

than 0.05 were considered to be statistically significant and those less than 0.01 highly significant.

3 Results

The results obtained are presented in Tables 1–4. Table 1 shows the clinical and pathohistological characteristics of the DTC patients included in the study.

Table 2 presents the mean counts of peripheral blood cells in control subjects and DTC patients before and after 131-I therapy. There were no statistically significant differences in the mean values for peripheral red blood cells (RBCs) (independent samples *t*-test, p = 0.859), platelets (independent samples *t*-test, p = 0.532), white blood cells (WBCs) (independent samples t-test, p = 0.094), and mononuclear cells (independent samples *t*-test, p = 0.882) between control subjects and DTC patients before 131-I therapy. The mean value for RBCs in DTC patients before 131-I therapy was 4.67 ± 0.50 x 10^{12} (range 3.97–5.52 x 10^{12}), whereas after therapy it was 4.37 ± 0.36 x 10^{12} (range 3.80–4.90 x 10^{12}). The difference between the RBC mean values was statistically significant (paired samples *t*-test, p = 0.012). The mean blood platelet count in DTC patients before 131-I therapy of 233.78 ± 59.02 x 10^9 (range 118–303 x 10^9) declined to 213.67 ± 56.26 x 10^9 (range 82–275 x 10^9) 7 days after the therapy (paired samples *t*-test, p = 0.042). Similarly, there was significant difference in mean counts of WBCs before and after 131-I therapy (7.03 ± 1.23 vs. 6.33 ± 1.29 x 10^9, paired samples *t*-test, p < 0.001), as well as for mean counts of mononuclear blood cells before and after the therapy (2.23 ± 0.63 vs. 1.74 ± 0.51 x 10^9, Wilcoxon test, p < 0.001).

Table 3 shows the relative expression of CD3, CD19, and CD56 in peripheral blood lymphocytes (PBLs) of DTC patients before 131-I therapy and in healthy controls. The mean value for CD3 in PBLs of DTC patients before therapy (72.49 ± 7.26%) was significantly lower than that for healthy controls (77.21 ± 6.80%) (Mann-Whitney U test, p = 0.036). There were no significant differences between the patients and control subjects concerning CD19-positive lymphocytes (5.82 ± 3.21% versus 6.04 ± 3.22%) (independent samples *t*-test, p = 0.808) and CD56 expression (16.79 ± 6.74% vs. 13.57 ± 4.67%, Mann-Whitney U test, p = 0.054).

Summarized results for CD3, CD19, and CD56-positive cells in PBLs of DTC patients (n = 24) before and after therapy are shown in Table 4. The relative number of CD19 positive cells in PBLs of DTC patients before 131-I therapy (5.82 ± 3.21%) was significantly higher than that in PBLs of the same patients 7 days after therapy (3.93 ± 2.60%) (Wilcoxon test, p = 0.008). There was no significant effect of 131-I therapy on the mean percentages of CD3-positive cells (72.49 ± 7.26 % vs. 73.41 ± 8.43%, Wilcoxon test, p = 0.265) and CD56-positive cells (16.79 ± 6.74% vs 17.26 ± 7.12%, Wilcoxon test, p = 0.710) in PBLs of DTC patients.

Moreover, there were no significant changes in the relative numbers of CD3, CD19, or CD56 positive cells in PBLs of DTC patients before and after therapy in relation to the applied dose of 131-I (binar logistic regression; p_{CD3} = 0.216, p_{CD19} = 0.973, p_{CD56} = 0.305). There were marked differences in CD3 (p = 0.024) and CD56 (p = 0.033) expression before therapy between the patients with and without metastases.

Table 1: Characteristics of 24 thyroid cancer patients treated with 131-I.

Patient no.	Sex (F/M)	Metastases (tumor stage)	Histology (P/F)*	Dose (GBq)
1	F	Yes (T1N1M0)	P/F	5.5
2	F	No (T2N0M0)	P	3.7
3	F	No (T1N0M0)	P	3.7
4	F	No (T1N0M0)	P	3.7
5	M	No (T2N0M0)	P	3.7
6	F	No (T1N0M0)	P	3.7
7	F	No (T1N0M0)	P/F	3.7
8	F	No (T1N0M0)	P	3.7
9	F	Yes (T1N1M0)	P	5.5
10	M	Yes (T1N1M0)	P	5.5
11	F	Yes (T1N1M0)	P/F	3.7
12	F	No (T1N0M0)	P	3.7
13	M	Yes (T3N1M1)	P	5.5
14	F	No (T1N0M0)	P	3.7
15	M	No (T1N0M0)	P	5.5
16	F	No (T1N0M0)	P	5.5
17	M	Yes (T1N1M0)	P	5.5
18	F	No (T1N0M0)	P/F	3.7
19	F	No (T2N0M0)	P	5.5
20	F	No (T1N0M0)	P/F	5.5
21	M	Yes (T3N1M1)	F	5.5
22	M	Yes (T3N1M0)	P	5.5
23	F	No (T1N0M0)	P	3.7
24	F	No (T1N0M0)	P	3.7

*P/F - follicular variant of papillary thyroid carcinoma

Table 2: Mean values for peripheral blood cell counts in control subjects and DTC patients before and 7 days after radioiodine therapy

	Controls	Patients before *131-I*	Patients after *131-I*
RBCs (x10^{12}/l)	4.62 ± 0.61	4.67 ± 0.50[a]	4.37 ± 0.36[a]
Platelets (x10^{9}/l)	250.25 ± 45.15	233.78 ± 59.02[b]	213.67 ± 56.26[b]
WBCs (x10^{9}/l)	7.66 ± 1.21	7.03 ± 1.23[c]	6.33 ± 1.29[c]
Mononuclear cells (x10^{9}/l)	2.13 ± 0.43	2.23 ± 0.63[d]	1.74 ± 0.51[d]

[a] significant difference in RBC count before and after 131-I therapy (p = 0.012)
[b] significant difference in platelet count before and after 131-I therapy (p = 0.042)
[c] highly significant difference in WBC count before and after 131-I therapy (p < 0.001)
[d] highly significant difference in mononuclear cell count before and after 131-I therapy (p < 0.001)

Table 3: CD3, CD19 and CD56 expression in peripheral blood lymphocytes of DTC patients before 131-I therapy and healthy controls

Analysis	Patients (before 131-I therapy)		Control subjects	
	n	mean % ± SD	n	mean % ± SD
	24		24	
CD3		72.49 ± 7.26[a]		77.21 ± 6.80[a]
CD19		5.82 ± 3.21		6.04 ± 3.22
CD56		16.79 ± 6.74		13.57 ± 4.67

[a] statistically significant difference

The difference between DTC patients before and after 131-I therapy in the extent of early apoptosis was significant (8.64 ± 4.9 vs. 11.7 ± 6.67 %., p = 0.037), as well as that for total apoptosis (8.99 ± 5.26 vs. 12.11 ± 6.75 %, p = 0.029). The change in mean value for late apoptosis in PBLs of DTC patients after therapy (0.34 ± 0.8 vs. 0.40 ± 0.48 %, p > 0.05) was not statistically significant.

Analysis of the relationships between 131-I-induced total apoptosis (total apoptosis after 131-I therapy minus total apoptosis before therapy) and 131-I-induced reduction of CD19 expression on PBLs (CD19 expression before 131-I therapy minus CD19 expression after therapy), detected a positive association between them (bivariate correlation test, Spearman r = 0.563, p = 0.013), indicating that apoptosis predominantly eliminates CD19-positive B cells.

Table 4: Summarized results for CD3, CD19 and CD56-positive cells in PBLs of DTC patients before and after 131-I therapy.

Analysis		Patients		
		n	before therapy mean ± SD	after therapy mean ± SD
CD3				
total		24	72.49 ± 7.26	73.41 ± 8.43
Metastases				
	No	16	75.33 ± 5.28[a]	74.48 ± 8.68
	Yes	8	66.82 ± 7.65[a]	71.26 ± 7.9
Therapy doses (GBq)				
3.7		13	74.6 ± 5.45	72.54 ± 7.71
5.5		11	69.54± 8.68	74.62 ± 9.63
CD19				
total		24	5.82 ± 3.21[b]	3.93 ± 2.60[b]
Metastases				
	No	16	5.76 ± 3.15	4.03 ± 2.81
	Yes	8	5.92 ± 3.54	3.7 ± 2.27
Therapy doses (GBq)				
3.7		13	6.37 ± 3.6	4.51 ± 2.89
5.5		11	5.03 ± 2,5	3.11 ± 1.97
CD56				
total		24	16.79 ± 6.74	17.26 ± 7.12
Metastases				
	No	16	14.13 ± 4.36[c]	16.13 ± 6.85
	Yes	8	22.11 ± 7.74[c]	19.5 ± 7.58
Therapy doses (GBq)				
3.7		13	14.21 ± 4.68	16.56 ± 7.24
5.5		11	20.39 ± 7.71	18.24 ± 7.22

[a] statistically significant difference in CD3 expression before therapy in the presence of metastasis (p = 0.024),
[b] statistically significant difference in CD19 expression before and after therapy (p = 0.008).
[c] statistically significant difference in CD56 expression before therapy in the presence of metastasis (p = 0.033).

4 Discussion

In this study we analyzed PBC counts and the expression of CD3, CD19, and CD56 molecules in PBLs of DTC patients before and 7 days after 131-I therapy. This is the first investigation on the possible role of apoptosis on the selected lymphocyte subpopulations in DTC patients after 131-I therapy.

Treatment of DTC patients with radioactive iodine is a standard procedure for the ablation of remnant thyroid

tissue following surgery and for the treatment of iodine-avid metastases [3]. Changes in peripheral blood cell counts occur early after 131-I administration [8] and may persist for months, indicating a possible suppressive effect of 131-I therapy on hematopoiesis [6,10]. Earlier work had shown decreases in the counts of all peripheral blood cells of radioiodine treated DTC patients: RBCs or hemoglobin [6], platelets [6,8], and WBCs [7]. Our results are consistent with this, as we found a significant reduction of all blood cells 7 days after the administration of 131-I. Considering the life span of RBCs (over 100 days), this decrease could not be explained by the effect of radioiodine on immature precursors in bone marrow. Moreover, it was probably not due to radiosensitivity of mature RBCs. Given that we also detected a similar reduction of blood protein concentrations (data not shown), we assume that the decrease of RBCs 7 days after 131-I administration might be a consequence of blood dilution. Namely, our patients were told to drink a lot of water to adequately eliminate 131-I.

We also found a significantly lower blood platelet count 7 days after 131-I treatment compared with the pretreatment value, which is consistent with previously published data [6,8]. However, the decrease was less pronounced than in other studies. This could be explained by differences in the time elapsed from 131-I therapy. Thus we determined changes in blood platelet counts early after 131-I administration, whereas in similar studies platelet counts were analyzed after several months. Molinaro et al. [10] reported that 131-I treatment is associated with a decline in platelet count that persists for at least 1 year after ablation, whereas Rosario et al. [13] demonstrated a transient platelet decrease in the first 3 months after therapy. Their results indicate that short term effects on the bone marrow may be more of a dose-related phenomenon, whereas the late persistent effects are predominantly influenced by the individual susceptibility of DTC patients. Because we did not examine platelet life span, we cannot say whether the decline in blood platelet count was due to increased removal from peripheral blood or not. If we consider our results for RBC and WBC counts in the peripheral blood of 131-I treated DTC patients, and the normal platelet life span of about 7 to 10 days, we can assume that the lower platelet counts obtained 7 days after 131-I treatment might be a result of the joint influences of blood dilution resulting from increased water intake and/or decreased production and/or increased removal of platelets in bone marrow and peripheral blood.

The most interesting, and so far the most studied, effect of 131-I therapy is on WBC counts and the distribution of lymphocyte subpopulations in the peripheral blood of DTC patients. Both earlier [7] and more recently published studies [10] showed decreases of WBCs after 131-I treatment of DTC patients. Our results confirm this, as there was a highly significant reduction of WBC counts 7 days after 131-I therapy that was more pronounced than the decreases in RBC or platelet counts.Lloyd and Dolphin [14] assumed that the rapid fall of the total lymphocyte count in the peripheral blood after whole body irradiation was not a consequence of intrinsic radiosensitivity and radiation-induced death of these cells. They assumed that the decrease in the lymphocyte count may reflect enhanced migration of cells out of the vasculature and into other tissues. However, the results of most published studies [15-17] indicate differences in the radiosensitivity of human lymphocyte subpopulations. By analyzing lymphocyte subpopulations in the peripheral blood of DTC patients before and after therapy, we wished to determine the subpopulation of cells that is the most affected.

Before 131-I therapy, our DTC patients had a significantly higher proportion of CD3+ T lymphocytes than control subjects but approximately equal proportions of CD19+ B lymphocytes and CD56+ NK cells as controls. Data in the literature on the presence of NK cells in the peripheral blood of patients with DTC are contradictory. One study (18) showed an increased percentage of NK cells in patients with DTC, whereas another found increased sensitivity of NK cells to apoptotic stimuli and a reduction in the number of NK cells in peripheral blood of DTC patients after thyroxine withdrawal [19]. Our DTC patients were severely hypothyroid after thyroxine withdrawal, but we did not detect a significant difference in the percentage of CD56+ NK cells between patients before 131-I therapy and healthy control subjects. Nevertheless, our DTC patients with metastases had a significantly greater percentage of CD56+ NK cells than patients without nodal or distant metastases. We did not analyze NK cell activity or the percentage of NK cells with regulatory and cytolytic phenotypes, but Wulff et al. [20] found a decreased proportion of regulatory NK cells in the peripheral blood of patients with head and neck carcinoma. As a subset of immune effectors, NK cells are responsible for tumor regression and elimination of blood-borne metastases [17, 21].

Seven days after the administration of 100 or 150 mCi 131-I, we demonstrated a significant reduction of CD19+ B cells in the peripheral blood of DTC patients. This result is in agreement with earlier published data that human B cells are more sensitive to radiation than T cells [15]. In patients treated with X-rays, Blomgren et al. [22] found that the percentage of presumed B cells in the total lymphocyte population was halved after treatment. Because we recently detected increased apoptosis of peripheral blood lymphocytes in DTC patients treated with 131-I [11],

we further analyzed whether this was correlated with the decrease of CD19+ B cells. Our results indicate that apoptosis contributes to the decline of B cells in the peripheral blood of 131-I treated DTC patients.

In conclusion, we can affirm that 131-I therapy of DTC patients leads to reduction of all peripheral blood cells. The decrease of CD19+ B cells directly correlates with apoptosis of PBLs, indicating that radiation damage to B cells may lead to their elimination by apoptosis.

Conflict of interest statement: Authors state no conflict of interest

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