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Functional features of neutrophils in subclinical hypothyroidism compared to euthyroid status

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Abstract

Neutrophils or polymorphonuclear leukocytes are critical to host defense against pathogens. Increasing evidence indicates that thyroid hormones also play an active role in immune mechanism while there is very little literature regarding effects of thyroid stimulating hormone on functional features of neutrophils. In this cross-sectional study, we evaluated the phagocytic, chemotactic, candidacidal, and respiratory burst activity of neutrophils in subclinical hypothyroid and euthyroid subjects. Phagocytosis and metabolic function of the neutrophils was assessed using Nitro-blue tetrazolium test. Chemotaxis, directional movement of the PMNs was measured using the N-Formyl methionyl-leucyl-phenylalanine as chemoattractant. Phagocytic activity was evaluated by exposing leukocytes to candida suspension and counting the number of candida ingested and killed. Microbicidal activity of neutrophils was assessed by the ability of leukocytes to kill Candida albicans in both subclinical hypothyroid and euthyroid subjects. Our study demonstrated that chemotactic and mean phagocytic activity was significantly less in those with subclinical hypothyroidism as compared to euthyroid control group (p = 0.005 and 0.045 respectively). Respiratory burst activities of both unstimulated and stimulated neutrophils were relatively reduced in subclinical hypothyroidism (p value of 0.08 and 0.07). Candidicidal activity was similar in both groups. We conclude that a raised TSH level is associated with altered functional characteristics of neutrophils.

Introduction

Neutrophils or polymorphonuclear leukocytes (PMNs) are an essential part of the innate immune system and the first line of defense in the body. They migrate to site of inflammation or infection by chemotaxis, and phagocytize the microorganisms to form phagosomes. Reactive oxygen species (ROS) and hydrolytic enzymes released by the phagosomes eventually cause lysis of ingested microorganism.

The thyroid stimulating hormone (TSH) has often been overlooked in its importance with respect to immune function. TSH is a key neuroendocrine mediator of the hypothalamus-pituitary-thyroid axis. TSH stimulates the release of thyroxine (T4) and triiodothyronine (T3) hormones that are essential for proper differentiation, growth and metabolism. Evidence indicates that these thyroid hormones play an active role in immunity **[1,2,3,4,5,6]**. Decreased levels of thyroid hormones are often associated with increased

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susceptibility to infections **[7,8,9]**. It is also observed that a high frequency of those with thyroid disorders had neutropenia **[10]**. A recent study conducted showed lower phagocytic capacity of the neutrophils in patients with abnormal thyroid functions in critical illness **[11]**. Some of the studies done on the effect of thyroid hormones on functional features of neutrophils concluded that hypothyroidism is associated with decreased bactericidal capacity **[12]**, reduced oxidative metabolism **[13]**, increased adherence and decreased spontaneous migration **[14]**. While it is known that thyroid hormones have a function in immunity, there is not much research with regards to the effect of TSH on immunity.

Few studies show that TSH stimulates splenic dendritic cells to significantly enhance the phagocytic activity and cytokine secretion **[5,6,15]**. Subclinical hypothyroidism (SCH) is considered as an early stage of hypothyroidism characterized by increased levels of TSH with thyroid hormone within normal reference range **[16]**. One study reported significantly reduced neutrophils in subclinical hypothyroidism; and after hormonal substitution, leucocytes had shifted to a favorable profile **[17]**. Evaluating neutrophil functions in SCH subjects provides us information about the role of TSH on the same. While studies indicate that elevated TSH seems to have some effects on immune functions, there exists very little evidence regarding its role on functional features of neutrophils. In this study, we evaluated the functional features of neutrophils in individuals with subclinical hypothyroidism compared to those in euthyroid status.

Materials and methods

This was a cross sectional study to compare the functional features of neutrophil between subclinical hypothyroid and euthyroid subjects. Institutional Ethical Committee granted approval for the study (IEC/2011/APP/070). Adult subjects in the age group of 19-50 years of both genders who satisfied the inclusion and exclusion criteria were recruited in this study. All participants gave written informed consent to be part of the study. The study was carried out in the department of Physiology in collaboration with the department of Endocrinology.

Inclusion criteria

Diagnosed cases of subclinical hypothyroidism based on well-established biochemical criteria: Elevated TSH level of > 5.0 mIU/L but < 10 mIU/L; with a normal free T_4 level (4.5-10.9 µg/dl) **[16,18]**. Individuals diagnosed with SCH had few or no apparent clinical features of hypothyroidism.

Exclusion criteria

Individuals with history of thyroid disease and/or on T4 hormone supplements were excluded from the study. In addition, patients with inflammatory conditions, infectious diseases, recurrent infections, asthma, allergy, atopic diseases, any suspected immunological disorders and on any immunomodulation treatment for the past six months were excluded from the study. Also patients should not have had malignancy, surgery and major trauma in the last six months. Individuals with history of cigarette smoking, alcohol consumption or use of any narcotics were excluded from study.

We intended to include 25 subjects with subclinical hypothyroidism and 25 age and gender matched euthyroid subjects as control group. Subjects who had gained weight or had difficulty in reducing it or who were either referred by clinician or self-referred for evaluating thyroid status were screened. Out of 147 individuals screened, 48 eligible participants agreed to be part of the study. Anthropometric measurements were undertaken and blood collected. Height and weight were measured and body mass index (BMI) calculated. After overnight fasting, venous blood (about 3-5ml each) was collected from brachial vein under aseptic precautions into separate vacutainers and stored at 4°C. Ethylenediaminetetraacetic acid (EDTA) vacutainers were used for complete blood count determination and to separate out white blood cells for the neutrophil function tests. Plain vacutainers were used to collect blood sample and to separate out serum using centrifuge which was then either used immediately or stored at 4°C. The plain blood sample was used for thyroid hormones assay and neutrophil function tests.

Thyroid profile

Serum T3 and total thyroxine (T4) was determined using the Access Total T3 and T4 assay which is a paramagnetic particle, chemiluminescent immunoassay. TSH was measured using reagent hypersensitive fTSH assay with Beckman Coulter, Inc, USA.

Neutrophil functions tests

Chemotaxis, phagocytosis, respiratory burst and candidacidal activity was assessed on the blood samples following standardized protocol mentioned briefly below. Neutrophil functions tests were performed for each blood sample individually within twelve hours of collection to avoid degeneration of neutrophils in the sample.

Phagocytosis and metabolic functions of neutrophils was assessed using yellow dye Nitro-blue tetrazolium (NBT) test. In this test, the neutrophils were exposed to NBT. Unstimulated

cells do not ingest this dye, but if stimulated, they phagocytize the dye. Intracellular reduction by superoxide free radical then converts them into insoluble, blue crystals of formazan. These crystals are visible under the light microscope and can be counted. The test gives information about both phagocytic and metabolic functions of the neutrophils. Metabolic function is the intracellular reduction which depends on hexose monophosphate (HMP) shunt activation, necessary for the microbicidal activity of PMNs.

Escherichia coli endotoxin was used as a neutrophil stimulant and Minimum Eagles' medium as a neutrophil preservative. Blood smear was prepared with endotoxin (stimulated) and without endotoxin (unstimulated) and then stained with Giemsa. The stained smear was then scanned under 100X light microscopy for stimulated neutrophils based on the characteristic morphology. Stimulated neutrophils were those with the characteristic dark blue NBT granules that nearly obscured the nucleus of the neutrophils. 100 consecutive neutrophils were counted and reported as percentage **[15]**.

Chemotactic activity of neutrophils was measured using the chemoattractant N-Formyl methionyl-leucyl-phenylalanine (fMLP). WBCs separated from the patients' blood were exposed to fMLP which is positively chemotactic to polymorphonuclear leucocytes. Agarose gel was cast on a glass slide and holes punched in gel to hold WBC's, fMLP, serum and medium. The slide was incubated for 2 hours, after which the slides were dipped in Methanol and formalin for 20 minutes each. Agarose mold was removed from slide and cells stained with Giemsa stain. The distance travelled by white blood cells in millimetre towards well containing fMLP was measured using a computerized light microscope **[19]**.

Phagocytosis: WBC's were exposed to candida suspension. The number of candida ingested and killed by the white blood cells was counted. In the test; WBC, candida suspension, serum and MEM were taken, similarly in the control all except serum were taken and both incubated at 37°C for 30 minutes. Smears are made using 10 µl from both test and control, fixed and stained in Giemsa stain for 30 minutes, washed, dried and observed under oil immersion (100X) objective. Number of 'ingested' candida associated with each cell was counted and reported as mean particle number associated with each cell.

Microbicidal activity of neutrophils was assessed by candidicidal assay, the ability of leukocytes to kill Candida albicans. First; leukocytes, candida, serum and MEM were incubated together for 37°C for 60 minutes. The leukocytes were then lysed with sodium desoxycholate. Methylene blue was added in a concentration that stained only nonviable C. albicans. In this test, the percentage of candida cells that were stained (with methylene blue) after 2.5 hour of incubation was taken to indicate the percentage of ingested organisms killed and partially degraded by neutrophils. The number of dead candida are counted and reported as percentage **[18]**.

Statistical analysis

Descriptive and inferential statistical analysis has been carried out using SPSS 15.0. Results on continuous measurements are presented as Mean ± Standard deviation (SD), Range (Min to Max) and results on categorical measurements are presented in number (%). Significance is assessed at 5 % level of significance.

Results

Overall, 48 participants were enrolled in the study of which 19 had subclinical hypothyroidism and 29 were euthyroid. Even though both genders were included in the study nearly 90% of subjects were females because thyroid dysfunction is more common in females and they are screened routinely for hypothyroidism. The demographic profile of the control and SCH subjects are shown in **Table 1**.

Age, gender distribution, diet pattern and built of subjects were comparable in both study and control groups. Body mass index (BMI) was significantly higher in the subclinical hypothyroid group compared to control (p=0.005).

Thyroid and hematological parameters of the subclinical hypothyroid and euthyroid individuals are shown in **Table 2**. TSH level were within normal limits (3.91±1.07) in the euthyroid control group compared to subclinical hypothyroid patients (7.17±1.57). T3 and T4 were within normal limits in both groups. Total WBC count and neutrophil count were within normal limits in both groups.

Both groups had almost similar clinical presentation. Salient clinical presentation observed in subclinical hypothyroid and euthyroid individuals is given in **Table 3.** Although females with subclinical hypothyroidism had increased (36.8%) menstrual irregularities as compared to euthyroid women (only 10.3%).

Functional features of neutrophils in euthyroid and subclinical hypothyroid subjects

Neutrophil function tests namely metabolic activity or respiratory burst activity, phagocytosis, chemotaxis and candidicidal assay have been variably effected in patients with subclinical hypothyroidism (**Table 4**).

Table 1: Demographic profile of the subclinical hypothyroid and euthyroid subjects

Parameters		Euthyroid (N=29)	Subclinical hypothyroidism (N=19)	p value
Age (years)		36.75±7.53	36.79±8.70	0.486
Gender	Female	26(89.7%)	17(89.5%)	0.560
	Male	3(10.3%)	2(10.5%)	
Occupation	Not working	11(37.9%)	8(42.1%)	0.830
	Working	18(62.1%)	11(57.9%)	
Diet pattern	Mixed	18(62.1%)	11(57.9%)	0.830
	Vegetarian	11(37.9%)	8(42.1%)	
Built	Moderate	25(86.2%)	17(89.5%)	0.498
	Well built	3(10.3%)	2(10.5%)	
	Poorly built	1(3.4%)	0(0%)	
BMI (kg/m^2)	<18.5	0(0%)	0(0%)	0.005**
	18.5-23.0	9(31%)	5(26.3%)	
	23.0-30.0	18(62.1%)	14(73.6%)	
	>30.0	2(6.9%)	0(0%)	

2-tailed independent sample t-test for scale variables; χ^2 / Fisher test for categorical variables; **Strongly significant (p value <0.01)

Table 2: Thyroid and hematological profile of subclinical hypothyroid and euthyroid individuals

Parameters	Euthyroid	Subclinical hypothyroidism	p value
TSH (mIU/L)	3.91±1.07	7.17±1.57	<0.001**
Triiodothyronine (T3) (µg/dl)	0.97±0.36	1.12±0.77	0.875
Total thyroxine (T4) (µg/dl)	7.17±2.90	6.13±3.16	0.631
RBC count (millions /mm^3 of blood)	4.57±2.18	5.31±0.53	0.769
Hemoglobin (g/dl)	10.92±4.64	12.20±0.69	0.930
WBC count (thousands/mm^3 of blood)	5.90±3.02	6.00±1.12	1.000
Neutrophil count (%)	50.21±26.51	62.80±6.03	0.469

**Strongly significant (p value <0.01)

Table 3: Clinical presentation of subclinical hypothyroid and euthyroid individuals

Parameters		Euthyroid	Subclinical hypothyroid	p value
Skin / Hair changes	Absent	20(69%)	12(63.2%)	0.759
	Present	9(31%)	7(36.8%)	
Emotional changes	Emotionally stable	28(96.6%)	18(94.7%)	1.000
	Emotionally unstable	1(3.4%)	1(5.3%)	
Menstrual history	Regular	24(82.8%)	10(52.6%)	0.060†
	Irregular	3(10.3%)	7(36.8%)	

†Suggestive significance (p value: 0.05<p<0.10)

Table 4: Mean and standard deviation of neutrophil function tests in subclinical hypothyroid (SCH) and euthyroid control groups

Neutrophil function tests	Subjects	N	Mean	SD	SEM
NBT-unstimulated (% cells)	Control	29	52.93	14.56	2.70
	SCH	19	46.21	9.66	2.22
NBT-stimulated with bacterial endotoxin (% cells)	Control	29	61.93	16.24	3.02
	SCH	19	54.79	10.67	2.45
Phagocytosis (mean particle number)	Control	29	4.59	0.83	0.15
	SCH	19	4.11	0.74	0.17
Candidicidal assay (% of dead Candida)	Control	29	26.79	17.06	3.17
	SCH	19	23.42	4.57	1.05
Chemotaxis (in mm)	Control	29	0.17	0.14	0.03
	SCH	19	0.08	0.06	0.01

SD=Standard deviation, SEM=Standard error of mean

Respiratory burst activity of neutrophils both unstimulated neutrophils and stimulated by Escherichia coli endotoxin were relatively reduced in subclinical hypothyroidism compared to normal but statistically was just suggestive significance (p value= 0.08 and 0.07 respectively). Chemotaxis or directional migration of neutrophil towards fMLP in subclinical hypothyroid subjects measured had mean and standard deviation of 0.08 ± 0.06 mm compared to the euthyroid group 0.17 ± 0.14 mm. Chemotaxis was significantly less in subclinical hypothyroidism compared to normal euthyroid subjects with a p value of 0.005.

Phagocytic activity as evidenced by mean particles number phagocytized by neutrophils was significantly less in (4.11±0.74) in subclinical hypothyroid patients compared to euthyroid group (4.59±0.83) with a p-value of 0.045. Microbicidal activity of neutrophils was not significantly different in those with subclinical hypothyroidism (23.42±4.57) compared to euthyroid group (26.79±17.06) with p-value of 0.32.

Discussion

Results of this study indicate that subclinical hypothyroid status may effect innate immunity as demonstrated by the presence of defective neutrophil function in individuals having SCH. Our study evaluated four functional features of neutrophils viz. chemotactic, metabolic, phagocytic and candidicidal activity. They are further discussed in detail in subsequent paragraphs. We did not find any studies regarding the neutrophil functional status in subclinical hypothyroid group. Hence, we have compared our results to those of neutrophil functional status in euthyroids.

Chemotactic activity

We noticed that chemotactic activity of neutrophils was significantly reduced in subclinical hypothyroid patients when compared to the control group. Few studies reported that chemotactic activity was reduced in patients with hypothyroidism and attributed it be part of metabolic and functional disturbances in the tissues of various organs in patients with hypothyroidism **[12,14,20]**. Marino et al studied neutrophil functions on athyrotic patients and found neutrophil migration to fMLP to be similar to cells from patients during hypothyroid status, post-replacement therapy and healthy controls **[21]**.

Metabolic activity

Our study documented metabolic and respiratory burst activities that were reduced, but not significant so, likely because thyroid hormone levels were within normal limits in SCH subjects. Studies have found a significant reduction in metabolic activity, specifically, oxygen radical production in hypothyroid patients that could be corrected by treatment with thyroxine **[21]**. They attributed this to abnormalities in the pattern of lysosomal enzymes and weakening of redox processes in these neutrophils. In another study, nitro-blue reduction was attributed to be part of the general metabolic and functional disturbances in various organs seen in hypothyroidism and not due to direct action of thyroid hormones.

Phagocytic activity

In our study, phagocytic function of neutrophils was significantly less in subclinical hypothyroidism when compared to normal euthyroid individuals. There are conflicting results regarding the effect of

hypothyroid status on phagocytic activity. According to one study, no significant deviationsof phagocytic activity of neutrophils was recorded in hypothyroid individuals when compared to euthyroid controls **[14]**. In another study, recombinant TSH beta was found to significantly enhance the phagocytic activity of dendritic cells from adult animals **[6]**. This conflicting activity could be because of different methods and different cell population studied.

Candidicidal activity

Candidicidal assay of the subclinical hypothyroid cases showed no change when compared to euthyroid group in our study. We found two conflicting reports on microbicidal activity of neutrophils in hypothyroid patients. One study found that PMNs had a decreased microbicidal activity in hypothyroid patients when compared to euthyroid control subjects. This microbicidal activity normalized after therapy with levothyroxine **[12]**. In another study, the ability of the neutrophils to ingest and kill Staphylococci was not impaired **[13]**.

Impairments of functional features of neutrophils seen in hypothyroidism have been attributed to general metabolic and functional disturbances. Other studies attribute impairments to abnormalities in the pattern of lysosomal enzymes and weakening of redox processes in these neutrophils. The reason for altered functional features of neutrophils in subclinical hypothyroid individuals in our study is not clear. These changes may mainly be due to increased levels of TSH seen in SCH or may be part of general impairment. During differentiation in the bone marrow these functional properties of the neutrophil are acquired sequentially **[22]**. We hypothesize this functional differentiation may be effected by the elevated levels of TSH. We further hypothesize that altered lipid metabolism change PMN lipid composition leading to altered cell membrane physiology, whereby the rate of oxygen and energy utilization by the cells may be effected **[23]**. These reasons may or may not be cause of altered neutrophil function in this study.

Conclusion

Individuals with subclinical hypothyroidism had normal thyroid hormone levels but increased TSH. In this study, it was seen that the neutrophils had defective chemotactic and phagocytic activity and could effect the physiological functions of the neutrophils. Further studies need to be done to evaluate the cause for altered neutrophil functions and its consequence on the health of the individual.

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