The role of Lymphocytes Apoptosis in Grave's Disease Patients

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Summary:

Background: Apoptosis is a physiological type of cell death; it is active, well-controlled genetic program of cell death that does not produce inflammatory process. It is involved in cell turnover in healthy adult tissues and it is responsible for focal elimination of unwanted cells during normal embryonic development, organ homeostasis, immune regulation and defense without causing stress to the neighboring cells.

Method: This study was carried on 30 Grave's disease female patients with a mean age of (29.8 ± 8.3) years. The study parameters were considered as: before and after treatment; patient becomes clinically and biochemically euthyroid after 4-6 weeks of starting treatment with antithyroid drug (carbimazole).From each patient 7 mL of blood were aspirated (2ml for detection of peripheral blood lymphocyte apoptosis 5ml for T3,T4andTSH).For each patient fine needle aspirate (FNA) from thyroid tissue to study the histopathological changes by Hematoxylene and eosin stain, and lymphocyte apoptosis by DNA binding dye Acridine orange.

Results: The percentage of peripheral blood lymphocytes apoptosis increased significantly after treatment (23.9 ± 5.06) than its percentage before treatment (11.1 ± 1.8) with P value < 0.00001, as detected by DNA-binding fluorescent stain (acridine orange). There was a significant increase of intrathyroidal lymphocytes apoptosis (21.7±4.2) and (34.7±5.6) before and after treatment respectively with a P value < 0.0001, detected by acredine orange and hematoxylin and eosin stains. T3, T4 and thyroid autoantibody levels decreased significantly, while TSH level increase significantly after treatment.

Conclusion: Grave's disease is associated with increased rate of apoptosis in both peripheral blood lymphocytes and intrathyroidal lymphocytes. After treatment with carbimazole there was significant Increase in peripheral blood lymphocytes and intrathyroidal lymphocytes apoptosis, this lead to significant decrease in the autoimmune reaction assessed by decrease in the anti thyroglobulin antibodies in serum.

Keywords: lymphocyte apoptosis, acredine orange, Grave's disease.

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Introduction:

Apoptosis is a physiological type of cell death that occurs in all multicellular organisms as a part of normal development (1). Increased apoptosis can cause disease, e.g. neurodegeneration, autoimmune diseases, AIDS and ischemia-associated injury while decreased apoptosis can also cause disease, e.g. Autoimmune Lympho-Proliferative Syndrome and cancer. The apoptotic processes can be stimulated by extrinsic signals such as: The binding of death inducing ligands (Fas-Fas ligands interaction) to cell surface receptors. (1). Induction of apoptosis by cytotoxic T-lymphocytes by granzyme B which is a serine protease that directly activates the target cell caspases. The latter occurs when T-cells recognize damaged or virus-infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic or virus-infected cells from spreading the infection (2). The morphological changes of apoptotic cell death involves cellular condensation, genomic DNA fragmentation, deposition of electron dense chromatin along the inner margin of the nuclear envelope, the formation of membrane blebs that contain portions of nucleus and intact organelles and, finally, formation of apoptotic bodies which are phagocytosed by the neighboring cells. (3). the thyroid gland is composed of large numbers of closed follicles (100-300 micrometers in diameter) filled with a secretory gelatinous protein-hormone substance called colloid which consists of large glycoprotein thyroglobulin, which contains the thyroid hormones. The follicles are lined with cuboidal epithelial cells that secrete the thyroglobulin into the interior of the follicles. (4). Thyroid hormones enter the cells; T_3 binds to receptors in the nuclei. ButT_4 binds less avidly to these receptors. The hormone-receptor complex then binds to DNA via zinc fingers and increases or in some cases decreases the expression of a variety of different genes that code for enzymes which regulate cell function. Thus, the nuclear receptors for thyroid hormones are members of relatively large family of hormone-sensitive nuclear transcription factors. In most of its actions, T_3 acts more rapidly and is 3-5 times more potent than T_4. Reverse T3 (RT_3) is inert. (5). the thyroid hormones increase the metabolic activities of almost all the tissues of the body. The

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rate of protein synthesis is increased, at the same time the rate of protein catabolism is also increased. This is achieved by two mechanisms: first increase the number and activity of mitochondria, which in turn increases the rate of formation of adenosine triphosphate (ATP) to energize cellular function; Second: Increasing the active transport of ions through cell membranes: by increasing Na^+-K^+ ATPase activity. This in turn increases the rate of transport of both sodium and potassium ions through the cell membranes of tissues. So increases the body's metabolic rate. (6). Thyroid hormones contribute to the development and maintenance of homeostasis in multicellular organisms to control cell growth and differentiation. It also induces apoptotic cell death of differentiating erythrocytic progenitor cells. (7). It also induces apoptotic cell death of many leukocytes and lymphocytes. Graves' disease is an organ-specific autoimmune thyroid disorder resulting in dysfunction (8), it was first described by Robert Graves in the early 19th century; Graves' disease is associated with diffuse enlargement of the thyroid gland to two –three times of its normal size, with tremendous follicular hyperplasia and enfolding of the follicular cell lining into the follicles, patchy lymphocytic infiltration and occasional formation of lymphoid germinal centers (8). Each cell increases its rate of secretion several-folds. Graves' disease is more common in women than in men (8:1), its onset is between the ages of 20 and 40. It has a familial tendency, and histocompatibility studies have shown an association with group HLA-B8, HLA-DR3 and HLA-DR2 . (9). The pathogenesis of the hyperthyroidism in Graves' disease involves the formation of autoantibodies that bind to the TSH receptor in thyroid cell membranes and stimulate the gland to hyperfunction by inducing continual activation of the cAMP system of the cells with resultant development of hyperthyroidism (10). TSH receptor antibodies (TSH-R Ab [stimulatory]) are demonstrable in the plasma of about 80% patients with Graves' disease. They have a prolonged stimulatory effect on the thyroid gland, lasting for as long as 12 hours, in contrast to a little over 1 hour for TSH. (4). The pathogenesis thyroid autoimmunity is as such: there is an increased number of intrathyroidal antigen presenting cells (APCs) appears to be the most prominent sign of the initiation of an autoimmune reaction (11). Lymphocytes interact with the presented autoantigens. Hence, immune tolerance is lost; the outcome of this interaction is the activation of antigen-specific T helper lymphocytes. These in turn stimulate, humoral immune response via B lymphocytes, hence generate an autoimmune reaction. Initially, the production of autoreactive T lymphocytes and antibodies takes place in the draining lymph nodes, but later lymphoid tissue develops locally in the thyroid itself. Thyroid cells may also express MHC II molecules that are required for antigen presentation to CD4+ T lymphocytes which can also act as APCs the generated autoreactive T and B lymphocytes accumulate in large numbers and infiltrate the thyroid parenchyma. (12). Role of apoptosis in autoimmune thyroid diseases: the pathway involved in thyroid autoimmunity is the death receptor-mediated apoptotic pathway; Fas/Fas Ligand (FasL) system. Fas is a type I transmembrane protein which belongs to the TNF receptor superfamily. FasL is a type II transmembrane protein that on ligation with the Fas receptor induces apoptosis on Fas-expressing target cells. The optimal conditions for the induction of Fas-mediated apoptosis in the thyroid cells require the presence of IFN-γ in combination with IL-1β or TNF-α (13).

Subjects and methods:

The study included (30) patients; their age range (15-54) year, all were females. All were diagnosed by specialist physician as cases of Grave's disease.The samples for the study were taken as: -Pre-treatment: Early diagnosed cases of Grave's disease who didn't have treatment with antithyroid medications (carbimazole). -Post-treatment: patients were controlled by treatment with antithyroid medication. The patients were usually clinically and biochemically euthyroid after 4-6 weeks after starting carbimazole. Seven milliliters of venous blood were aspirated from each patient; the sample was divided into two parts: First part (2ml) was processed for peripheral blood lymphocytes separation (PBL) to study: Lymphocytes count and viability; Morphology of apoptotic lymphocytes by DNA-binding fluorescent dyes (acredine orange). Second part (5 ml) was used for serum separation to estimate: Thyroid hormones level (T3, T4, and TSH). Thyroid auto-antibodies. Fine needle aspirate cytology (FNAC) from the thyroid gland of each subject to detect the apoptotic intrathyroidal lymphocytes in the aspirated thyroid material. All these parameters were detected in Graves' disease patients before and after treatment with antithyroid drugs. Peripheral blood Lymphocytes (PBL) separation was done according to the method of Goldrosen (14). The number and viability were counted according to Doyle and Griffiths (15) expressed as cell/mm^3 Trypan blue exclusion test was done to asses' cell viability. The principle of this test is that the viable cells exclude Trypan blue dye (i.e. does not be stained); while the dead cells accept the dye (stained blue). Fresh slide smear was prepared from lymphocyte suspension. The smear was allowed to dry at room temperature, fixed for 30 minutes. After that slides were stained with DNA-binding fluorescent dye (acredine orange) and examined by fluorescent microscope according to procedure of Vacca (16).The morphological characteristics of lymphocyte apoptosis were assessed according to the method of Willingham (17). These changes include: Cell shrinkage, membrane blebbing, Chromatin condensation and fragmentation of nuclear material and DNA fragmentation. Estimation of thyroid hormones level: Serum T3,T4 and TSH was estimated by

Radio Immuno Assay (RIA) system . Anti-TG (anti-thyroglobulin) antibodies were detected by enzyme immunometric assay (ELISA) in Grave's disease patients before and after treatment with antithyroid drugs. Fine- needle aspiration biopsy cytology (ABC): The procedure involves penetration of the lesion which is surrounded by a zone of normal tissue, by a fine needle. The specimen thus obtained consists of a minute amount of tissue or evacuated fluid, After the spread of specimen on slides, first group the slides fixed immediately in 95% ethyl alcohol for 30 minutes and not more than 7 days, Stained by Hematoxylin and Eosin stain, examined under light microscope. (18). The second group stained by acredine orange; after fixation of the slides in formal acetone, the staining procedure follows the same steps of peripheral blood lymphocytes staining by acredine orange and examined under fluorescent microscope.(16)

Statistical analysis: The data were analyzed by Microsoft Excel program and were presented as mean ± standard deviation. A paired sample T-test was used to compare between the data before and after treatment and the difference was considered statistically significant when P value was less than 0.05. (19).

Results:

Thirty cases of Grave's disease, all were females, their age range (15-43) years with a mean of (29.8 ± 8.3), all were diagnosed recently as cases of Grave's disease without receiving any treatment with anti-thyroid medications. The parameters of this study were estimated in these patients before and after treatment with carbimazole. The dose of treatment was 5-20 mg daily. The patients become euthyroid after 6 weeks of treatment. The whole duration of treatment was 18-24 months. The percentage of apoptosis in peripheral blood lymphocytes of Grave's disease patients after treatment with carbimazole was significantly more than that detected before treatment (23.9 ± 5.06) , (11.1 ± 1.8) respectively using paired samples T test, with (P value ≤ 0.0001). As illustrated in table (1). The morphological features of lymphocyte in peripheral blood are shown in figure 1.

Table.1: comparison between the percentage of apoptosis of peripheral blood lymphocytes in Grave's disease patients before and after treatment with carbimazole (By acredine orange staining).

Parameter	mean % ± SD	P-value
Apoptosis of PBL before treatment	11.1 ± 1.8	0.00001
apoptosis of PBL after treatment	23.9 ± 5.06	

*** P value is significant at 0.05**

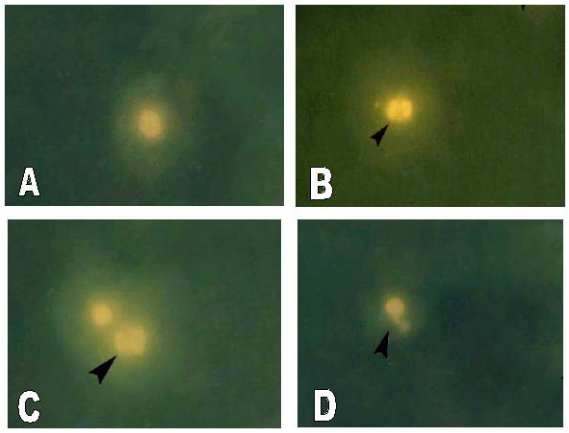


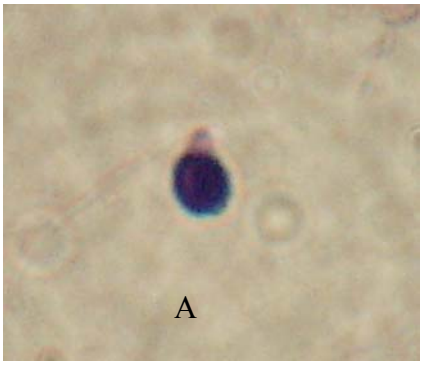
Figure 1: Peripheral blood lymphocytes stained by acridine orange in Grave's disease patient, magnifications of 400: A Normal lymphocyte. , B Apoptotic lymphocyte showing division of the nucleus into two parts. , C Apoptotic lymphocyte showing division of the nucleus into three parts. , D Apoptotic lymphocyte showing apoptotic body formation.

Detection of intrathyroidal lymphocytes apoptosis: The percentage of apoptosis of the lymphocytes that infiltrate the thyroid glands of Grave's disease patients after treatment with carbimazole was significantly more than that detected before treatment (34.7 ± 5.6) (21.7 ± 4.2) respectively as compared by paired T test as illustrated in table (2).The apoptotic intrathyroidal lymphocytes are shown in figure (2) stained by hematoxyllin and eosin .

Table 2: comparison between the percentage of apoptosis of intrathyroidal lymphocytes of Grave's disease patients, before and after treatment with carbimazole.

Parameter	Mean% ± SD	P value
Intrathyroidal lymphocytes apoptosis before treatment	21.7 ± 4.2	0.0001
Intrathyroidal lymphocytes apoptosis after treatment	34.7 ± 5.6	

*** P value is significant at 0.05**



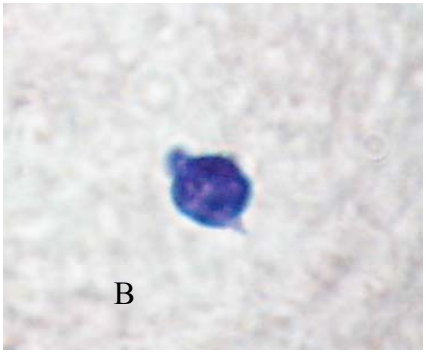


Figure (2): FNAC of thyroid gland of Grave's disease patient showing: A: Apoptotic intrathyroidal lymphocyte showing bleb formation.B: bleb and spike on lymphocyte surface. (Stained by Haematoxylene and Eosin 100X).

Serum T3, T4, TSH and thyroid autoantibodies levels: Serum triiodothyronine (T3) level estimated in Grave's disease patients after treatment was(2.4 ± 0.4 nmol/L) which was significantly lower than that estimated before treatment (3.4 ± 0.5 nmol/L) using paired samples T test. Serum thyroxine (T4) level also decreased significantly after treatment as compaired with its level before treatment (136.9 ± 25.9 nmol/L) (204.2 ± 26.9 nmol/L) respectively. Serum level of thyroid stimulatory hormone increased significantly after treatment as compared with its level before treatment (1.3 ± 0.4 µIU/ml) (0.3 ± 0.2 µIU/ml) respectively. Serum anti-thyroglobulin (ani-TG) antibodies level decreased significantly after treatment as compared with its level before treatment (478 ± 158 IU/ml) (277.4 ±104.1 IU/ml) respectively (table 3)

Table (3): Comparison between serum levels of T3, T4, TSH and anti-TG abs, in Grave's disease patients, before and after treatment with carbimazole (by paired samples T test).

Parameter	mean ± SD before treatment	mean ± SD after treatment	P value
serum T3 in nmol/L	3.4 ± 0.5	2.4 ± 0.4	0.00001
serum T4 in nmol/L	204.2 ± 26.9	136.9 ± 25.9	0.00001
serum TSH in µIU/L	0.3 ± 0.2	1.3 ± 0.4	0.00001
serum anti TG abs in IU/ml	478 ± 158	277.4 ± 104.1	0.00001

*** P value is significant at 0.05**

Discussion:

The term apoptosis is used to describe the mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of body cell populations. Its morphological features suggest that it is an active, inherently programmed phenomenon. It has been shown that it can be stimulated or inhibited by a variety of environmental stimuli, both physiological and pathological (4). This study has showed that in Grave's disease patients who didn't receive any treatment with antithyroid medication, the percentage of apoptosis in peripheral blood lymphocytes was (11.1% ± 1.8%) which is more than that estimated in normal subjects. This is in agreement with a study done by Mihara. (20). these results may be due to the effect of high level of circulatory thyroid hormones in these patients. The status of peripheral blood lymphocytes after treatment: the Grave's disease patients have become clinically and biochemically euthyroid 4-6 weeks after starting treatment with carbimazole. The percentage of apoptosis in the peripheral blood lymphocytes increased to (23.9% ± 5.06%). This increase is possibly due to the effect of the antithyroid drug on the peripheral blood lymphocytes by the following mechanism: Carbimazole action is via the upregulation of Fas ligand expression, which may then attenuate the autoimmune response of Fas-expressing T lymphocytes by inducing apoptosis of these cells. (21). The status of intrathyroidal lymphocytes before treatment: It is generally accepted that Grave's disease is a complex and polygenic organ-specific autoimmune disorder. Interaction of environmental, genetic and endogenous factors may play a role in the initiation, progression and clinical outcome of the disease. Potential antigen presenting cells (APCs) take up and present the relevant autoantigens to immune cells in the draining lymph nodes of the thyroid gland. Aberrant regulation of immune response determined by genetic or endogenous factors lead to inappropriate and excessive immune reaction instead of reinforcing immune tolerance towards self-antigens. (22). consequently, autoreactive T and B lymphocytes accumulate in large numbers, infiltrating the thyroid parenchyma. Thus, the thyroid gland is converted into a battlefield with the invading lymphocytes on one side and the defending thyroid cells on the other, fighting for survival. (11) The outcome of this battle is largely dependent on the balance between T helper-1 lymphocytes (Th1) and T helper-2 lymphocytes (Th2) response and the different profile of inflammatory cytokines(IL-4, IL-5, IL-10) released in the microenvironment. In Grave's disease, a predominant Th2 response favors humoural immunity that may induce B lymphocytes to produce anti- TSH receptor antibodies. The prevailing type of anti-TSH antibodies is stimulatory for TSH receptor, leading to thyroid cell hyperplasia and hyperfunction. These antibodies create an antiapoptotic potential for the thyroid cells thus protecting thyroid cells from apoptosis by decreasing Fas expression in normal thyrocytes and pro-apoptotic milieu for the intrathyroidal lymphocytes. (12). In addition, Th2 type cytokines may up regulate antiapoptotic molecules, including Bcl-2 protein and thus protecting thyrocytes from

apoptotic cell death (23). The status of intrathyroidal lymphocytes after treatment: In this study, the estimated percentage of apoptosis of the lymphocytes that infiltrate the thyroid glands of Grave's disease patients from the blood, was (34.7% ± 5.6%) which is significantly more than that estimated before treatment (21.7% ± 4.2%). This increment can be attributed the effect of the drug on Fas/ FasL system.Carbimazole up regulates Fas ligand expression in thyroid epithelial cells which in turn will ligate with Fas- bearing T- lymphocytes that infiltrate the thyroid gland, leading to stimulation of death receptor pathway of apoptotic cell death of these lymphocytes, which may then attenuate the autoimmune response of Fas-expressing T cells. (21). In this study, there is clear evidence that apoptosis provides a stringent and highly effective "quality control mechanism " that limits the accumulation of harmful cells such as the self reactive lymphocytes, since accumulation of these cells acts as the major cause in the pathogenesis of Grave's disease as an autoimmune thyroid disorder. Thyroid function test before and after treatment: The estimated high serum levels of tri-iodothyronine (T3), thyroxin (T4) and low level of serum TSH in grave's disease patients is due to the effect of the autoantibodies that bind to the TSH receptor in thyroid cell membranes and stimulate the gland to hyperfunction by inducing continual activation of the cAMP system of the cells with resultant development of hyperthyroidism (Stassi and Maria, 2002). TSH receptor antibodies have a prolonged stimulatory effect on the thyroid gland, lasting for as long as 12 hours, in contrast to a little over 1 hour for TSH and this will feedbacks to the anterior pituitary and to the hypothalamus decreasing the serum level of TSH. (4) Thyroid autoantibodies of anti-TG type show high serum levels in Grave's disease patients before treatment as compaired with normal values. This is due to effect of autoreactive Th2 lymphocytes which produce the inflammatory cytokines (IL-4, IL-5 and IL-10), These cytokines stimulate B lymphocytes to produce this high titre of autoantibodies. (24). After treatment with carbimazole, serum T3 and T4 levels declined because this drug has reduced the synthesis of new thyroid hormones by inhibiting the iodination of tyrosine. It also has an immunosuppressive action, leading to diminution in autoantibodies synthesis by B cells. (21).

Conclusion:

From this study we conclude that Grave's disease is associated with: Increase in peripheral blood lymphocytes apoptosis than normal subjects. Increase in peripheral blood lymphocytes and intrathyroidal lumphocytes apoptosis after treatment with carbimazole as compared with their percentages before treatment, this in turn will decrease the autoimmune reaction caused by these autoreactive cells which modifies the outcome of this autoimmune disorder.

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