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The Role of Lactoferrin and Adenosin De Aminase Enzyme in Thyroiditis (single and multiple nodule goiters) Iraqi Pateints

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Abstract :

Lactoferrin is a multifunctional iron-binding protein present in several mucosal secretions as well as in secondary granules of polymorphonuclear leukocytes. Anti-lactoferrin antibodies, which belong to anti neutrophil cytoplasmic antibodies have been described in several immunomediated diseases.
A total of 60 nodular and multi-nodular goiter patients, their age ranged from (22-71) years, attending the Medical City\Surgery department and Dijlah Private Hospital, during the period from August 2010 -April 2011. In addition, twenty healthy (controls), matched patients for age, were also included in the study. The study was conducted for the detection of serum level of anti- lactoferrin antibodies by using immunometric enzyme immunoassay. While (ADA) enzyme activity was measured by the method of (Giusti, 1981), also Protein ratio was measured by the method of (Bradford, 1976).
Anti- lactoferrin antibodies were detected in 14 (23.33%) patients, which was significantly higher than that in normal individuals (p value 0.01) and the mean of positive cases was (4.473 ± 0.206) compared to the negative cases which was (23.676 ± 2.137) (P value 0.001). (ADA) enzyme showed an increasing in mean value (4.56^{A} ± 1.27) in patients, compared to controls (1.18^{B} ± 0.49). Total protein was detecting with high values and significant difference between patients (21.99^{B} ± 0.33 gm/100ml) and control group (8.37^{A} ± 1.31 gm/100ml). Where the normal value of the total protein is (6.4–8.3) gram per deciliter (g/dL). We conclude that anti-lactoferrin antibodies could be detected in patients with thyroiditis (single and multiple nodule goiters) in addition to elevatet level of (ADA) enzyme and often total protein in the same patients.
Keywords: Thyroiditis, Single and multiple nodule goiters, Lactoferrin, Adenosin De Aminase enzyme, protein.

Introduction:

Nodular goiters are clinically recognizable enlargements of the thyroid gland characterized by excessive growth and structural and/or functional transformation of one or several areas within the normal thyroid tissue. In the absence of thyroid dysfunction, autoimmune thyroid disease, thyroiditis, and thyroid malignancy, they constitute an entity described as simple nodular goiter (1). Over the past few decades, clinical and mechanistic studies have indicated many relations between nutrition and health, thus evidence that diet is a key environmental factor affecting the incidence of many chronic diseases is overwhelming (2),(3). Indeed, several food derived compounds we eat are among the most promising chemo-preventive agents being evaluated.
Chemo-prevention is defined as using chemicals with the goal of preventing, interrupting or reversing the carcinogenic process. Since carcinogenesis is a multistage process, which usually takes many years in humans, there is ample opportunity to intervene and prevent the development of cancer (4). Cancer is a major killer in today's world accounting for around 13% of all deaths according to the World Health Organization (W.H.O.).
A promising field of research is clinical studies with cancer preventive proteins existing in milk (5), namely lactoferrin (LF). It is a natural forming iron-binding glycoprotein, and known for its inhibitory action on cell proliferation, as well as for its anti-inflammatory and antioxidant abilities, has been described to have anti-carcinogenic properties in several *in vivo* and *in vitro* studies (5), (6), (7), (8), (9).
LF is produced in exocrine glands located in the gateways of the digestive, respiratory, and reproductive systems, and is secreted in many external fluids as a first line of defense. LF also resides in the specific granules of polymorph nuclear neutrophil leukocytes (PMN) and becomes exocytose upon PMN activation. During active inflammatory disease, raised serum levels of (LF) can be measured (10). Several physiological roles have been attributed to LF, namely regulation of iron homeostasis, host defense against infection and inflammation, regulation of cellular growth, and differentiation and protection against cancer development and metastasis by inducing apoptosis and inhibit proliferation in cancer cells as well as restore white and red blood cell levels after chemotherapy. These findings have suggested LF's great potential therapeutic use in cancer disease prevention and/or treatment, namely as a chemo preventive agent (11), (12). So LF is a multifunctional protein and an essential element of innate immunity.
LF participates in acquisition of iron from food and its storage in the body, and to a certain degree also in iron transport to cells and the effect of LF in combating microorganisms by chelating iron. The iron-chelating property of LF renders iron inaccessible to the pathogens, thus restricting their growth. Iron, due to its

participation in many metabolic processes, is an essential element for almost all microorganisms. Iron is not easily accessible for pathogens within the host. Since iron is crucial for normal function of both pathogens and the host, an ability to acquire iron during infection is regarded as an important virulence factor. Higher vertebrates have evolved a complicated protection system of iron storage and LF is an important element of this system. Low iron-saturated LF effectively combats bacteria and fungi, acting in a bacteri static and fungi static way. The degree of iron saturation also influences antiviral activity of LF.

Adenosine deaminase (also known as adenosine aminohydrolase, or (ADA) is an enzyme (EC 3.5.4.4) involved in purine metabolism. It is needed for the breakdown of adenosine from food and for the turnover of nucleic acids in tissues (13), the enzyme has been found in bacteria, plants, invertebrates, vertebrates, and mammals, with high conservation of amino acid sequence (14). Primarily, ADA in humans is involved in the development and maintenance of the immune system. However, ADA association has also been observed with epithelial cell differentiation, neurotransmission, and gestation maintenance (15). It has also been proposed that ADA, in addition to adenosine breakdown, stimulates release of excitatory amino acids and is necessary to the coupling of A1 adenosine receptors and heterotrimeric G proteins (16). There are 2 isoforms of ADA: ADA1 and ADA2. ADA1 is found in most body cells, particularly lymphocytes and macrophages, where it is present not only in the cytosol and nucleus but also as the ecto- form on the cell membrane attached to dipeptidyl peptidase-4 (aka, CD26). ADA2 was first identified in human spleen (17). It was subsequently found in other tissues including the macrophage where it co-exists with ADA1. The two isoforms regulate the ratio of adenosine to deoxyadenosine potentiating the killing of parasites. ADA2 is the predominant form present in human blood plasma and is increased in many diseases, particularly those associated with the immune system: for example rheumatoid arthritis, psoriasis and sarcoidosis. The plasma ADA2 isoform is also increased in most cancers. ADA2 is not ubiquitous but co-exists with ADA1 only in monocytes-macrophages (18). Deficient levels of ADA have also been associated with pulmonary inflammation, thymic cell death, and defective T-cell receptor signaling (19).
Cancer is a complex disease that is dictated by both cancer cell-intrinsic and cell-extrinsic processes. Adenosine is an ancient extracellular signaling molecule that can regulate almost all aspects of tissue function. As such, several studies have recently highlighted a crucial role for adenosine signalling in regulating the various aspects of cell-intrinsic and cell-extrinsic processes of cancer development (19). Accordingly, the present study was designed to inspect the role of ADA enzyme in this type of goiter as well as lactoferrin.

Subjects, Material and Methods:

Subjects:

This study includes sixty nodular and multi-nodular goiter patients attending the Medical City\Surgery department and Dijlah Private Hospital, during the period from August 2010 -April 2011.
They were clinically examined and evaluated by the consultant medical staff at the two hospitals and they were subjected to a personal interview and full history was taken including age, disease history in the family, other associated disease and drug intake. The result of neck scan, analysis of thyroid hormone, clinical feature, were taken from the case sheet. All patients were investigated in teaching laboratories of medical city /immunology department. In addition, twenty healthy (controls), matched patients for age, were also included in the study.

Blood Collection:

Five milliliter (ml) of venous blood was collected from each patient. Sera were separated after leaving the sample at room temperature for about 15-30 minutes, and then centrifuged at 1500 rpm (revolution per minute) for 5 minute, serum were dispensed into 4-5 Eppendrof tubes, frozen at -20 ℃, and were used for assessment of (LF), (ADA) enzyme and specific protein.

Serum Level of Anti-Lactoferrin Antibodies:

By using immunometric enzyme immunoassay for quantitative determination of auto antibodies against lactoferrin (20).
Highly purified lactoferrin is bound to micro wells. Antibodies against this antigen, is present in diluted serum or plasma, bind to the respective antigen. Washing of the micro wells removes unspecific serum and plasma components. Horseradish peroxidease (HRP) conjugate anti-human IgG immunologically detects the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the micro wells removes unbound

conjugate. An enzyme substrate in the presence of bound conjugate hydrolysis to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photo metrically at 450 nm. The intensity of color is directly proportional to the concentration of IgG antibodies present in the original sample.

Calculation of Sample Results:

The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for samples by using standard curve fitting equations for anti-lactoferrin (Figure -1).

Assessment of ADA enzyme:

(ADA) enzyme activity was measured by the method of (Giusti, 1981). This method depending on an indirect measurement of ammonia production when (ADA) enzyme acting on adenosine.
Ammonia forms an intensely blue indophenols with sodium hypochlorite and phenol in alkaline solution, ammonia concentration is directly proportional to the absorbance of the indophenols at 630nm (21).

Estimation of Total Protein:

Protein ratio was measured by the method of (Bradford, 1976). Using (Biurate) solution. This method depending on the direct interaction of carbomyl group in proteins with alkaline cupper solution to configure a purple compound which was spectro photo metrically assessed at 540 nm (22) .

Statistical Analysis:

The Statistical Analysis System- SAS (2010) was used to effect of different factors in study parameters. The student T test and Chi-square test was used to compare the level among patients and control group. Duncan multiple range test was used to significant compare between means in this study.

Results:

The study involved sixty nodular and multi- nodular goiter patients. Their age ranged from (22-71) years. A twenty healthy individuals matched group served as control.

Anti- Lactoferrin Ab. In nodular and multi- nodular goiter patients:

We analyzed the prevalence of anti-lactoferrin antibodies in a number of patients with thyroiditis (single and multiple nodule goiters) Iraqi Patients. The main clinical and immunological features of the patients are reported in table 1, table 2 and figure 2.
Lactoferrin antibodies were elevated above normal level (10 U/ml) in 14 (23.33%) patients, which was significantly higher than that in normal individuals (p value 0.01) and the mean of positive cases was (4.473 ± 0.206) compared to the negative cases which was (23.676 ±2.137) (P value 0.001) table1.

(ADA) enzyme in nodular and multi- nodular goiter patients with control:

Our results revealed that there are elevation in (ADA) enzyme and also total protein in serum of (single and multiple nodule goiters) Iraqi patients as compared with control group. Table 3, showed an increased in mean value of (ADA) enzyme (4.56^{A} ±1.27) in patients, compared to controls (1.18^{B} ±0.49).

Total Protein in nodular and multi- nodular goiter patients with control:

The table 4, showed high values of total protein with significant difference between patients (21.99^{B} ± 0.33 gm/100ml) and control group (8.37^{A} ±1.31 gm/100ml).

Discussion:

As in table1, table 2 and figure 2, lactoferrin antibodies were elevated above normal level (10 U/ml) in 14 (23.33%) patients, which was significantly higher than that in normal individuals (p value 0.01) and the mean of positive cases was (4.473 ±0.206) compared to the negative cases which was (23.676 ±2.137) (P value 0.001).

Elevated levels of lactoferrin antibodies based on molecular science that have shown that lactoferrin is multitasking protein, exhibit antibacterial, antiviral, anti inflammatory activity and that anti-LF antibodies binding to LF block the anti-inflammatory activity of it, which implies a deterioration in host defense against microorganisms and inflammation (23). Such findings agree with the findings of investigators that lactoferrin immune reactivity was strongly associated with neoplastic proliferation and may be used as a useful auxiliary marker to distinguish malignant from benign thyroid lesions in cytological smears and biopsy samples (24).

In another Immuno histochemical study of lactoferrin in follicular adenomas and thyroid carcinomas, follicular adenomas showed a consistent negativity, whereas follicular and papillary carcinomas exhibited various degrees of positivity for lactoferrin. Incorporated organoid structures observed in anaplastic carcinomas were strongly stained; the spindle cell parts of these cancers were always negative for this iron-binding protein. Medullary carcinomas were also unstained (25). Due to its iron binding properties and interactions with target cells and molecules, lactoferrin can both positively and negatively influence immune system cells and cells involved in the inflammation reaction. In one way, lactoferrin may support the proliferation, differentiation, and activation of immune system cells and strengthen the immune response. On the other hand, lactoferrin acts as an anti-inflammatory factor. Thanks to its antimicrobial activity and capability of binding components of bacterial cell walls (LPS) or their receptors, lactoferrin may prevent the development of inflammation and subsequent tissue damage caused by the release of pro-inflammatory cytokines and reactive oxygen species (26). The protective effect of lactoferrin is manifested in a reduced production of some pro-inflammatory cytokines such as tumor necrosis factor (TNF α) or interleukins IL-1β and IL-6 (27), (28).

Our results revealed that there are elevation in (ADA) enzyme in serum of (single and multiple nodule goiters) patients as compared with control group. Table 3, showed an increased in mean value of (ADA) enzyme (4.56^{A} ± 1.27) in patients, compared to controls (1.18^{B} ± 0.49).In a study of Roberts EL and Roberts OT , 2012, in which paid study on patients with different tumors, Plasma AD2 was measured before and seven to 10 days after the first dose of chemotherapy. The changes in plasma AD2 were then compared with the tumor regression score, following first-dose chemotherapy, plasma AD2 was decreased. The percentage decrease in plasma AD2 correlated with the tumor regression score. These data suggest that plasma AD2 may have a role in determining tumor response to treatment (29). Therefore, our results agree with the researchers study that the level of (ADA) enzyme was raised and the activity is increased in conditions associated with tumor growth. Such findings may highlight the importance of Plasma AD for the screening and monitoring of cancer, as AD2 activity is increased in conditions associated with tumor growth.

Proteins are large, complicated molecules that are vital to the function of all cells and tissues. They are made in many places throughout the body and circulate in the blood. Proteins take a variety of forms, such as; albumin, antibodies and enzymes and have many different functions including; helping to fight disease, regulating body functions, building muscles and transporting drugs and other substances throughout the body and the possible causes of high blood protein include: bone marrow disorder and chronic inflammatory conditions. Certain proteins in the blood may be elevated as the body fights an infection or some other inflammation (30).
In many cases, it results from excessive activity of the thyroid gland, with a pathologically increased production of thyroid hormones (31). A total serum protein test measures the total amount of protein in the blood. It also measures the amounts of two major groups of proteins in the blood: albumin and globulin (32). The results of this study, showed high values of total protein with significant difference between patients (21.99^{B} ±0.33 gm/100ml) and control group (8.37^{A} ± 1.31 gm/100ml). Where the normal value of the total protein is (6.4–8.3) gram per deciliter (g/dL), table 4.

Our results, disagree with another study of Al byati, R. K. M. *et. al.*, 2009, on patients with (Breast, Ovary and Uterus) cancer, Their results indicated the presence of significant decrease ($P<0.05$) in albumin concentration in uterus cancer patients and in pregnant women and non significant difference ($p>0.05$) in breast and ovary cancer patients as compared to normal subjects, also a non significant difference ($p>0.05$) in total protein and globulin concentrations in all patients groups and pregnant women as compared to normal subjects (33). However there is another study support our results that it can be concluded that the total serum protein was higher in female patients with breast cancer, compared to the healthy female group and the results of their study indicate that serum protein levels may be used for the diagnosis of breast cancer (34).

This increase in total serum protein concentration can be due to the fact that total serum protein is composed of albumin and other proteins, collectively termed as globulins, and it is known that the serum albumin concentration may change under oxidative stress, such as the stress associated with cancer (35). In addition, as

the plasma circulates through the tissues, it collects proteins that are released from their original locations due to certain physiological events, including tissue remodeling, trauma and cell death, which lead to an increase in total serum protein (36).

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Table 1: Anti-Lactoferrin Ab in In Thyroiditis (single and multiple nodule goiters) Patients Comparison to Healthy Control.

Study groups	**Anti-Lactoferrin Ab.**			
	Positive (≥10U/ml)		**Negative(<10U/ml)**	
	No.	%	**No.**	**%**
Thyroiditis (single and multiple nodule goiters)	14	23.33	46	76.66
Healthy control	0	0	20	100
Total	14		66	
P-value	0.01*			

Table 2: Mean value of Anti-Lactoferrin Ab in positive and negative Thyroiditis (single and multiple nodule goiters) Patients.

Study groups	**Positive patients**	**Negative patients**	**All patients**
	Mean ±S.E	**Mean ±S.E**	**Mean ±S.E**
	4.473 ±0.206	23.676 ±2.137	7.092 ±0.398
P value	0.001*		

Table 3: (ADA) enzyme activity in serum of single and multiple nodule goiters patients and control.

Groups	(ADA) Activity Unit/mg protein Mean ± SD.
Patients	4.56 ^{A} ± 1.27
Control	1.18 ^{B} ± 0.49

- **Significant differences between groups (P≤ 0.05).**

Table 4: Mean of Total protein value in serum of single and multiple nodule goiters patients and control.

Groups	Total Protein gm/100ml Mean ± SD.
Patients	21.99^{B} ± 0.33
Contrl	8.37 ^{A} ± 1.31

- **Significant differences between groups (P≤ 0.05).**

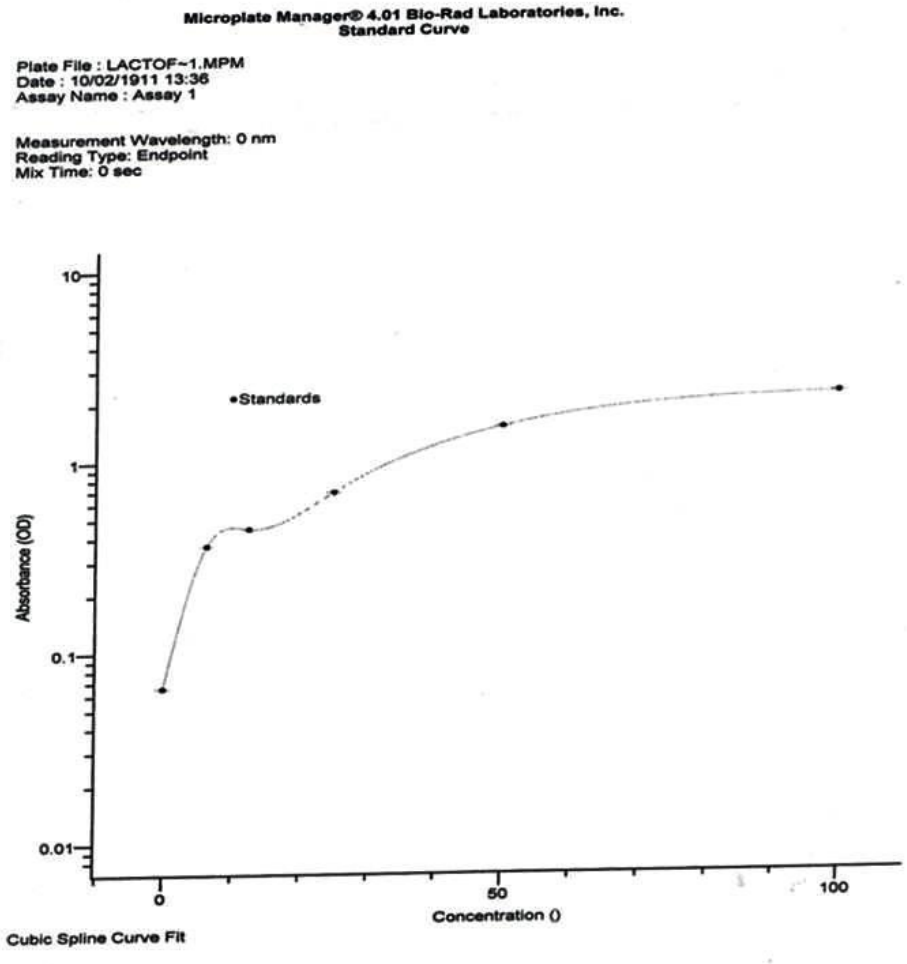

Figure1: ELISA standard curve of Lactoferrin.

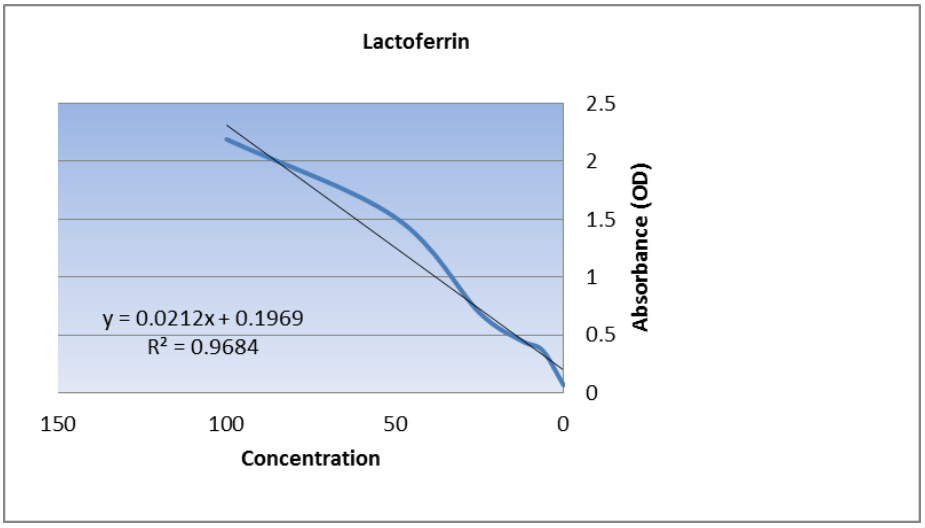

Figure 2: Lactoferrin concentration in serum of single and multiple nodule goiters patients.