Plasma Growth Arrest Specific-6 and Axl Levels in Patients with Hyperthyroidism / Hypothyroidism

Hipertiroidizm/Hipotiroidizm Hastalarında Plazma Growth Arrest Spesific-6 ve Axl Düzeyleri

Ali Eraydın[1], Tuba Taşkan[2], Taylan Turan[1], Reyhan Ersoy [3], Aymelek Gönenç[1]

[1] Gazi University Faculty of Pharmacy, Department of Biochemistry, 06330, Ankara, Turkiye
[2] Afyonkarahisar Health Sciences University Faculty of Pharmacy, Department of Biochemistry, Afyonkarahisar, Turkiye
[3]Yıldırım Beyazıt University Faculty of Medicine, Department of Endocrinology and Metabolism, Ankara, Turkiye

ABSTRACT

Objective: Growth Arrest Specific-6 is the ligand of the Tyro, Axl, and Mer (TAM) receptors subfamily of the receptor tyrosine kinase family. Gas6/Axl signaling pathway is highly regulated in several pathological conditions. The role of Gas6/Axl signaling in hypothyroidism and hyperthyroidism has not been explored previously. In this study, we aim to investigate possible changes in plasma Gas6 and Axl levels in patients with hyperthyroidism and hypothyroidism.
Methods: In the study, 33 patients who were diagnosed with hypothyroidism, 35 patients who were diagnosed with hyperthyroidism and 36 healthy volunteers were included as the control group. Gas6, Axl, IL-6, TNF-α, and AOPP levels in plasma were measured with ELISA kits, MDA and TAC levels were measured with colorimetric kits.
Results: The plasma Axl level of the hyperthyroid patient group was significantly higher than control group ($p<0.05$). MDA levels were significantly higher in both hyperthyroid and hypothyroid patient groups compared to the controls ($p<0.01$, $p<0.01$, respectively). Plasma TAC level were found to be significantly lower, while AOPP were higher than controls in hyperthyroid group ($p<0.05$, $p<0.05$ respectively). In all groups, Gas6 and Axl have positive correlations, both among themselves and with IL-6 and TNF-α levels.
Conclusion: Our results support the hypothesis that the Gas6/Axl signaling pathway may be involved in the pathogenesis of these diseases. In addition, it can be thought that lipid peroxidation increases and antioxidant defense is impaired in both hyperthyroidism and hypothyroidism.

Keywords: Gas6; Axl; IL-6; MDA; hyperthyroidism; hypothyroidism

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ÖZET

Amaç: Growth Arrest Spesifik-6, reseptör tirozin kinaz ailesinin Tyro, Axl ve Mer (TAM) reseptör alt ailesinin ligandıdır. Gas6/Axl sinyal yolu, çeşitli patolojik koşullarda yüksek düzeyde düzenlenir. Gas6/Axl sinyalinin hipotiroidizm ve hipertiroidizmdeki rolü daha önce araştırılmamıştır. Bu çalışmada hipertiroidi ve hipotiroidisi olan hastalarda plazma Gas6 ve Axl düzeylerindeki olası değişiklikleri araştırmayı amaçladık.
Yöntemler: Çalışmaya hipotiroidi tanısı alan 33 hasta, hipertiroidi tanısı alan 35 hasta ve 36 sağlıklı gönüllü dahil edildi. Plazmadaki Gas6, Axl, IL-6, TNF-α ve AOPP seviyeleri ELISA kitleri ile, MDA ve TAC seviyeleri kolorimetrik yöntemlerle ölçülmüştür.
Bulgular: Hipertiroidi hasta grubunun plazma Axl düzeyi kontrol grubuna göre anlamlı derecede yüksekti ($p<0.05$). MDA düzeyleri hem hipertiroidi hem de hipotiroidi hasta gruplarında kontrollere göre anlamlı derecede yüksekti (sırasıyla $p<0.01$, $p<0.01$). Plazma TAC düzeyi hipertiroid grubunda kontrollere göre anlamlı olarak daha düşük, AOPP ise daha yüksek bulundu (sırasıyla $p<0.05$, $p<0.05$). Tüm gruplarda Gas6 ve Axl hem kendi aralarında hem de IL-6 ve TNF-α seviyeleri ile pozitif korelasyona sahiptir.
Sonuç: Sonuçlarımız Gas6/Axl sinyal yolunun bu hastalıkların patogenezinde rol oynayabileceği hipotezini desteklemektedir. Ayrıca hem hipertiroidizmde hem de hipotiroidizmde lipid peroksidasyonunun arttığı ve antioksidan savunmanın bozulduğu düşünülmektedir.

Anahtar Sözcükler: Gas6; Axl; IL-6; MDA; hipertiroidizm; hipotiroidizm

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ORCID IDs: A.E.0000-0001-7530-3362, T.T.0000-0003-1677-5356, T.T.0000-0001-7335-1213, R.E.0000-0002-7437-1176, A.G.0000-0001-9661-8291

Address for Correspondence / Yazışma Adresi: Aymelek Gönenç,MD Gazi University Faculty of Pharmacy, Department of Biochemistry, 06330, Ankara, Turkey E-mail: aymelek@gazi.edu.tr

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INTRODUCTION

Growth Arrest Specific-6 (Gas6) is a protein found in many cell types such as vascular smooth muscle cells, mesangial cells, endothelial cells, bone marrow, stromal cells, macrophages, platelets (1, 2). Studies show that Gas6 protein is associated with various cancer types as well as diseases (3, 4). It has also been stated that plasma Gas6 concentration can be considered as a potential biomarker for early inflammation detection and insulin resistance (5). Gas6 is the ligand of the Tyro, Axl, and Mer (TAM) receptors, a sub-member of the receptor tyrosine kinase family and performs its function by binding these receptors. The Gas6/TAM interaction is involved in the regulation of many functions, including inflammatory cytokine release, stabilization of blood clots, cell adhesion, cell survival, proliferation, apoptosis, migration and invasion (6, 7). It is known that the Gas6/Axl signaling pathway, which is the most studied, among these. The Gas6/Axl signaling pathway is thought to contribute to the formation of atherosclerosis and restenosis lesions (8). For this reason, the possible effect of the Gas6/Axl signaling pathway on atherosclerosis formation in hypothyroidism is remarkable. On the other hand, Axl and Gas6 interaction leads to the production of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and type I interferons (9). There are various studies showing that these cytokines are related to the pathogenesis of thyroid diseases (10, 11). Reactive oxygen species (ROS) and their intermediates are also thought to be effective in the development of autoimmune diseases in some endocrine glands, including thyroid diseases. Oxidative stress has been reported as an important factor in the pathogenesis of Graves' disease, one of the most important autoimmune diseases of the thyroid gland (12). Also, it is known that there is a close relationship between oxidant-antioxidant balance and thyroid disorders. Advanced oxidation protein products (AOPP) are the tyrosine containing cross-linked protein that is a biomarker to evaluate oxidant-mediated damage (13). There is no study to evaluate the plasma Gas6 and Axl levels in overt hypothyroidism and hyperthyroidism. In the present study, we aimed to measure the levels of Gas6, Axl, IL-6, TNF-α, total antioxidant capacity (TAC), malondialdehyde (MDA), and AOPP in patients have thyroid dysfunction.

METHODS

Collection of Blood Samples

The study included 35 patients with hyperthyroidism and 33 patients with hypothyroidism who were newly diagnosed and not yet started to be treated and 36 healthy volunteers without any systemic or metabolic disease who applied to endocrinology outpatient clinics of Ankara Yildirim Beyazıt University Faculty of Medicine between March 2018 and June 2019. In all study groups, patients with thyroid disease, diabetes and cancer, or people who have been treated for cancer, pregnant women, liver or kidney disease, chronic heart failure, existing inflammation were excluded, and individuals younger than 18 years of age and older than 65 years were selected within the scope of exclusion criteria. Clinical studies on Gas6, Axl and IL-6 related diseases were considered in the selection of exclusion criteria. Within the scope of the study, biochemical tests (TSH, fT3 and fT4) were performed on the control group and it was determined that there was no subclinical hyperthyroidism or hypothyroidism. Biochemical measurements (TSH, fT3 and fT4), thyroid ultrasonography and thyroid scintigraphy were used in the diagnosis of hyperthyroidism and hypothyroidism. Plasma was separated by centrifuge for 15 min at 2300 g, from the blood samples taken into the tubes with K3EDTA. Samples were stored at -80°C until analysis.

In accordance with the Helsinki declaration, Gazi University Clinical Research Ethics Committee approved this study and obtained informed consent from both patients and controls.

Gas6, Axl, IL-6, TNF-α and AOPP Measurements

Gas6, IL-6, TNF-α and AOPP measurements in plasma were done by ELISA method using Bioassay kits (Bioassay Technology Laboratory, China), and Axl measurement in plasma was performed by ELISA method using SunRed kit (SunRed Biotechnology, China) according to the manufacturer's instructions.

50 µL of standard and the blank solution were added into the wells of the plate. Then 40 µL of sample and 10 µL of antibody solution were added to the remaining wells. 50 µL of streptavidin HRP solution was added on all wells (except blank) and incubated at 37°C for 60 min. After incubation step, plate was inverted to empty all contents and were washed 5 times with 350 µL washing buffer. Afterward, 50 µL of solution A and then 50 µL of solution B were added to each well and incubated at 37°C for 10 min. Optical density was measured at 450 nm wavelength after adding 50 µL of stop solution to each well.

Mean intra-assay CVs were 2.24% for Gas6; 2.99% for Axl; 2.97% for IL-6; 8% for TNF-α and 8% for AOPP. Mean inter-assay CVs were 5% for Gas6; 5.45% for Axl; 3.55% for IL-6; 10% for TNF-α and 10% for AOPP.

MDA Measurement

MDA in plasma was measured using the method based on the conjugation reaction of MDA with thiobarbituric acid (14). After taking 100 µL of the standard solutions prepared, 500 µL of 20% (w/v) trichloroacetic acid and 1000 µL of 0.67% (w/h) thiobarbituric solution were added to water and kept in a 100°C water bath for one hour. The solution was then cooled in an ice bath. While the solution was at 50°C, it was centrifuged at 12000 g for 5 min. The optical density of the solution after centrifugation was measured at a wavelength of 532 nm. The intra-assay CV is <4.04% and inter-assay precision CV is <5.11%.

TAC Measurement

TAC measurement in plasma was performed by colorimetric method using Rel Assay kit (Relassay diagnostics, Turkey) (15). 18 µL of distilled water, 18 µL of standard (1mmol/L Trolox) and 18 µL of the sample were added to the 96-well plate. Then, 300 µL reagent 1 (Acetate Buffer, 0.4 M, pH 5.8) was added onto each well and the absorbance at 660 nm was measured. After the first measurement, 45 µL of reagent 2 (prochromogen solution, 30 mM) was added to each well and mixed for 5 min at 37°C. Finally, absorbance was measured at 660 nm. The intra-assay CV is 2.45% and the inter-assay precision CV is 2.60%.

Measurement of Routine Biochemical Parameters

Fasting blood glucose, total cholesterol and triglyceride levels were measured in Roche device (Roche Diagnostics, Mannheim, Germany) and Roche Cobas e501 analyzer kit using competitive ELISA-based electrochemiluminescence immunoassay (ECLIA) method; while TSH, fT3 and fT4 levels were measured the ECLIA method using the Roche Cobas e6000 analyzer kit in same device.

Statistical Analysis

Statistical analysis of the data was evaluated using the SPSS 22.0 statistical package program. Descriptive statistics were given with arithmetic mean±standard error values. The distribution of the data was examined with the Kolmogorov-Smirnov normality test. " Independent sample T-test " was used for the analysis between the means of two independent groups and "one-way analysis of variance" (ANOVA) was used for the comparison of more than two groups. The effect of more than one factor on a variable was examined by "analysis of multiple variances". "Pearson correlation coefficient" was used for the relationship between the levels of the measured parameters.

RESULTS

The clinical characteristics and some biochemical parameters of the study group were given in Table 1. In patients with hyperthyroidism, fT3 and fT4 levels were higher than the control group ($p<0.01$, $p<0.01$, respectively) and TSH levels were lower ($p<0.01$). Total cholesterol, fT3 and TSH levels were found to be higher in patients with hypothyroidism compared to healthy controls ($p<0.01$, $p<0.05$, $p<0.01$, respectively), and fT4 levels were found to be lower ($p<0.05$).

Table 1. The clinical characteristics and some biochemical parameters of the study group.

Parameters	Hyperthyroidism (N=35)	Hypothyroidism (N=33)	Control (N=36)
Female / Male	19/16	25/8	23/13
Age (years)	39.77±2.12	44.55±2.63	42.11±2.03
BMI (kg/m²)	24.66±0.51	27.84±1.28	24.03±0.50
Glucose (mg/dL)	92.29±1.43	91.52±1.86	91.69±1.67
Total cholesterol (mg/dL)	151.82±5.42	179.36±8.51**	140.25±7.04
Triglyceride (mg/dL)	128.67±12.78	119.82±12.05	124.55±6.52
fT_3 (pg/mL)	7.53±0.94**	3.08±0.13*	2.54±0.13
fT_4 (ng/dL)	2.45±0.24**	1.09±0.05*	1.26±0.05
TSH (µIU/mL)	0.02±0.006**	12.77±3.47**	2.22±0.16

*Significant difference from control group (p<0.05),
**Significant difference from control group (p<0.01)

In Figure 1 plasma Axl level of the hyperthyroid and hypothyroid patients and the control group were given. The hyperthyroid patient group (10.19±2.35 ng/mL) was found to be significantly higher than the healthy controls (3.71±1.22 ng/mL; p<0.05).

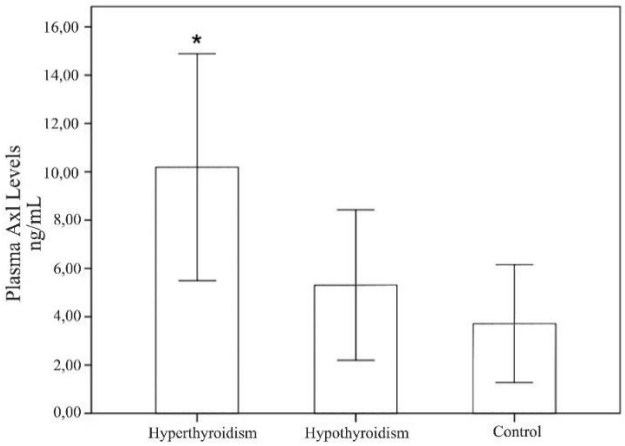

Figure 1. Comparison of plasma Axl levels in study groups.
*Significant difference from control group (p<0.05)

Plasma Gas6 levels, which were found as 74.79±15.89 ng/mL in patient with hyperthyroidism, 41.01±11.73 ng/mL in patient with hypothyroidism, 65.96±15.03 ng/mL in the control group (p>0.05). No significant difference was found between patient groups and controls.In Figure 2, plasma MDA levels of the hyperthyroid and hypothyroid patients and the control group were given. Plasma MDA levels of the hyperthyroid patients (5.72±0.20 µmol/L) and the hypothyroid patients (5.28±0.23 µmol/L) were found to be significantly higher than healthy controls (1.44±0.09 µmol/L) (p<0.01, p<0.01, respectively).

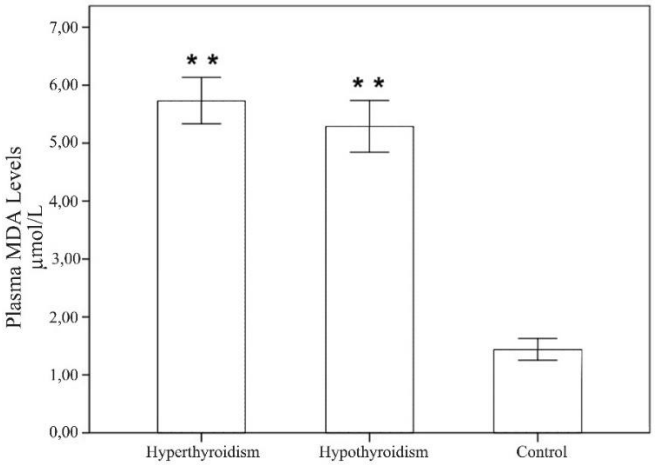

Figure 2. Comparison of plasma MDA levels in study groups.
**Significant difference from control group (p<0.01).

Figure 3 demonstrates the plasma TAC levels in the study groups. Plasma TAC levels were found to be significantly lower in patients with hyperthyroidism (1.71±0.12 mmol/L) than healthy controls (2.06±0.09 mmol/L; p<0.05).

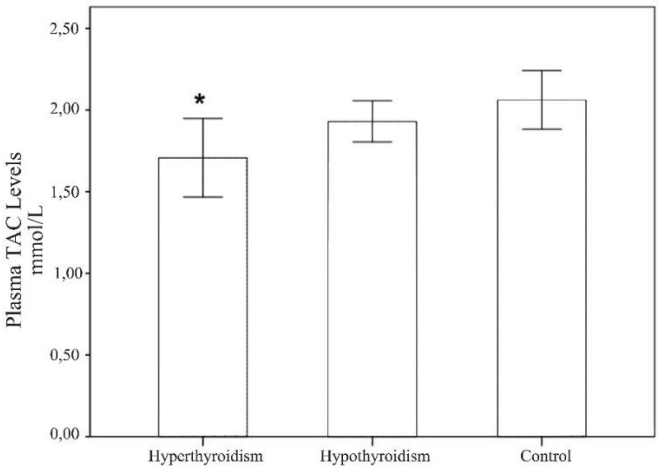

Figure 3. Comparison of plasma TAC levels in study groups.
*Significant difference from control group (p<0.05)

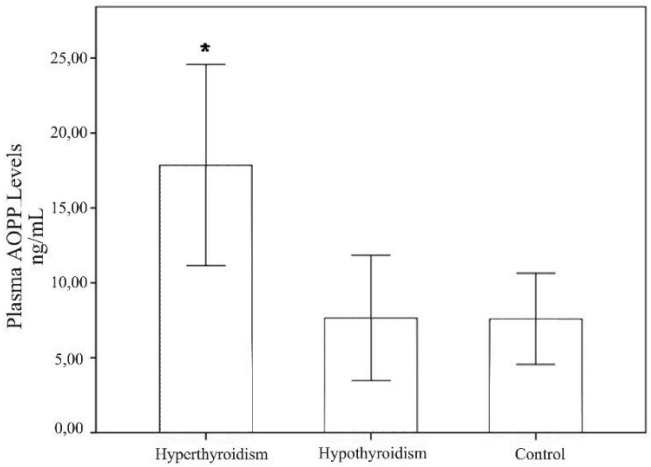

Figure 4. Comparison of plasma AOPP levels in study groups.
*Significant difference from control group (p<0.05)

The plasma AOPP level of the hyperthyroid patients (17.84±3.31 ng/mL) was found to be significantly higher than healthy controls (7.57±1.51 ng/mL; p<0.05). (Figure 4).

No significant differences were found among the study groups in terms of the plasma levels of IL-6 and TNF-α. IL-6 level was 4.23±0.54 pg/mL in hypothyroidism, 3.24±0.46 pg/mL in hyperthyroidism, 3.48±0.50 pg/mL in control and TNF-α level was 367.09±69.90 ng/mL in hypothyroidism, 179.22±60.96 ng/mL in hyperthyroidism, 268.30±68.04 ng/mL in control. There were found correlations in patients with hypothyroidism, hyperthyroidism, and healthy controls. Correlations between Gas6 and Axl in patient groups and controls were given in Figure 5.

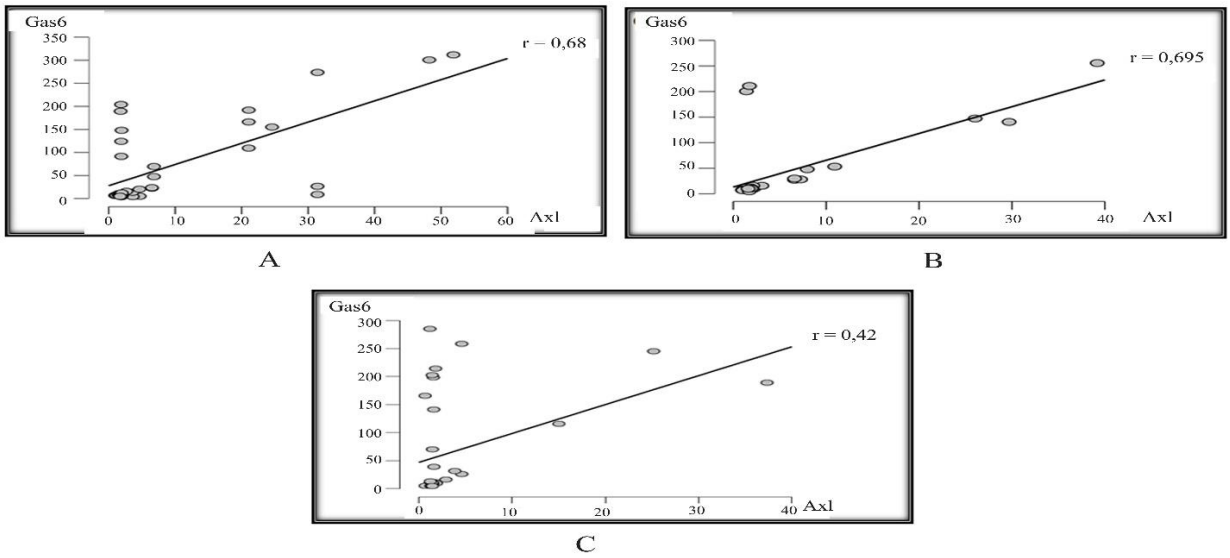

Figure 5. Gas6&Axl correlations in A) Hyperthyroidism B) Hypothyroidism C) Control group.

DISCUSSION

The Gas6/Axl signaling pathway is involved in the realization of many cellular functions such as cell growth, survival, migration, apoptosis, cell adhesion and proliferation. Although there have been studies in relation with thyroid cancer, there are no studies in the literature on thyroid dysfunction. In this study, we aimed to investigate plasma Gas6 and Axl concentrations as well as oxidative stress parameters MDA, TAC and AOPP and inflammation parameters IL-6 and TNF-α levels in patients with hyperthyroidism and hypothyroidism.

Gas6/Axl pathway is involved in inflammation, viral infection, immune response, angiogenesis, cancer development, metastasis, thromboembolic diseases, atherosclerosis, sepsis, multiple sclerosis and other autoimmune diseases (16).

There are many studies showing that the expression or blood levels of Axl and its ligand, Gas6. Researchers found that Gas6 increases endothelial cell activation, Axl mediates the endothelial response to Gas6, and Gas6 increases the accumulation of platelets and leukocytes in the activated endothelium (17). In a study which is investigating the activation and relationships of Gas6 and TAM receptors by Gong et al., serum Gas6, Axl and Mer levels of lupus erythematosus were found to be higher (18). In another study, myocardial expression and serum level of Axl increased in patients with heart failure (19). The relationship between plasma Gas6 levels and carotid atherosclerotic plaque formation was investigated in patients with high cardiac risk, and Holden et al. were reported that Gas6 may play a protective role in the formation of atherosclerotic plaque in humans (20). Similarly, Clauser et al. reported that increased secretion of Gas6 by muscle cells in atherosclerotic plaques and pointed to the potential role of Gas6 as a protective factor for atherosclerosis (21).

Contrary to these studies, Hung et. al. was found decreased plasma Gas6 concentrations in diabetic patients (22). There are few studies investigating the Gas6/Axl pathway in thyroid diseases. It has been found that Gas6 and Axl are co-expressed in cancerous thyroid tissue, the survival and proliferation of Gas6-stimulated cancer cells are increased, and the reduction of Axl activity through siRNA stops the viability of cancer cells (23). Possible effects of Gas6 gene expression on iodine-induced autoimmune thyroiditis were investigated, it was stated that Gas6 was reduced in inflamed thyroid tissue and an anti-inflammatory effect occurred in mice treated with recombinant Gas6, and recombinant Gas6 may have a possible therapeutic value for patients with autoimmune thyroiditis (24). In our study, although the plasma Gas6 level was higher in patients with hyperthyroid compared to healthy subjects, there was not found significance. However, the plasma Axl level of the hyperthyroid patient group was higher than healthy subjects. The high level of Axl can be explained by the increase in activation of Gas6-mediated Axl signaling pathway in hyperthyroidism in parallel with the increase of Gas6. In addition, the correlations observed between Gas6 and Axl in the study group support this finding.

Gas6 is involved in cell protection and tissue formation and can also inhibit inflammatory responses. Gas6 treatment inhibits the activation of the NF-κB signaling pathway and decreases the production of IL-6, TNF-α in mice with acute liver injury (25). Jia et al. investigate the expression level of IL-6 in patients with goiter and hyperthyroidism and determined that IL-6 is expressed at a high level in the thyroid follicles of the patients (28). Serum IL-6 levels were found to be significantly higher in both the hyperthyroid and hypothyroid patients compared to the controls (27). Sekeroğlu et al. compared the serum cytokine of hyperthyroid patients and found that IL-6 level was higher in the hyperthyroid patient group (11). Decrease in serum thyroid hormone concentration has been reported with the increase in serum IL-6 concentration. (28). It has been reported that serum levels of TNF-α are significantly higher in patients with hyperthyroidism and primary hypothyroidism compared to the control group. These results are not compatible with the data we obtained (29, 30). In our study, any differences were found between the groups in terms of IL-6 and TNF-α. In hyperthyroidism and hypothyroidism, we found correlations of IL-6 and TNF-α with both Gas6 and Axl concentrations.

Various mechanisms such as oxidative stress induced Ca^{2+} influx, which is responsible for inflammatory processes, and overexpression of endothelium that induces ROS production in endothelial cells by increasing NADPH oxidase activity, prove the relationship between oxidative stress and inflammation (31). Axl is activated by ROS to regulate signal transduction, increasing its interaction with myosin heavy chain-IIB, thereby mediating migration in vascular dysfunction (32). It has been suggested that ROS induced by high levels of thyroid hormones cause an increase in the lipid peroxidation product of MDA and normalization of MDA levels with treatment (33). Tejovathi et al. investigate the effects of lipid peroxidation together with endothelial dysfunction in patients with hypothyroidism and stated that increased MDA levels indicate the formation of ROS and oxidative stress may have an effect on endothelial dysfunction in hypothyroidism (34). Serum MDA levels of patients with overt hyperthyroidism and subclinical hyperthyroidism were reported to be significantly higher than the control group (35). The results obtained in our study are consistent with the studies (33, 35) that showed high plasma MDA levels in both hyperthyroidism and hypothyroidism. Similarly, increased plasma MDA levels were measured in the hypothyroid patient group compared to healthy individuals. This situation is consistent with literature and indicates the increased lipid peroxidation in both disorders. It can be thought that this increase is related to the disruption of oxidant-antioxidant balance AOPP, which occur as a result of the effect of free radicals on proteins, is reliable marker used to determine the extent of oxidant-mediated protein damage (36). Serum AOPP levels are higher in thyroid cancer than healthy controls (36, 37). On the other hand, there are no studies in overt hyperthyroidism and hyperthyroidism related with AOPP. As seen in our results, higher AOPP in patients with hyperthyroidism suggests that it may be an indicator of the presence of progressive protein damage in hyperthyroidism. Also, correlations of AOPP with Gas6 and Axl in both patient groups suggest that protein damage may be associated with the Gas6/Axl signaling pathway. Aslan et al. evaluate the antioxidant status in patients with hyperthyroidism and reported that serum TAC levels in hyperthyroid patients were found to be significantly lower than healthy individuals (38).

It has been reported that ROS are increased, intracellular and extracellular antioxidant systems are impaired in Graves' disease (12). Patients with both overt and subclinical hyperthyroidism were found to have low serum TAC levels associated with T_3 concentrations (39). In our study, plasma TAC level of the hyperthyroid patient group was found to be significantly lower than healthy controls. The data we have obtained are consistent with the literature studies (38, 39) demonstrating that increased thyroid hormone level increases oxidative stress and impairs the oxidative-antioxidative balance in the direction of oxidation in hyperthyroidism. Our results support that the decrease of antioxidant may expose patients with hyperthyroidism to free radical product damage.

The limitations of our study are that it cannot be divided into sub-study groups according to the diseases that cause hyperthyroidism and hypothyroidism due to the relatively low number of samples collected, and the changes in these parameters cannot be examined according to the etiopathogenesis of these diseases. Although one of the limitations of the study was one of the exclusion criteria is pregnancy, breastfeeding status, menstrual cycle periods in women, and menopause/andropause conditions were not taken into account.

CONCLUSION

The fact that the Axl levels of hyperthyroid patients are higher and the strong relationship between Gas6 and Axl in both hyperthyroid and hypothyroid patients support the hypothesis that the Gas6/Axl signaling pathway may be involved in the pathogenesis of these disorders. It is thought that lipid peroxidation increases and antioxidant defence is impaired due to changing thyroid hormones in both hyperthyroidism and hypothyroidism. We think that the effects of the Gas6/Axl signaling pathway on the thyroid hormone mechanism should be supported by new studies in this issue.

Conflict of interest
No conflict of interest was declared by the authors.

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