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The role of fetuin-A in vascular aging in the rat aorta

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ABSTRACT

Objectives: In this study, we aimed to investigate the relationship between vascular system changes due to the increased inflammatory response and oxidative stress with advanced age and fetuin-A levels in aortic tissues of a naturally aged rat model.

Materials and methods: A total of 16 Wistar albino rats were equally divided into two groups as the young group and the elderly group. The thoracic aorta was excised. The effect of aging on the aorta, proliferation, oxidative stress, and inflammation markers were evaluated using the real-time polymerase chain reaction method. Histological examination was performed to confirm the findings related to aging, and serum sampling was performed to determine fetuin-A levels.

Results: Interleukin-6 levels were lower in the elderly group (1.007 *vs.* 0.099-fold decrease, respectively; p=0.000). There was a moderate, positive correlation between fetuin-A and interleukin-6 levels (r=0.56; p=0.03). There was no significant difference in the antioxidant capacity as assessed by superoxide dismutase-1, while the oxidative stress markers were significantly higher in elderly rats (inducible nitric oxide synthase: 1 *vs.* 812.3-fold increase, respectively; p=0.006; endothelial nitric oxide synthase: 0.98 *vs.* 3.65-fold increase, respectively; p=0.001). There was a moderate, negative correlation between fetuin-A and endothelial nitric oxide synthase (r=-0.56; p=0.024).

Conclusion: Vascular senescence causes cell damage through inflammatory responses. Fetuin-A may indicate early vascular damage.

Keywords: Atherosclerosis, fetuin-A, inflammation, rat, senescence.

Progressive decrease in cellular functions in aging is seen due to the increased oxidative stress and increased inflammatory response.[1] The underlying mechanisms leading to the development of vascular damage due to aging have been elucidated in recent years. Some signaling networks, with increased oxidative stress due to aging and triggering of inflammation, can explain the development of cardiovascular diseases in animal models in the *in vitro* and *in vivo* settings due to vascular stress and inflammatory response. Furthermore, inhibitions of these signaling networks have been shown to delay the onset of these cardiovascular diseases and aging.[2]

Increased permeability, deterioration of the extracellular matrix, impaired intravascular laminar flow and endothelial damage, increased protease activity, damaged endothelium-thrombus aggregation relationship, leukocyte adhesion, formation of foam cells and atherosclerotic plaque formation are the triggering factors in many cardiovascular pathologies.[3] Intimal layer

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changes as a result of increased collagen deposition and weakening of vascular elasticity are similar to the changes that occur during atherosclerosis. With smooth muscle proliferation and increased elastase activity in the vascular media layer, it is difficult to distinguish endothelial apoptosis and endothelial dysfunction in old age from endothelial damage seen only in cases such as atherosclerosis, hypercholesterolemia, and metabolic syndrome secondary to hypertension.[4,5]

Depending on vascular aging, genetic predisposition, family history and smoking, it may show symptoms at early ages, in the fourth and fifth decades of life. As a result, fetuin-A, also called alpha2-Heremans-Schmid glycoprotein, is a prevalent anti-inflammatory glycoprotein that resists the release of proinflammatory cytokines.[6,7] Decreased levels of fetuin-A indicate an increased risk of cardiovascular disease accompanied by metabolic syndrome.[8,9] As fetuin-A levels decrease, the production of inflammatory cytokines increases as a result of adipocyte-related inflammation.[10] This condition is associated with the increased rate of cardiovascular disease.[11,12] Recent studies have shown that low fetuin-A levels have a prognostic value in acute coronary syndromes.[13]

Inflammatory cytokines are suppressed by keeping high grades of fetuin-A. Low grades of fetuin-A may indicate many cardiovascular pathologies triggered by increased inflammation. Currently, the increased inflammatory response and related outcomes in the presence of peripheral arterial disease, family history, diabetes mellitus, and increased cardiovascular risk are a matter of interest for many researchers. In this study, we aimed to investigate the relationship between vascular system changes due to the increased inflammatory response and oxidative stress with advanced age and fetuin-A levels in aortic tissues of a naturally aged rat model.

MATERIALS AND METHODS

Experimental model

A total of 16 male adult Wistar-Albino rats weighing 325 to 425 g supplied from the Experimental Medicine Research and Application Unit of Kocaeli University were used in this study. First, a naturally aged rat model was created during a 24-month period. The rats were maintained under standard controlled humidity laboratory conditions (45%), temperature (22±2˚C), and lighting (from 07:00 AM to 07:00 PM). Water and food were given ad libitum. The experimental protocol in the current study was conducted in accordance with the National Institutes of Health (NIH) Guidelines (NIH publication No. 8023), the European Communities Council Directive of November 24, 1986; 86/609/EEC), and Regulation of Animal Research Ethics Committee in our country (July 6, 2006; Number 26220). The study protocol was approved by the Animal Experiments Local Ethics Committee of Kocaeli University, Faculty of Medicine (No: 4/2-2020; Date: 25/06/2020). All rats were randomly divided into two equal groups: young group (8-week-old) and elderly group (24-month-old).

The rats were first anesthetized with ketamine hydrochloride (Ketalar®; Pfizer, Istanbul, Turkey), 100 mg per kg, intraperitoneally. After anesthesia, the rats were euthanized with a lethal dose of sodium thiopental injection (pentothal sodium; Abbott Laboratories, Italy). After the thoracic aortas were explored with a median sternotomy, they were removed and placed in 0.9% saline for tissue examinations.

Hematoxylin and eosin (H-E) staining

Aortic tissues obtained after sacrification of animals were rapidly washed with physiological saline and fixed in 10% neutral buffered formalin for 72 h for light microscopic studies. Tissues were, then, dehydrated by passing through a series of rising alcohol (70%, 90%, 96%, 100%), cleared by holding in xylene for 2×10 min, kept in paraffin overnight in a 60˚C oven and, then, blocked in cassettes with an embedding device.

The H-E staining was applied to the sections taken from paraffin blocks with a thickness of about 4 μm for morphological evaluation. The sections were, then, examined and photographed with a light microscope (LEICA DM 1000; Leica Microsystems GmbH, Wetzlar, Germany) (Figure 1a, b).

Gene expression analysis

Thoracic aorta was excised for the analysis. The effects of aging in the aortic tissues were evaluated by the gene expression analysis of proliferation, oxidative stress, and inflammation markers (Table 1). Real-time polymerase chain reaction (RT-PCR) method was used for the analysis of gene expression. The Maxima H Minus First Strand complementary deoxyribonucleic acid (cDNA) synthesis kit (Thermo Scientific, Waltham, MA, USA) was used for cDNA synthesis.

Biochemical analysis of fetuin-A

Blood samples were collected from terminally anaesthetized rats. The serum was prepared by centrifugation at 1,000 g for 15 min at 4°C and stored at -40°C for biochemical analysis. The circulating levels of fetuin-A were analyzed with rat fetuin-A enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory; Cat. No. E0580Ra) according to the manufacturer's instructions. All measurements were performed by the VersaMax microplate reader at a wavelength of 450 nm (Molecular Device, CA, USA).

Statistically analysis

Statistical analysis was performed using the IBM SPSS for Windows version 20.0 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was used to check the normality of data distribution. Continuous variables were expressed in mean ± standard deviation (SD) or median (25^{th}-75^{th} percentiles). Comparisons of nonnormally distributed continuous variables between the groups were performed using the Mann-Whitney U test. The relationship between numerical variables was evaluated using the Spearman and Pearson correlation analyses. A p value of <0.05 was considered statistically significant.

RESULTS

Compared to the tissues of the young rats, the expression of transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-1β increased in the elderly group; however, it did not reach statistical significance (p>0.05). The anti-oxidative capacity was slightly decreased in the older tissues. In the histological study, inflammatory responses with degenerate cardiomyocytes with irregular myofibrils were confirmed in the elderly group (Figure 1a, b). The mean fetuin-A level was 15.0±2.9 ng/mL in young rats, while it was 11.1±2.1 ng/mL in elderly rats, indicating a statistically significant difference (p=0.007) (Table 1).

The IL-6 levels were also lower in the elderly group (1.007 *vs.* 0.099-fold decrease, respectively; p=0.000). There was a moderate, positive correlation between fetuin-A and IL-6 levels (r=0.56; p=0.03). There was no significant difference in the antioxidant capacity as assessed by superoxide dismutase-1 (SOD1), while the oxidative stress markers were significantly higher in elderly rats (inducible nitric oxide synthase [iNOS]: 1 *vs.* 812.3-fold increase, respectively; p=0.006; endothelial nitric oxide synthase [eNOS]: 0.98 *vs.* 3.65-fold increase, respectively; p=0.001). There was a moderate, negative correlation between fetuin-A and endothelial nitric oxide synthase (r=-0.56; p=0.024).

DISCUSSION

Aging is a physiological process characterized by the increased oxidative stress and vascular senescence that causes cell damage through inflammatory responses. In the study of Merx et al.,[14] the increased cardiovascular mortality was observed in dialysis patients with fetuin-A deficiency. In this study, myocardial calcification and diffuse soft tissue calcification were noted in fetuin-A-knockout

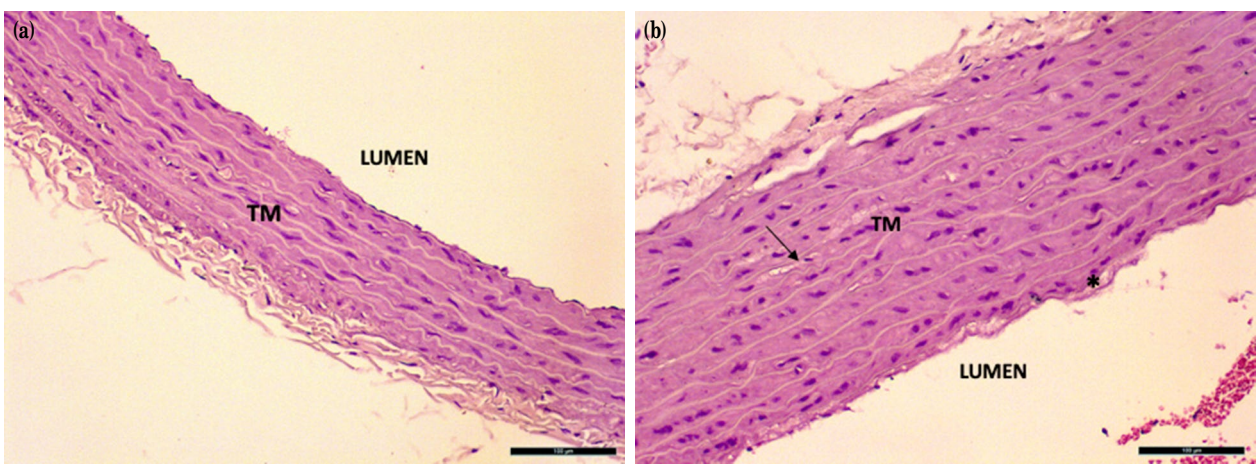

Figure 1. Aortic tissue of **(a)** young rat **(b)** elderly rat (100 μm scale).
TM: Tunica media.

Table 1. The results of the gene expression analysis with biochemical analysis of fetuin-A

	Young rats			Elderly rats				
	Mean±SD	Median	25th-75th percentiles	Mean±SD	Median	25th-75th percentiles	Exact p	*p* value
Fetuin-A	14.5±2.9			11.1±2.1			NA	0.007
SOD-1		1.06	0.58-1.78		0.44	0.16-2.08	0.279	0.248
TNF-α		1.014	0.55-1.77		1.127	0.771-2.173	0.442	0.401
IL-1β		1.001	0.822-1.264		1.68	0.991-3.035	0.065	0.059
IL-6		1.007	0.373-3.183		0.099	0.000-0.198	<0.001	<0.001
NF-κB		0.969	0.694-1.594		1.074	0.531-2.023	0.878	0.834
TGF-β		0.969	0.916-1.139		2.739	0.804-6.276	0.279	0.248
i-NOS		1.00	1.00-2.313		812.287	6.039-19555.483	0.005	0.006
e-NOS		0.983	0.780-1.337		3.65	2.526-8.474	<0.001	0.001
Ki-67		0.969	0.898-1.117		1.59	0.833-2.687	0.798	0.753
PDGFR-β	1.0±0.2			0.8±0.7			NA	0.46

SD: Standard deviation; SOD-1: Superoxide dismutase-1; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; NF-κB: Nuclear factor-kappa B; TGF-β: Transforming growth factor-beta; i-NOS: Inducible nitric oxide synthase; e-NOS: Endothelial nitric oxide synthase; Ki-67: A protein known as a proliferation marker; PDGFR-β: Platelet-derived growth factor receptor-beta.

(fetuin-KO) mice. Isolated myocardial calcification was also detected in fetuin-KO mice independent of arterial stiffness. In the study of Wang et al.,[15] peripheral administration of fetuin-A was shown to alleviate early cerebral ischemic damage in rats. Its protective effect from ischemic injury suggests that fetuin-A can be investigated in many ischemia-reperfusion models in the future. Contrary to most fetuin-A studies, there are few studies in the literature showing that high fetuin-A levels may cause increased myocardial infarction and ischemic stroke.[16] In our study, we showed that fetuin-A played a role as a reverse marker in the presence of oxidative damage and inflammation.

Fetuin-A, a protein produced extensively by liver cells and circulating in high concentrations, has a protective effect against insulin resistance. It suppresses the increase in tyrosine kinase activity in insulin resistance and, in this way, it exerts a protective effect from insulin resistance and metabolic syndrome.[17] Recent studies have shown that it is also excreted in the adipose tissue, and this excretion is richer in adipose tissue. The presence of such a protective effect of an organism in metabolic processes, as in our study, suggests that anti-inflammatory and antioxidant effects are pronounced at high levels and low levels of fetuin-A indicate pathologies such as insulin resistance, endothelial damage, and atherosclerosis.

In conclusion, based on our study findings, fetuin-A, which can be easily measured in blood, may be used as a marker of vascular aging. We believe that these preliminary findings may further enlighten the principles of prevention of vascular aging and cardiovascular protection. With the early detection of vascular aging, it may be easier to take precautions. Therefore, fetuin-A may be a marker of pathologies that need to be evaluated and clarified in early vascular damage.

Declaration of conflicting interests

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