Endothelin Receptor-Independent Correlation Between HSP47 and Collagen in Rabbit Model of Early Atherosclerosis

Tavşan Erken Ateroskleroz Modelinde HSP47 ile Kolajen Arasındaki Endotelin Reseptöründen Bağımsız İlişki

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ABSTRACT Objective: Collagen in the extracellular matrix (ECM) plays an important role in modulation of response to the vascular injury during the progression of atherosclerosis and restenosis. Collagen can regulate smooth muscle cell (SMC) proliferation, migration and matrix metalloproteinase (MMP) production. Therefore, collagen turnover in the arteries is an important determinant of intimal thickening. Heat shock protein 47 (HSP47), a collagen-specific molecular chaperone, is thought to be essential for the processing and secretion of procollagen molecules. Endothelin (ET) is a strong chemoatractant and mitogen promoting SMC proliferation and migration. The aim of this study was to investigate the possible role of HSP47 and its relation to collagen synthesis, and the effects of a nonselective ETA/ETB receptor antagonist, TAK-044 in collar-induced early atherosclerosis model. **Material and Methods:** New Zealand white rabbits were divided into two groups. Both groups received vehicle (0.9% NaCl 0.8 ml/kg/day, s.c.) or TAK-044 (5 mg/kg/day, s.c.) for three weeks. On 8th day, a non-occlusive silicon collar was placed around the left carotid artery. The right carotid artery was sham-operated. HSP47 expression in carotid arteries were determined immunohistochemically. Furthermore, total collagen levels, collagen expression and type I procollagen expression were established. **Results:** HSP47 expression correlated with collagen expression did not change in collared arteries. TAK-044 treatment did not affect HSP47 and collagen levels. **Conclusion:** There was a correlation between HSP47 expression and collagen expression in carotid arteries. However, intimal thickening did not induce HSP47 expression and early collagen development. The ineffectivenes of TAK-044 suggests that ET-1 signaling may not be implicated in HSP47 and collagen in this model.

Key Words: HSP47 heat-shock proteins; atherosclerosis; collagen; endothelin-1; rabbits

ÖZET Amaç: Ekstrasellüler matriksteki (ESM) kolajen ateroskleroz ve restenozun gelişimi sırasında vasküler hasara yanıtın modülasyonunda önemli bir rol oynar. Kolajen düz kas hücresi proliferasyonunu, migrasyonunu ve matriks metalloproteinaz (MMP) üretimini düzenleyebilir. Bu nedenle arterlerdeki kolajen döngüsü intimal kalınlaşmanın önemli bir belirtecidir. Kolajene özgü moleküler bir şaperon olan ısı şoku proteini 47'nin (HSP47) prokolajen moleküllerinin işlenmesi ve sekresyonu için temel olduğu düşünülmektedir. Endotelin (ET) düz kas hücre proliferasyonunu ve migrasyonunu teşvik eden güçlü bir kemoatraktan ve mitojendir. Bu çalışmanın amacı yaka ile oluşturulan erken ateroskleroz modelinde HSP47'nin olası rolünü ve kolajen senteziyle ilişkisini, ayrıca seçici olmayan ETA/ETB reseptör antagonisti olan TAK-044'ün etkilerini araştırmaktır. **Gereç ve Yöntemler:** Yeni Zelanda beyaz tavşanlar iki gruba ayrıldı. Her iki gruba üç hafta boyunca taşıyıcı (0.9% NaCl 0.8 ml/kg/day, s.k.) veya TAK-044 (5 mg/kg/gün, s.k.) verildi. Sekizinci günde sol karotit arter çevresine sıkıştırıcı olmayan silikon bir yaka yerleştirildi. Sağ karotit artere yalancı girişim uygulandı. Karotit arterlerdeki HSP47 ekspresyonları immünohistokimyasal olarak belirlendi. Ayrıca toplam kolajen düzeyleri, kolajen ve tip 1 prokolajen ekspresyonları saptandı. **Bulgular:** Kolajen ekspresyonu ile ilişki halindeki HSP47 ekspresyonu yaka uygulanan arterlerde değişmedi. TAK-044 tedavisi HSP47 ve kolajen düzeylerini etkilemedi. **Sonuç:** Karotit arterlerde HSP47 ile kolajen ekspresyonları arasında ilişki vardır. Bununla birlikte, intimal kalınlaşma gelişimi HSP47 ekspresyonunu ve erken kolajen gelişimini uyarmadı. TAK-044'ün etkili olmaması bu modelde ET-1 sinyal iletiminin HSP47 ve kolajen ile ilişkili olmayabileceğini düşündürmektedir.

Anahtar Kelimeler: HSP47 ısı-şok proteinleri; ateroskleroz; kollajen; endotelin-1; tavşanlar

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Intimal thickening, resulting from the proliferation and migration of vascular smooth muscle cells, is an early step of atherosclerosis and a common cause of restenosis after balloon angioplasty.[1] In the collar model, which was also used in the present study, intimal thickening has been achieved by periadventitial placement of a nonoccclusive and soft silicon collar around the carotid artery of the rabbit.[2,3]

The heat shock proteins (HSPs) which are called molecular chaperones are a highly conserved family of stress response proteins. HSPs function primarily as molecular chaperones, facilitating the folding of other cellular proteins, preventing protein aggregation.[4] HSPs have been shown to be highly expressed in cardiovascular tissues, and induce inflammatory responses and progression of atherosclerosis.[5,6] HSP47, a stress-inducible protein located in the endoplasmic reticulum, acts as a molecular chaperone and participates in the intracellular processing, folding assembly and secretion of procollagens.[7,8]

Collagen is the major constituent of extracellular matrix (ECM) in arteries and myocardium. The collagen matrix plays a key role in the maintenance of structural integrity, geometry and elasticity of the vessels.[9] Collagen synthesis is regulated by HSP47 at the level of procollagen synthesis.[10,11] Type I collagen comprises approximately two-thirds of the total collagen in atherosclerotic tissues.[12] Indeed, previous studies has shown that HSP47 and collagen have essential roles in advanced atherosclerotic lesions.[5] However, the exact nature of their roles and possible interactions have not been understood in the early atherosclerotic process. On the other hand, the degradation of newly synthesised procollagen and mature collagen is primarily carried out by matrix metalloproteinases (MMPs).[9,13] Degradation of interstitial collagen scaffold by MMPs enables SMC proliferation and migration, and contributes to progression of cardiovascular pathologies such as neointima or atherosclerotic plaque formation.[9,14]

In our previous studies we demonstrated that endothelin contributed to the development of intimal thickening, MMP upregulation and activation. We also showed that a nonselective endothelin receptor antagonist, TAK-044, inhibited intimal thickening, MMP upregulation and activation in this model.[15,16] Thus, in this study, we aimed to investigate the possible role of HSP47, the relation between HSP47 and collagen sythesis, and also the effects of a nonselective ETA/ETB receptor antagonist, TAK-044 in collar-induced early atherosclerosis model.

MATERIAL AND METHODS

MATERIAL

Material sources were as follows: Sodium pentobarbital from Sigma-Aldrich (Cat # P3761, Sigma-Aldrich, St Louis, MO, USA), silicone (MED-4011) from Nusil Silicone Technology, Anglet, France. TAK-044 was kindly provided by Takeda, Osaka, Japan. All histochemical materials with their sources were stated whereever mentioned in the text.

METHODS

Tissue sections available from our previous study were used.[16] This study was approved by the Local Ethics Committee of the Faculty of Pharmacy, Ege University, Izmir, Turkey. All animals received care complied with the "Principle of Laboratory Animal care" formulated by the National Society for Medical Research and the "Guide for Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources. New Zealand white rabbits were obtained from Animal Supply Unit of Ege University. Rabbits of either sex (n= 20; 2.0-2.5 kg) were randomly divided into two groups. Throughout the 3-week treatment period, the first group (n= 10) received TAK-044 (5 mg/kg/day, s.c.).[16,17] The second (placebo) group (n= 10) received only the vehicle (0.9% NaCl 0.8 ml/kg/day, s.c.). Each rabbit was kept in a separate cage and allowed access to a regular diet (standard rabbit chow and tap water ad libitum). After the seventh day of treatment with TAK-044 and placebo, the rabbits were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). Subsequently, a non-occlusive, flexible silicone collar (2 cm in length) was positioned around the left carotid artery, as descri-

bed.[2,16] The contralateral carotid artery was sham-operated. The carotid arteries were then returned to their original positions and the incisions sutured. After recovery from the anesthesia, all rabbits were kept in their individual cages for a further two weeks before tissue isolation. After 14 days treatment period (placebo or TAK-044), the rabbits were sacrificed with overdose sodium pentobarbital, and both carotid arteries were isolated and dissected.

HISTOLOGY

One pair of segments were cut from both the collared and sham-operated arteries for morphometry.[16] Then, the segments were immediately placed in 10% neutral buffered formalin for 24 hours, dehydrated in a graded series of isopropyl alcohol (60 to 100%) and followed by xylol before being embedded in paraffin.

Paraffin sections (5 μm) of each specimen were stained with hematoxylin-eosin, Masson's trichrome and Sirius red. Masson's trichrome and Sirius red were used to stain total and fibrillar collagen, respectively. Images of randomly chosen transverse sections from samples stained with two different stains were recorded. Then the sections were examined under a light microscope for Masson's trichrome staining or under polarized light microscope for Sirius red staining. Images were processed using a computerized image analysis system consisting of a microscope (Olympus BX-50) equipped with a high-resolution video camera (JVC TK-890E, Japan) and image processing program (UTSCSA; Image tool version 3,0).

IMMUNOHISTOCHEMISTRY

HSP47 expression and type I procollagen expression were determined in paraffin sections immunohistochemically using a commercial kit (Vectastain ABC Elite Kit P-6102, Vector Laboratories Inc. Burlingame, USA) according to the manufacturer's protocol. Tissue sections from sham-operated and collared carotid arteries from each group were incubated with diluted normal blocking serum (1% BSA for 30 min) to block nonspecific binding, and then they were incubated overnight at 4°C with 1:100 diluted specific primary monoclonal antibodies for HSP47 (anti-HSP47, cat. # SC8352 Santa Cruz Biotechnology, Santa Cruz, CA, USA) or for type I procollagen (anti-Procol 1A1, cat. # SC8782 Santa Cruz Biotechnology, Santa Cruz, CA, USA). The following day, appropriate biotinylated IgG secondary antibody (Vector, Burlingame, CA, USA) was applied. The bound secondary antibody was then amplified with Vector Elite ABC® Reagent, followed by chromogenic detection of the antibody-biotin-avidin-peroxidase complexes by using 0.02% diaminobenzidine (DAB) substrate (Roche Diagnostics GmbH, Mannheim, Germany) for five minutes. The sections were counterstained with Harris-hematoxilin, cleared and mounted. Negative control samples in which an equal amount of IgG was substituted for the primary antibody (anti-eNOS antibody) were included in each assay, and were uniformly negative. Appropriate positive controls were also stained. For immunoscoring of HSP47 antibody, the degree of positive cytoplasmic staining of cells in intima, media and adventitia layers was evaluated by semiquantitative scoring on a scale of the intensity of positively stained cells in the following range 0: negative, +/-: 1; very weak, +: 2; weak, ++: 3; moderate, +++:4; strong.[18]

TOTAL COLLAGEN DETERMINATION

Total collagen levels were measured in frozen carotid artery tissues available from previous study, which were stored at -80°C until use. For this purpose, a modified version of the method of Reddy and Enwemeka was used for determining hydroxyproline levels in biological tissues utilizing alkaline hydrolysis.[16,19] Hydroxyproline is found almost exclusively in collagen, constituting about 12.5% of the dry weight of the protein. The rate of this imino acid formation is therefore considered to be a good indication of the rate of collagen biosynthesis.[20] Hydroxyproline was quantified as described. Briefly, the frozen tissue samples (~10 mg) were homogenized in an ultrasonic sonicator at approximately 4°C for 30 s in Eppendorf tubes. Samples were hydrolysed with 100 uL in 2 mol/L NaOH for 15 min at 121°C (70 kPa) in autoclave. Hydroxy-

proline was converted to pyrole-2-carboxylic acid by reducing the sample with 900 uL of a 0.056 mol/L chloramine T solution (Sigma-Aldrich, USA) at room temperature for 25 min. After the oxidation step, 1mL Ehrlich's reagent (Sigma-Aldrich) was added to derivatize pyrole-2-carboxylic acid and vortexed. The samples were then placed on the water bath at 65°C for 20 min. Optical densities of coloured samples were read using a UV Varian Cary 50 spectrophotometer at 550 nm. The sensitivity of the assay was 0.025 mmol/L. The hydroxyproline concentrations were calculated by standard curve using standard solutions of l-hydroxyproline (Sigma-Aldrich). Assuming that 12.5% of collagen is formed by hydroxyproline, a converting factor was used to transform the results. The collagen content was expressed as μg/mg wet tissue.

STATISTICAL ANALYSIS

Statistical analyses of data of total collagen levels (μg/mg tissue) were performed for drug treatments (two levels, TAK-044 or placebo) as between rabbit factor; and collar (two levels, present or not) as within rabbit factor with a factorial analysis of variance (ANOVA). Chi-square test was used to evaluate the statistical difference of immunostaining between TAK-044-treated group and placebo group. Statistical analyses of paired data (collared vs sham) from immunscoring were performed with Wilcoxon signed ranks test. All data were expressed as mean ± SEM. "n" indicates the number of animals in each group. $p < 0.05$ was considered to be statistically significant.

RESULTS

HISTOLOGY

Consistent with the result of previous studies, intimal thickening was observed in collared arteries as compared to those in sham-operated arteries in placebo group, as a characteristic feature of the model (Figure 1).[16]

In order to determine collagen expression of the vessels, Masson's trichrome and Sirius red stainings were carried out. Masson's trichrome and Sirius red stainings marked remarkable stained areas in adventitia layer of each vessel segments (Figure 2, 3). However, collagen expression in the vessels were affected by neither collar placement nor TAK-044 treatment.

IMMUNOHISTOCHEMISTRY

Immunostainings with HSP47 and type I procollagen antibodies revealed that positive immunostainings for HSP47 and type I procollagen were observed in intima, media and adventitia layers of both collared and sham-operated arteries in the placebo group (Figure 4, 5 and Table 1). Immunoscoring for HSP47 and observation of immunopositivity for type I procollagen showed that the HSP47 expression and type I procollagen expression were not affected by collaring. TAK-044 treatment did not change the expressions of these two proteins in either collared or sham-operated arteries (Figure 4, 5 and Table 1).

TOTAL COLLAGEN LEVELS

Total collagen levels were measured by hydroxyproline assay in extracts of sham operated and collared arteries from both groups. Total collagen levels were not affected by collar placing in placebo group (Table 2). TAK-044 treatment did not change these levels either in collared or in sham-operated arteries (Table 2).

DISCUSSION

Collagen provides mechanical strength to the arterial wall and plays a vital role in the maintenance of structural and functional integrity of the arterial wall.[21] Furthermore, collagen functions as a signaling molecule and regulates many cellular responses including proliferation, migration, and matrix degradation.[9,22]

HSP47, known as a collagen-specific molecular chaperone, plays an essential role in collagen biosynthesis.[23,24] Previous studies have shown a marked increase in HSP47 expression in parallel with increased collagen production and type I procollagen expression during the progression of atherosclerosis in balloon-injured rat and rabbit carotid arteries, and in atheromatous plaques of human coronary arteries.[5,25,26] All these studies revealed the major roles of HSP47 and collagen ex-

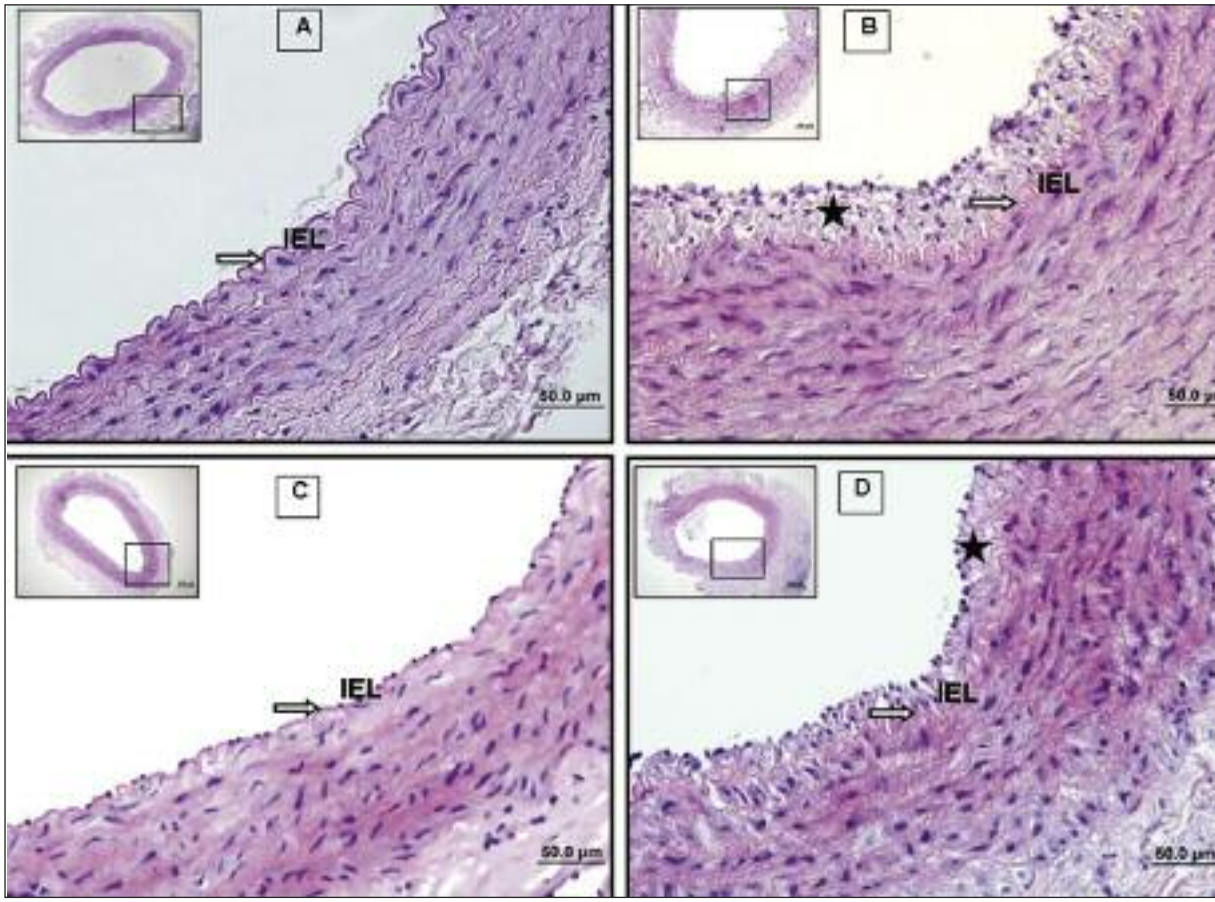

FIGURE 1: Representative photomicrographs of paraffin transverse sections of carotid arteries stained with haematoxylin-eosin.A) Sham-operated artery from placebo group, B) Collared artery from placebo group, C) Sham-operated artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group, D) Collared artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group. Stars indicate intimal thickening. White arrows point out the internal elastic lamina (IEL).

pression in the progression of advanced atherosclerotic lesions. However, the possible role of HSP 47 remained unclear in the regulation of secretion of newly formed procollagen molecules in early atherosclerosis.

In the present study we have demonstrated that there is a correlation between HSP47 expression and collagen production in collar-induced early atherosclerosis model. However, intimal thickening does not induce HSP47 expression and early collagen development. Consistent with our findings, Goto et al. showed that there were prominent collagen bundles in the media and adventitia of ballon-injured carotid arteries of dogs, but not in dog carotid arteries with external collar-mediated intimal thickening.[27] It is known that balloon-mediated neointima is caused by endothelial denudation, deep medial injury and proliferation of medial SMC, and ECM deposition.[1] However, collar-induced intimal thickening leads to minimal medial SMC injury under an uninterrupted endothelial cell layer.[3,28] Therefore, our evidence suggests that the degree of the medial injury and the activation of SMCs may be important determinants of HSP47 expression and collagen matrix synthesis in the development of vascular injury.[21]

Furthermore, we observed that bundles of total collagen and fibrillar collagen were more distinctive in adventitia of the arteries. This finding is consistent with our previous evidence that discoidin domain receptors (DDRs), which are non-integrin collagen receptors were considerably co-expressed with collagen in adventitia, but not intima and media in both collared and sham operated arteries from

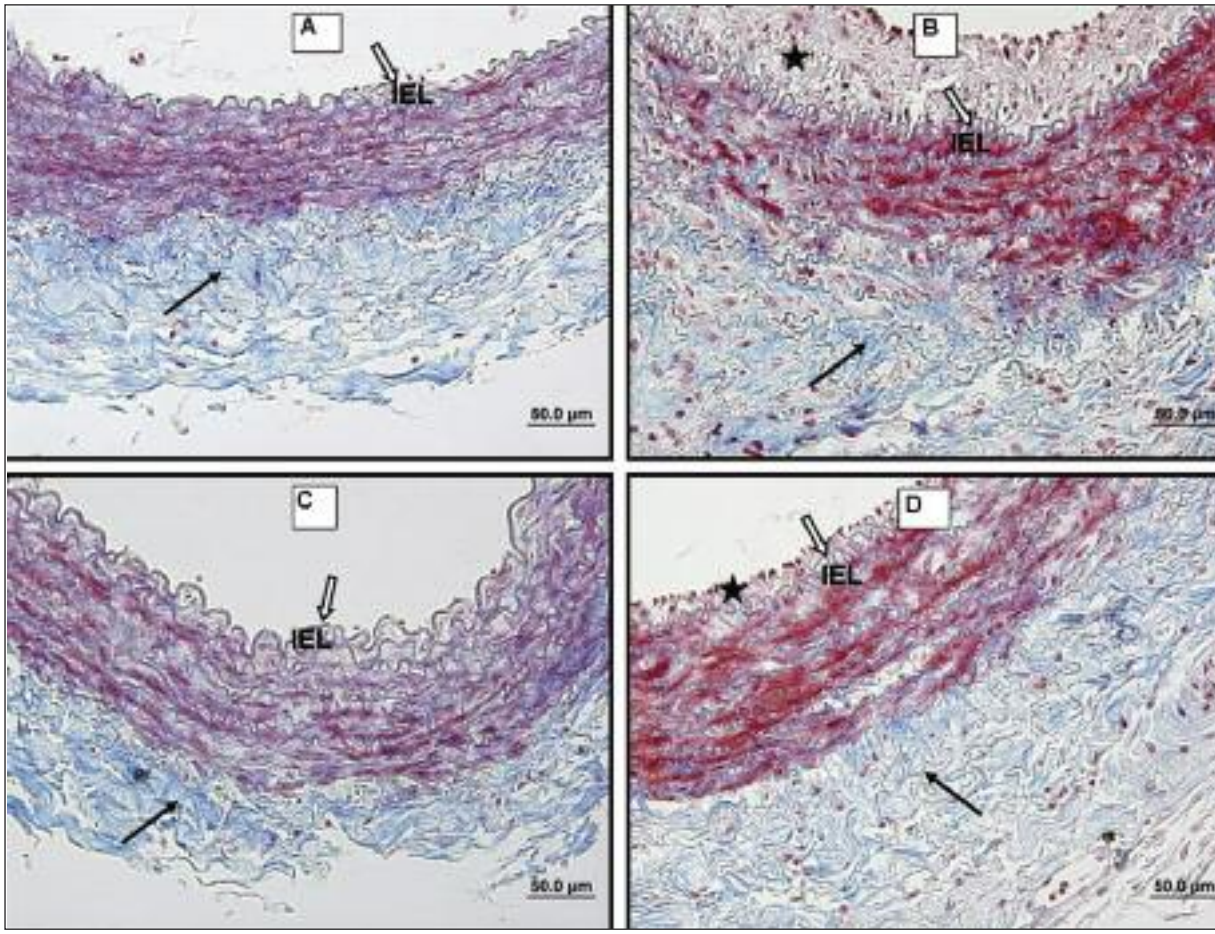

FIGURE 2: Representative photomicrographs of paraffin transverse sections of carotid arteries stained with Masson's trichrome . A) Sham-operated artery from placebo group, B) Collared artery from placebo group , C) Sham-operated artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group, D) Collared artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group. Stars indicate intimal thickening. White arrows point out the internal elastic lamina (IEL) and black arrows show collagen.

placebo group (unpublished data). This evidence is not surprising since DDRs activated by collagen are expressed in adventitial fibroblasts.[29]

In this study, we showed that HSP47 and type I procollagen were co-expressed in intima, media and adventitia of both collared and sham-operated arteries from placebo group. However, HSP47 expression and type I procollagen expression appear to be more abundant in adventitia compared to intima and media. It has been known that type I collagen is a major collagen secreted by fibroblast, and constitutive expression of HSP47 is always accompanied by the collagen expression under non-stressed conditions.[30] Therefore our findings suggest that the HSP47 is correlationally expressed with type I procollagen by adventitial fibroblasts. However, our observations revealed that intimal thickening did not induce HSP47 expression or early collagen development in the collar-induced early atherosclerosis model.

It is known that MMPs, in particular gelatinases (MMP-2 and MMP-9) degrade ECM components and contribute to the intimal thickening process. The reason for the similarity of collagen content between collared and sham-operated arteries may be attributed to an increased MMP activation in collared arteries. Similarly, Sluijter et al. demonstrated that during flow-induced arterial remodeling in rabbits, collagen I synthesis increased as shown with mRNA levels, but collagen fiber content did not change, since collagen was degraded by activated MMP-2.[26] Indeed, in our previo-

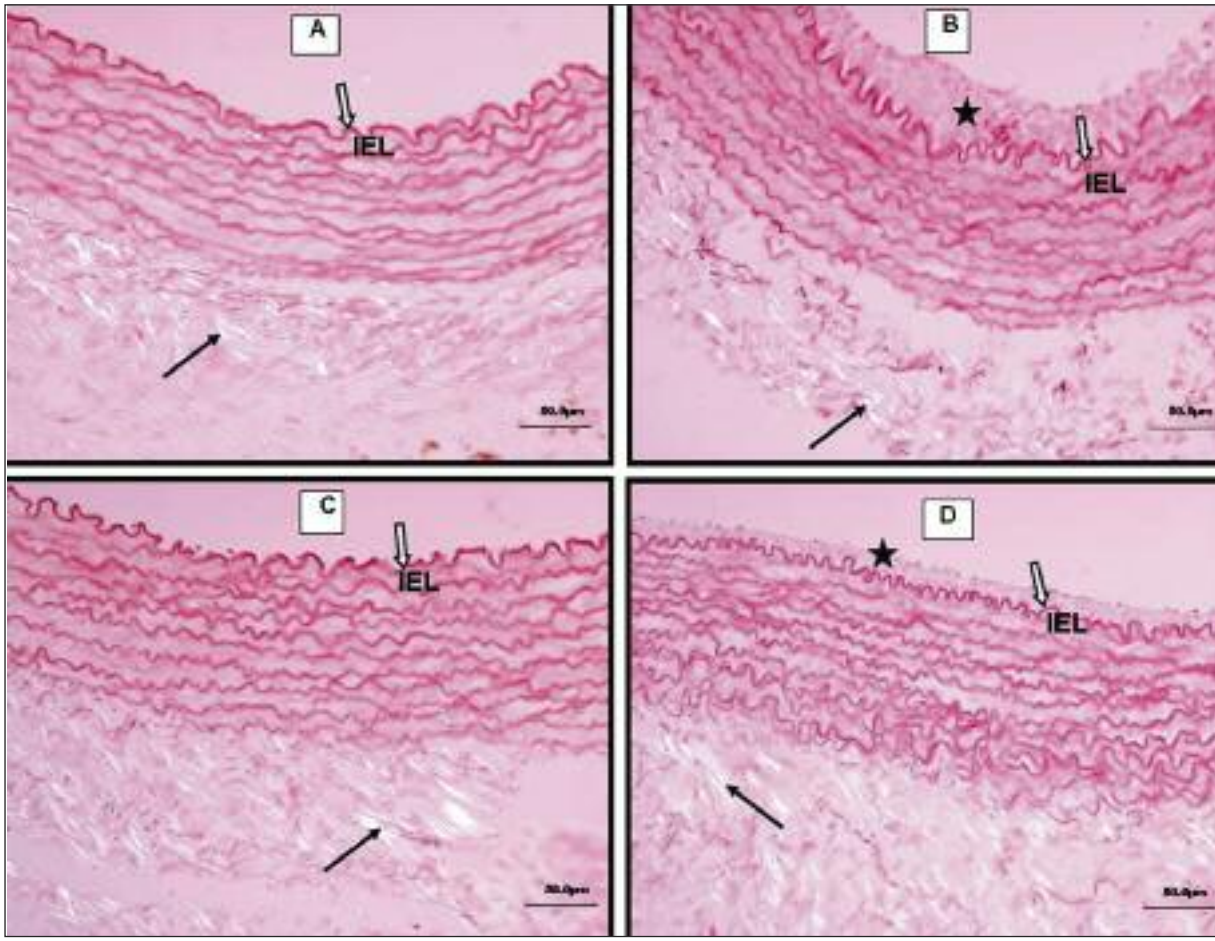

FIGURE 3: Representative photomicrographs under polarized light of paraffin transverse sections of carotid arteries stained with Sirius red. A) Sham-operated artery from placebo group, B) Collared artery from placebo group, C) Sham-operated artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group, D) Collared artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group. Stars indicate intimal thickening. White arrows point out the internal elastic lamina (IEL) and black arrows show bright stained collagen.

us studies in this model, we have shown that the gelatinase activities increased in collared arteries compared to sham-operated arteries.[16] However, we have not been able to determine the mRNA levels of collagen I in order to evaluate collagen synthesis of the arteries in this study. This point requires further investigation.

On the other hand, endothelin (ET) was shown to stimulate collagen synthesis and to increase collagen content in vivo and in vitro advanced atherosclerosis models.[31,32] Similarly, ETA antagonism inhibited ET-induced collagen stimulation in porcine aortic smooth muscle cell culture.[33] However, the possible effects of ET antagonism on collagen levels and HSP47 expression in early atherosclerosis model have not been studied yet. In this study, we found that treatment with TAK-044, a nonselective ETA/ETB receptor antagonist did not change HSP47 expression, type I procollagen expression or collagen levels in collar-induced early atherosclerosis model. Furthermore, in our previous study in this model we demonstrated that endothelin contributed to the development of intimal thickening, gelatinase upregulation and activation. We have also shown that ET has an important role in the progression of intimal thickening in this model and the nonselective endothelin receptor antagonist TAK-044 inhibited intimal thickening, gelatinase upregulation and activation in collared arteries.[15,16] It is known that gelatinase upregulation induces degradation of collagen. However, in this study, collagen content was not af-

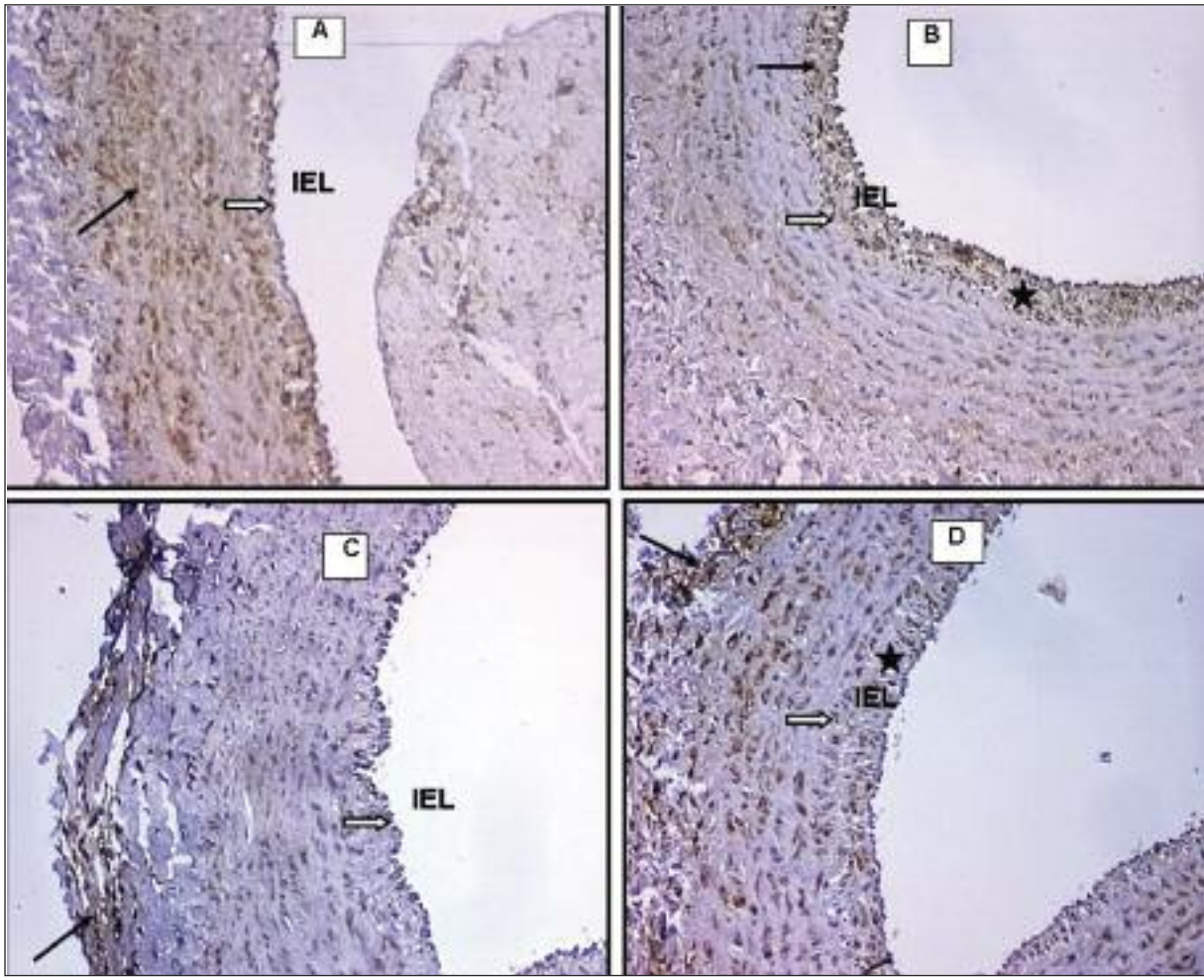

FIGURE 4: Representative photomicrographs of paraffin transverse sections of carotid arteries stained with HSP 47 antibody immunohistochemically. A) Sham-operated artery from placebo group, B) Collared artery from placebo group , C) Sham-operated artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group, D) Collared artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group. Stars indicate intimal thickening. White arrows point out the internal elastic lamina (IEL) and black arrows show dark brown stained HSP 47 immunopositive areas.

fected by TAK-044 treatment. Therefore, this evidence suggests that HSP47 and collagen are expressed independently from ET receptor-mediated cell signaling pathways in the collar induced early atherosclerosis model.

CONCLUSIONS

In conclusion, in the present study we have demonstrated that there is a correlation between HSP47 expression and collagen expression in collar-induced early atherosclerosis model. However, HSP47 expression and collagen expression were not affected by collar-induced intimal thickening. This evidence suggests that the intensity of vascular injury designates HSP47 expression and collagen synthesis.

Furthermore, the ineffectiveness of TAK-044 suggests that regulation of HSP47 and collagen expression after endothelial injury is independent from ET receptor signaling in the collar induced early atherosclerosis model.

Combined data from all of the studies related to early atherosclerosis may help to provide the rationale for the development and use of drugs that interfere with collagen synthesis which may negatively influence the outcomes of cardiovascular diseases.

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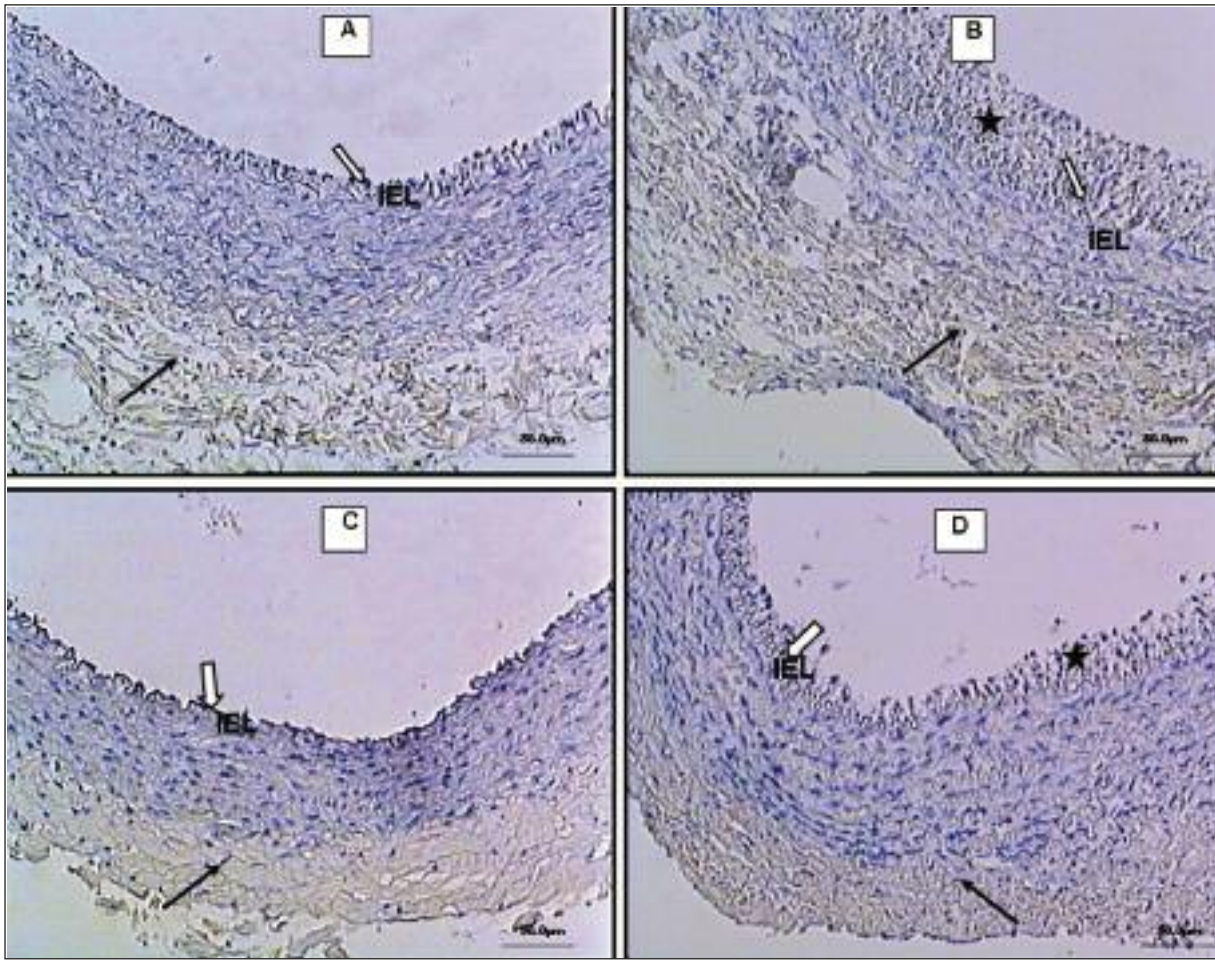

FIGURE 5: Representative photomicrographs of paraffin transverse sections of carotid arteries stained with type I procollagen antibody immunohistochemically. A) Sham-operated artery from placebo group, B) Collared artery from placebo group , C) Sham-operated artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group, D) Collared artery from nonselective ETA/ETB receptor antagonist TAK-044-treated group. Stars indicate intimal thickening. White arrows point out the internal elastic lamina (IEL) and black arrows show dark brown stained type I procollagen immunopositive areas.

TABLE 1: HSP47 immunoreactivity of collared and sham-operated carotid arteries.

Immunoscoring	0	1	2	3	4
Intima					
Placebo sham	1	4	4	1	0
Placebo collared	0	3	5	2	0
TAK-044 sham	0	7	2	1	0
TAK-044 collared	0	6	3	1	0
Media					
Placebo sham	0	3	2	5	0
Placebo collared	1	4	1	2	2
TAK-044 sham	1	2	6	1	0
TAK-044 collared	1	4	2	3	0
Adventitia					
Placebo sham	0	1	2	5	2
Placebo collared	1	0	2	3	4
TAK-044 sham	0	0	5	3	2
TAK-044 collared	0	1	2	5	2

Placebo group (n=10), nonselective ETA/ETB receptor antagonist TAK-044-treated group (n=10). Immunscoring scale was accepted as 0: negative, +/-: 1; very weak, +: 2; weak, ++: 3; moderate, +++:4; strong.

TABLE 2: Total collagen levels in collared and sham-operated carotid arteries.

	Placebo (n= 9)	TAK-044 (n= 8)
Total collagen (µg/mg protein)		
Sham	8.88 ± 1.25	9.97 ± 1.02
Collared	7.74 ± 1.21	8.92 ± 1.11
Significance of factors in analysis of variance:		
Collar	n.s.	
TAK-044	n.s.	
Interaction:		
-044 by Collar	n.s.	

Placebo group (n=9), nonselective ETA/ETB receptor antagonist TAK-044-treated group (n=8). Shown are mean±S.E.M. n represents the number of animals in each group. n.s. Not significant (ANOVA).

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