

Effects of Defibrotide on Morphological and Contractile Properties of Rabbit Carotid Artery

Defibrotidin Tavşan Karotid Arterinin Morfolojik ve Kontraktıl Özellikleri Üzerine Etkisi

Gülnur Sevin¹, Necmettin Özdemir², Aslı Özer¹ and Levent Üstünes^{1*}

Departments of Pharmacology, Faculty of Pharmacy* and Pathology, Faculty of Medicine*, Ege University, Izmir, Turkey

Abstract

The effects of defibrotide, a polydeoxyribonucleotide, were investigated on the morphological and contractile properties of normal as well as collared carotid arteries. Intimal thickening induced by the perivascular application of a silicone collar was not prevented by defibrotide. However, interestingly, a significant medial thickening was observed both in sham or collared arteries from defibrotide-treated rabbits. In addition, this medial thickening was not accompanied by any luminal narrowing. Defibrotide-induced medial thickening without any luminal narrowing in rabbit carotid artery represents an outward hypertrophic vascular remodeling and may contribute to the effectiveness of the drug in the treatment of peripheral obliterative arterial disease.

Key words: carotid arteries, smooth muscle, hypertrophy, remodeling, medial thickening, silicon collar.

Introduction

Intimal thickening is considered not only an adaptive change in a normal blood vessel to physical stimuli (i.e. changes in luminal blood flow) but also a susceptible site for atherosclerosis in uncompensated disease states (Stary *et al.*, 1992). This event is usually accompanied by alterations in vascular reactivity (Vrints *et al.*, 1992). Intimal thickening can be evoked experimentally by perivascular application of a flexible silicone collar *per se* (Huth *et al.*, 1975). In a modified intimal thickening model (Booth *et al.*, 1989), polymorphonuclear leukocytes (PMNLs) were also found to infiltrate into the vascular wall from the lumen of the collared arteries during the early stages of collar-induced intimal thickening (Kockx *et al.*, 1993). Although mononuclear leukocytes are assumed to be involved in the development of atherosclerotic lesion by releasing cytokines and growth factors, they received only minor attention in collar-induced intimal thickening model, suggesting that the leukocytes subpopulations and their time-dependent occurrence in plaque formation are dependent on the experimental model (Kling *et al.*, 1993). The role of PMNLs infiltration in progression of the atherosclerotic lesions, on the other hand, is not well defined.

* Corresponding author

Defibrotide, a polydeoxyribonucleotide obtained from a single-stranded mammalian DNA, has been developed for the treatment of a number of vascular disorders (Palmer *et al.*, 1993, Pegram *et al.*, 2001). Oral defibrotide treatment was shown to have potent and potentially valuable antiatherosclerotic effects, possibly in part, by reducing the platelet hyperreactivity, increasing the sensitivity to prostacyclin (PGI₂) as well as stimulating PGI₂ production in cholesterol-fed rabbits (Lobel *et al.*, 1989, Rossoni *et al.*, 1999). It was also suggested that oral defibrotide treatment reduce the development of atherosclerosis in aorta by decreasing the number of leukocytes and platelets as well as deactivating the previously infiltrated blood cells (Pescador *et al.*, 1995). On the other hand, in one study, the drug increased the protein content of cultured human umbilical endothelial cells and also slightly induced the cell proliferation (Bilsel *et al.*, 1989). Radiolabeled defibrotide was also found in the plasma membrane and the nucleus of the endothelial cell, suggesting the internalization of the drug into the cell (Bilsel *et al.*, 1992). Therefore, the purpose of this study was to investigate, in intact rabbit carotid artery, whether defibrotide 1) prevents the collar-induced intimal thickening and accompanied changes in vascular reactivity and 2) yields any morphological changes in vascular wall.

Methods

Treatments: The protocol was approved by the Animal Care Committee of Faculty of Pharmacy, Ege University. White rabbits of either sex (1.7–1.9 kg, n=20) were divided into 2 groups. The treatment group (n=10) received 120 mg kg⁻¹ day⁻¹ defibrotide (dissolved in isotonic NaCl solution) via gastric gavage. The placebo group (n=10) received only saline (2.5 ml kg⁻¹ day⁻¹, p.o.) for 3 weeks. Throughout the 3 week-treatment period, rabbits were kept in separate cages and allowed to access regular diet (standard rabbit chow and tap water *ad libitum*).

Induction of intimal thickening: After 7 days of treatment with or without defibrotide, the rabbits were anesthetized with sodium pentobarbital (30 mg kg⁻¹ i.v.). Subsequently, the left carotid artery was dissected free and, then, surrounded by a non-occlusive, flexible silicone collar as described (Booth *et al.*, 1989). The collar was 2 cm in length slightly touching the outer surface of the artery on each end. In all experiments, the contralateral carotid artery served as control (sham), i.e. separated from the surrounding connective tissue and the vagus nerve, also receiving a similar stretch as the left collared artery. Upon manipulation, arteries were returned to their original position and incisions sutured. After the recovery from anesthesia, the rabbits received their respective treatment for 2 weeks.

Morphometry: Following heparin (150 U kg⁻¹, i.v.) administration for anticoagulation, the rabbits were killed with overdosed sodium pentobarbital. A standard protocol was used for isolation and fixation of tissue segments. For this purpose two segments (4 mm each) were isolated from both sham and collared arteries, one for morphometry and the other for isometric tension recordings (see below). The former tissue was immediately placed in formalin fixative solution (0.4%) for 24 hours, dehydrated in a graded series of isopropyl alcohol (60 to 100 %) followed by toluol before being embedded in paraffin. Transverse sections were cut and stained with sirius red haematoxylin. Two transverse sections from each artery ring were randomly chosen and their video images recorded by using a video-camera (JVC Color Video Camera, Head Model No. TK-890E, Japan) connected to a light microscope (Olympus BH-2, Japan). Intimal and medial cross-sectional areas were measured by using a computerized system. In brief, video (Sony VCR SL-C6E) images of each segment were captured via a video-card

(Video Blaster SE, Creative Labs, Creative Labs Inc., USA), intimal and medial cross-sectional areas marked (CorelDraw, Version 4.00.A5, Corel Corporation 1993, USA) and measured (AutoCAD, release 12-cl, 1993, Autodesk, Inc., USA) as previously reported (Üstünes *et al.*, 1996). In each segment, the luminal area (A) was simply calculated ($A=C^2/4\pi$) by using luminal perimeter (C) that is not affected by the shape of the vessel segment.

Cell counting: The number of smooth muscle cells (SMCs) in medial cross-sectional area was determined by counting the nuclei in the media in cross sections of each segment by using a light microscope. The point counting were done in duplicate and mean values used. The cell number per unit area (μm^2) of the medial layer was also calculated.

Vascular Reactivity: The two remaining rings from the right (sham) and the left (collared) carotid arteries were used in contractility studies. The arterial rings were mounted in organ chambers filled with 25 ml physiological salt solution maintained at 37°C and continuously gassed with 95%—O₂5% CO₂ (Üstünes *et al.*, 1996). The physiological salt solution contained the following composition (mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25 and glucose 11.1. Isometric force measurements were done (Grass FT3 force transducer) and recorded (IOSlab version 3.23 MS8, EMKA Technologies, Paris, France) via a personal computer as described previously (Üstünes *et al.*, 1996). After 15 min-equilibration period, the preparations were gradually stretched to a resting tension of 7 g, a previously determined optimal tension based upon the length-tension relationship for both sham and collared arteries. The arterial rings were then allowed to equilibrate a further 45 min at their optimal length along with changing the bath solution every 15 min. At the end of the equilibration period, tissues were contracted with phenylephrine (3×10^{-7} mol/L). During the phenylephrine-induced steady state contraction, acetylcholine (ACh) was administered in a cumulative manner (10^{-9} to 10^{-4} mol/L) and concentration-response relationship constructed for each preparation. ACh-induced relaxation (due to nitric oxide release) also confirmed the presence of functional endothelium. The arterial rings relaxed in response to ACh more than 40% of the initial phenylephrine contraction (indicative of functional presence of endothelium) were used for a further evaluation. The rings were washed out three times between cumulative administration of 5-HT (10^{-9} to 3×10^{-5} mol/L), and histamine (10^{-9} to 10^{-4} mol/L). At the end of each experiment, tissues were contracted maximally with 120 mmol/L KCl (equimolar replacement of NaCl with KCl).

Materials

Crinos S.p.A. (Como, Italy): defibrotide; Nusil Silicone Technology (Anglet, France): Silicone (MED-4011); Sigma (St. Louis, MO, USA): acetylcholine chloride, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate (5-HT); Psyphac (Brussels, Belgium): sodium pentobarbital; Roche (Istanbul, Turkey): heparin. 5-HT was dissolved in distilled water including ascorbic acid (0.01 %). The other drug solutions were prepared in distilled water.

Data Analysis

All data are given as mean \pm SEM. "n" represents the number of animals. Statistical analysis was performed on drug treatment (defibrotide or placebo, 2 levels, between rabbit factor) and collar (present or not, 2 levels, within rabbit factor) with a factorial analysis of variance (ANOVA) (SPSS/PC+, Chicago, IL, USA). Statistical differences between two means corresponding each agonist concentration were determined by Wilcoxon matched pairs signed-ranks test (sham vs. collar, paired data) and by Mann-Whitney U test (placebo vs. defibrotide,

unpaired data). Pearson's correlation test was used where appropriate. Significance was accepted at $P=.05$.

Values of 50% effective concentration (EC_{50}) were derived for each cumulative concentration-response curve with an iterative nonlinear curve fitting (Polywin95, Commat Ltd., Ankara, Turkey). Geometric means of the EC_{50} values (pD_2) were compared. ACh-induced relaxations were expressed as % initial phenylephrine contraction.

Results

Survival: All rabbits in both the placebo and defibrotide treatment groups survived with no apparent side effects. The weight gain was similar in both placebo- and defibrotide-treated rabbits.

Morphometry: Perivascular application of silicone collar caused significant increase in intimal cross-sectional area (intimal thickening) comparable to those of sham arteries from placebo group (Fig 1A). Defibrotide treatment did not affect the intimal thickening caused by the collar (Fig 1A). Collar did not alter the medial cross-sectional area and the calculated luminal area (Fig 1, B and C). Defibrotide treatment, however, caused a significant medial thickening without affecting the calculated luminal cross-sectional area in both sham and collared arteries (Fig 1, B and C).

Medial SMC count: Defibrotide significantly increased the number of SMCs in medial cross-sectional area in both sham and collared arteries (Table 1). However, SMC count per unit area ($SMCs/\mu m^2$) was not altered by defibrotide treatment in either arteries (Table 1). In addition, since collar did not significantly affect the number of medial SMCs and medial the cross sectional area either in placebo- and defibrotide-treated groups, measurements in sham and collared arteries from the same rabbit in both treatment groups were accepted as duplicate and their mean values used in the correlation. Upon a statistical comparison, no statistical correlation was observed between the number of medial SMCs and the medial cross-sectional area in placebo group ($r=.15$, $P=.72$, $n=8$, Pearson's test), whereas defibrotide-induced significant increase in medial cross-sectional area was found fairly correlated ($r=.73$, $P=.039$, $n=8$, Pearson's test) with the significant increase in the number of medial SMCs.

Table 1: Effects of collar and defibrotide ($120 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$) on the number of SMCs in medial cross-sectional area of rabbit carotid artery.

	PLACEBO (n=8)	DEFIBROTIDE (n=8)
Number of SMCs in medial cross-sectional area		
sham	1076 ± 64	1408 ± 184
collar	984 ± 50	1328 ± 108
Significance of factors in analysis of variance		
defibrotide	$P=.006$	
collar	NS	
interaction: defibrotide by collar	NS	
SMCs/ μm^2		
sham	3.7 ± 0.2	3.6 ± 0.1
collar	3.6 ± 0.2	3.6 ± 0.3
Significance of factors in analysis of variance		
defibrotide	NS	
collar	NS	
interaction: defibrotide by collar	NS	

Shown are means \pm SEM. n represents the number of animals in each group. NS: not significant.

Vascular reactivity: KCl (120 mmol/L)-induced maximal contractions (E_{max}) were significantly diminished in collared arteries (E_{max} , g: 3.4 ± 0.4 and 0.5 ± 0.1 , placebo-treated sham and collared arteries, respectively, $P < .01$, $n=6$). Although not significant, the defibrotide treatment increased the KCl contractions in both arteries (E_{max} , g: 4.2 ± 0.4 and 0.8 ± 0.2 , defibrotide-treated sham and collared arteries, respectively, $n=6$).

5-HT (10^{-9} to $3 \times 10^{-5} \text{ mmol/L}$) induced concentration-dependent contractions in sham and collared arteries (Fig 2). In collared arteries, E_{max} was significantly diminished. Despite the reduced E_{max} , the concentration-response relationship of 5-HT-induced contraction significantly shifted to the left in collared arteries (pD_2 values: 7.20 ± 0.10 and 7.60 ± 0.15 , placebo-treated sham and collared, respectively, $P < .01$, $n=7$). Defibrotide did not alter the pD_2 values significantly (7.20 ± 0.13 and 7.80 ± 0.17 , defibrotide-treated sham and collared, respectively, $n=6$). Although defibrotide treatment increased the 5-HT-induced maximal contractions of sham and collared arteries, the data did not attain statistical significance (Fig. 2).

Histamine (10^{-9} to 10^{-4} mol/L) also induced concentration-dependent contractions. While there was no effect on the pD_2 values (5.49 ± 0.04 and 5.45 ± 0.09 , placebo-treated sham and collared arteries, respectively, $n=8$), the E_{max} was significantly diminished in collared arteries (Fig 3). Although defibrotide did not significantly affect the histamine-induced contractions (pD_2 values: 5.33 ± 0.06 and 5.33 ± 0.10 , defibrotide-treated sham and collared arteries, respectively, $n=8$), maximal contractions tended to increase in both sham and collared arteries (Fig 3) as observed with KCl and 5-HT.

Acetylcholine (10^{-9} to 10^{-4} mol/L) induced concentration-dependent relaxations in sham and collared arteries precontracted with phenylephrine ($3 \times 10^{-7} \text{ mol/L}$). The collar significantly increased the E_{max} value for acetylcholine-induced relaxations without affecting the pD_2 values (Fig. 4). Defibrotide treatment did not alter either values (Fig. 4).

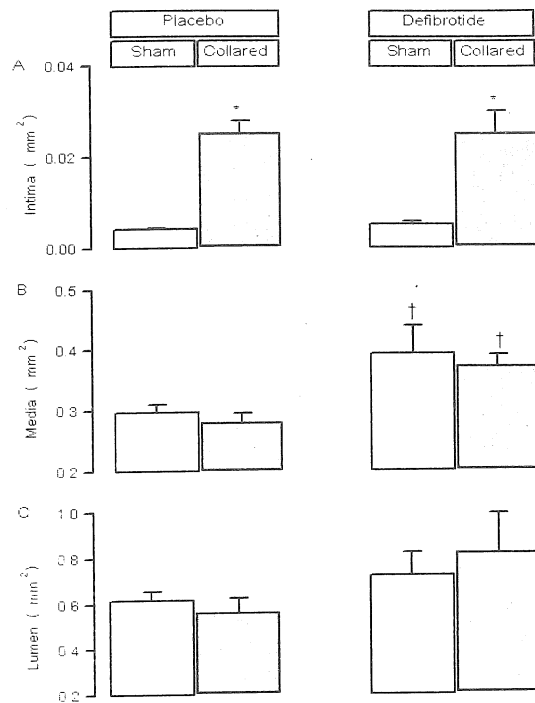


Fig 1: Effects of defibrotide on the medial and luminal areas and the collar-induced intimal thickening in rabbit carotid artery. Cross-sectional areas of intima (A), media (B), and lumen (C) are shown. Sham- (open columns) and collared arteries (solid columns) are isolated from the rabbits received only the vehicle (placebo) or 3-week treatment of defibrotide ($120 \text{ mg kg}^{-1} \text{ day}^{-1}$, p.o.). Shown are means \pm SEM (defibrotide-treated group, $n=8$; placebo group, $n=8$, each measurement is an average of two transverse sections from the same tissue). † Placebo vs. defibrotide ($P<.01$, ANOVA), * sham vs. collared ($P<.001$, ANOVA)

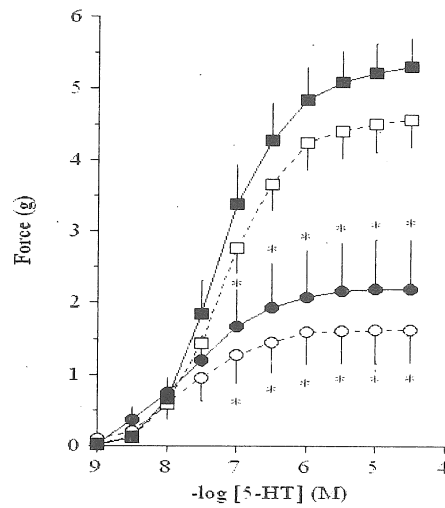


Fig 2: Effects of defibrotide and collar on the 5-HT-induced contractions. Concentration-response relationship for the 5-HT-induced contractions were constructed both in placebo-treated sham (\square) and collared (\circ) arteries, and defibrotide-treated sham (\blacksquare) and collared (\bullet) arteries. The data are expressed as gram (g) contraction. Shown are means \pm SEM (placebo group, n=7; defibrotide group, n=6). * Sham vs. collared in both placebo and defibrotide-treated groups ($P < .05$, Wilcoxon matched-paires signed-ranks test).

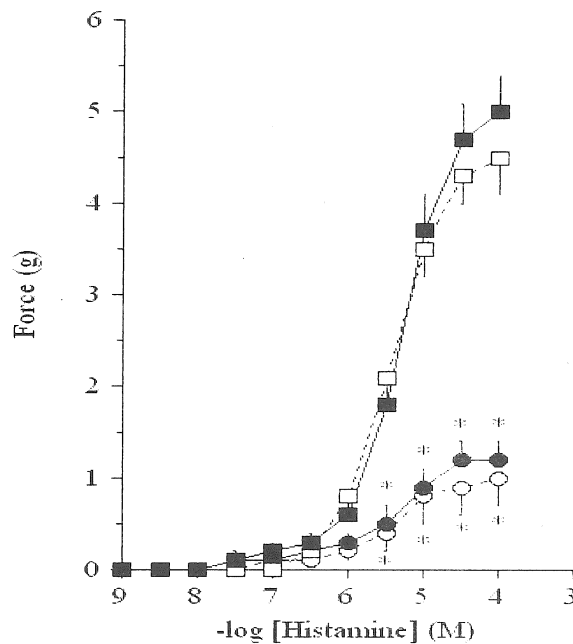


Fig 3: Effects of defibrotide and collar on the histamine-induced contractions. Concentration response relationship for the histamine-induced contractions were constructed both in placebo-treated sham (\square) and collared (\circ) arteries and, defibrotide-treated sham (\blacksquare) and collared (\bullet) arteries. Data are expressed as g contraction. Each point represents means \pm SEM (placebo group n=8 and defibrotide group n=8). * Sham vs. collared in both placebo and defibrotide treated groups ($P < .05$, Wilcoxon matched-paires signed-ranks test).

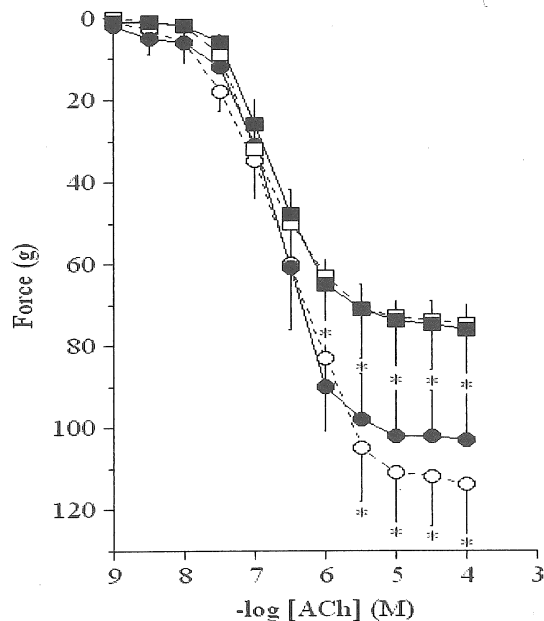


Fig 4: Effects of defibrotide and collar on the acetylcholine-induced relaxations. Concentration-response relationship for the acetylcholine-induced relaxations were constructed both in placebo-treated sham (□) and collared (○) arteries, and defibrotide-treated sham (■) and collared (●) arteries precontracted with 3×10^{-7} mol/L phenylephrine. The each point represents means \pm s.e.mean (placebo group $n=6$ and defibrotide group $n=7$) and expressed as % of initial contraction to 3×10^{-7} mol/L phenylephrine. * Sham vs. collared in both placebo- and defibrotide- treated groups ($P < .05$, Wilcoxon matched-paires signed-ranks test).

Discussion

Defibrotide and intimal thickening: The present results demonstrate that defibrotide, a polydeoxyribonucleotide sodium salt extracted from mammalian organs, did not prevent the collar-induced intimal thickening. This result appears to be conflicting with the antiatherosclerotic effectiveness of the drug in cholesterol-fed rabbits in which the frequency of intimal thickening was decreased reportedly due to the defibrotide-inhibited leukocyte activity (Pescador *et al.*, 1995). This discrepancy could be best explained by the fact that the collar-induced intimal thickening appears to an adaptive response of the vessel to the perivascular presence of the silicone collar and the diet-induced intimal thickening, however, represents an atherosclerotic process. It is also known that the leukocyte subpopulation as well as their temporal occurrence in intimal thickening process are different in diet-induced atherosclerosis compared to that caused by physical stimuli such as, balloon catheter injury or perivascular manipulation (Kling *et al.*, 1993). The failure of defibrotide to suppress the intimal thickening in collared carotid artery may not be resulted from an inadequate dosage of the drug since atherosclerotic plaque formation was significantly reduced even by lower doses of oral defibrotide (60 and $100 \text{ mg kg}^{-1} \text{ day}^{-1}$ p.o.) (Lobel *et al.*, 1989; Rossoni *et al.*, 1999). On the other hand, the three week-drug treatment was also long enough to cover the triphasic sequence of intimal thickening in rabbit collared carotid artery (Kockx *et al.*, 1992). Moreover, the present study demonstrates that the drug significantly increased the medial cross-sectional area in both sham and collared arteries at a given dose that did not exert any effect on intimal

thickening (discussed below).

Defibrotide was shown to inhibit polymorphonuclear leukocyte (PMNL) activity on endothelial cells (Di Perri *et al.*, 1988, Pellegatta *et al.*, 1996). In conjunction with this concept, Kockx *et al.* (Kockx *et al.*, 1993, 1992) demonstrated that PMNLs infiltrated into the vascular wall during initial stages of collar-induced intimal thickening in rabbit carotid artery. In the present study, defibrotide did not prevent intimal thickening. Therefore, the present study suggests that the PMNL infiltration into the vascular wall may not contribute to the development of intimal thickening, at least in the rabbit collared carotid artery model. This result also supports the recent suggestion of van Put *et al.* (van Put *et al.*, 1998) utilizing the same experimental model.

Defibrotide and medial thickening: Vascular hypertrophy is reportedly an indication of essential hypertension (Tice *et al.*, 1996). It is generally known that hypertrophy and/or hyperplasia yields medial thickening as well as the luminal narrowing in blood vessels (Lever, 1986). In contrast, in one rare case, the medial thickening was not accompanied by luminal narrowing in the esophageal veins of rabbits after partial occlusion of the portal vein (Juhl *et al.*, 1989). The proliferative effect of defibrotide ($75 \mu\text{g ml}^{-1}$) has been previously demonstrated on cultured human umbilical vein endothelial cells (Bilsel *et al.*, 1989). Present study demonstrates a novel finding that defibrotide caused a significant medial thickening in both sham and collared rabbit carotid artery without any apparent luminal narrowing. In contrast, although not significant, the luminal area tended to increase by defibrotide treatment in both sham and collared arteries. This medial thickening should be resulted from numerical hypertrophy (hyperplasia) since defibrotide increased the total number of SMCs in medial cross-sectional area of both sham and collared arteries without altering the number of SMCs per unit area. In support of this conclusion, defibrotide-induced increase in medial cross-sectional area showed a direct correlation with the increase in the number of medial SMCs. Based on these findings, the data may support the outward hypertrophic model of vascular remodeling in which lumen diameter and wall thickness are both increased (Mulvany *et al.*, 1996).

Effects of collar on vascular reactivity: Force development in response to contractile agents, KCl, 5-HT, and histamine were diminished in collared arteries consistent with previous reports (Üstünes *et al.*, 1996, De Meyer *et al.*, 1994). The possible underlying mechanisms that were discussed in some detail earlier, briefly, may include 1) the opposing force generation by longitudinally oriented intimal SMCs (Kockx *et al.*, 1992, De Meyer *et al.*, 1991) and 2) the alteration of medial SMCs from contractile to synthetic phenotype (Manderson *et al.*, 1989). The possibility that the collar may mechanically damage the medial layer would be unlikely since the histological examinations showed no mechanical damage or necrosis on the medial layer of the collared arteries.

The leftward shift in 5-HT concentration-response curve was also a typical effect of collaring as discussed earlier (Üstünes *et al.*, 1996).

ACh was used to induce endothelium-dependent vasorelaxant responses mediated by the release of nitric oxide. In contrast to the previous observations (Üstünes *et al.*, 1996, De Meyer *et al.*, 1991), the sensitivity to ACh did not change in collared arteries, whereas ACh-induced maximal relaxation increased significantly. Although the effect of collar on the pD_2 value of ACh in the present study is in the same line with van Put *et al.* (van Put *et al.*, 1995), currently, we have no explanation on these conflicting ACh-mediated responses.

Effects of defibrotide on vascular reactivity: Although not significant, KCl-, 5-HT- and histamine-induced maximal contractions were enhanced in carotid arteries from defibrotide-treated rabbits. Increased contractility in response to KCl appeared to be highly correlated ($r=.75$, $n=6$, Pearson's test) with the increased number of SMCs in medial cross-sectional area

of both sham and collared arteries. The reason why agonist-induced maximal contractions of carotid artery rings from defibrotid treated rabbits did not reach the statistical significance, could be due to the phenotypical alterations of contractile SMCs as discussed above.

Defibrotide was shown to inhibit the effects of endothelin by acting directly on the endothelial cells or increasing the levels of PGI₂ and/or EDRF (Fareed *et al.*, 1990, Rossoni *et al.*, 1991). A specific defibrotide-binding site on the vascular endothelial cells was also reported (Bilsel *et al.*, 1990). On the contrary, it was also reported that defibrotide did not modify the vasodilator effect of ACh in the precontracted mesenteric vascular bed (Peredo, 2002). In addition, most of actions of defibrotide have been observed in *in vivo* test models and very little pharmacological actions of this agent have been noted in *in vitro* systems (Fareed *et al.*, 1988). Our results are also in agreement with these earlier-obtained findings of others (Fareed *et al.*, 1988).

Defibrotide was reported to improve the physical performance by restoring the defective fibrinolysis, which ameliorates the blood flow, in patients with peripheral arterial disease, coronary artery disease, and Raynaud's phenomenon (Cimminiello, 1996). The drug is a deoxyribonucleic acid derivative and internalized into the nucleus (Bilsel *et al.*, 1989). Therefore, it is likely that the drug incorporates into the DNA. In addition, defibrotide was shown to induce the proliferation of vascular endothelial cells in human umbilical vein (Bilsel *et al.*, 1989). Additionally, present study demonstrates that defibrotide induces medial thickening in a conduit vessel, rabbit carotid artery. Therefore, the effects of defibrotide should be evaluated based on the vessel type considering that the medial thickening caused by a chronic defibrotide treatment may lead to luminal narrowing in different blood vessels. However, in the present study, the defibrotide-induced medial thickening was not accompanied by luminal narrowing and needs to be evaluated further in order to elucidate the effectiveness of the drug in peripheral obliterative arterial diseases, such as intermittent claudication.

Özet

Bu çalışmada bir polideoksiribonükleotid olan defibrotidin, normal ve silikon yaka uygulanmış tavşan karotid arterinde vasküler morfolojik özellikler ve kontraktıl yanıtlar üzerindeki etkileri araştırılmıştır. Defibrotid silikon yaka modelinde oluşan intimal kalınlaşmayı engellememiştir. Ancak beklenmedik bir şekilde, defibrotid tedavisi gören tavşanların gerek kontrol gerekse yaka arterlerinde istatistiksel olarak anlamlı medyal kalınlaşma gözlenmiştir. Defibrotidin luminal daralma olmaksızın yapmış olduğu medyal kalınlaşma, dışı doğru hipertrofik bir vasküler yeniden modellenmeyi temsil etmektedir. İlacın bu özelliği periferik obliteratif arter hastalığının tedavisinde daha önce bildirilen yararlı etkilerini açıklayabilir.

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