Angiogenesis in Vulnerable Atherosclerotic Plaques in Apparently Healthy Human Hearts

ERNESTO A. DURONTO[MTSAC, 1], CARLOS VIGLIANO[2], PATRICIA CABEZA MECKERT[2], ALEJANDRO BERTOLOTTI[3], RUBÉN LAGUENS[2], ENRIQUE GURFINKEL[†4]

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Address for reprints:
Dr. Ernesto A. Duronto
Hospital Universitario, Fundación Favaloro
Av. Belgrano 1746
(1093) CABA, Argentina
Phone number: (+5411) 4378-1293
Fax number: (+5411) 4378-3181
e-mail: eduronto@ffavaloro.org

ABSTRACT

Background
Angiogenesis or neovascularization involves the formation of new blood vessels adjacent to preexisting vessels. This vascular proliferation is prevalent in various clinical conditions, such as atherosclerosis. Microvessels in coronary artery atherosclerotic plaques may contribute to plaque instability.

Objectives
The aim of this study was to correlate the presence of angiogenesis in atherosclerotic plaques with the criteria of plaque vulnerability used by the American Heart Association (AHA).

Methods
One hundred and twenty one hearts from non-diabetic and apparently healthy transplant donors older than 40 years were selected. The coronary arteries were examined and all areas of cross-sectional luminal narrowing underwent histological, immunohistochemical and morphometric studies. A semi-quantitative score (scale 0-3) was used to identify of angiogenesis. Univariate and multivariate logistic regression analysis was performed to identify angiogenesis-related risk factors.

Results
On hundred and forty three high-risk lesions (AHA type IV, V and VI) in the left anterior descending coronary artery (46.3%), the circumflex coronary artery (28.9%) and the right coronary artery (43%) were identified. Angiogenesis had a statistically significant association with the severity of vascular occlusion, inflammatory cell infiltration, presence of a lipid core, fibrosis and periarteritis. A history of hypertension (HT) was associated with angiogenesis only in lesions of the left anterior descending coronary artery (LAD). According to the AHA classification angiogenesis was detected in 1 Type II, 5 Type III, 21 Type IV, 22 Type V, and 7 Type VI plaques.

Conclusions
Angiogenesis in vulnerable plaques was associated with the severity of vascular occlusion, inflammatory cell infiltration, fibrosis and presence of a lipid core, and with a history of HT in LAD lesions. There was no association between angiogenesis and plaque hemorrhage or calcification, suggesting that angiogenesis may anticipate plaque rupture.

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Key words > Angiogenesis, Pathologic - Aorta - Plaque, Atherosclerotic - Hearth

Abbreviations >

EAHA	American Heart Association	**INCUCAI**	Unique Central National Institute for the Coordination of Ablations and Implantations (Instituto Nacional Central Único Coordinador de Ablación e Implante)
RCA	Right coronary artery		
LCx	Left circumflex coronary artery		
LAD	Left anterior descending coronary artery		
HIF	Hypoxia-inducible factor	**VEGF**	Vascular endothelial growth factor
HT	Hypertension		

[MTSAC] Full Member of the Argentine Society of Cardiology
[1]Chief of the Coronary Care Unit, Fundación Favaloro
[2]Department of Pathology, Fundación Favaloro
[3]Homograft and Tissue Bank, Fundación Favaloro
[4]Director of the Department of Cardiovascular Medicine, Fundacion Favaloro, National Scientific and Technical Research Council (CONICET)
[†] Deceased.

Early in embryonic development, blood vessels develop by a process known as vasculogenesis, in which endothelial cell precursors called angioblasts and, in occasions, hematopoietic precursor cells or hemangioblasts form a network of primitive tubules.

In the extra-uterine life, formation of new vessels is a process known as angiogenesis or neovascularization. Angiogenesis refers to the formation of new conduits from pre-existing vessels. Circulating endothelial progenitor cells may also participate in the process.

Vascular proliferation is common in several clinical conditions as cancer (1) and atherosclerosis. (2)

Previous autopsy studies of subjects dying from coronary events reported the presence of neovessels in the intima, media and adventitia, suggesting that neovascularization from the adventitial vasa vasorum also participates in the development of the disease. (3, 4)

It is still unclear whether angiogenesis is the cause or the consequence of plaque progression leading to coronary events such as infarction or sudden death. (5, 6)

We investigated the presence of neovessels in atherosclerotic coronary plaques non-complicated by previous thrombotic events in human hearts from apparently healthy middle-aged transplant donors provided by the Unique Central National Institute for the Coordination of Ablations and Implantations (INCUCAI, Instituto Nacional Central Único Coordinador de Ablación e Implante)

METHODS

Patient selection

From January 1996 to December 2007, INCUCAI provided 652 hearts to the Fundación Favaloro .

One hundred and twenty one hearts of brain dead patients due to stroke or traumatic brain injury were selected for this study. The inclusion criteria were beating hearts excluded for heart transplantation due to inappropriate vital conditions but suitable for providing valves for homograft transplantation.

Exclusion criteria were: a) hearts appropriate for transplantation or heart and lung transplantation; b) age < 40 years; c) history of symptomatic atherosclerotic disease; d) myocardial infarction or any other heart disease found during the histopathological study of the heart; e) any prior chronic condition; f) any prior acute or chronic infective condition; and g) history of type 1 and type 2 diabetes (unacceptable for organ transplantation according to the local law).

Tissue preparation

Heart weight, interventricular septum thickness, left ventricular anterior, lateral and posterior wall thickness, and right ventricular free wall thickness were measured. After fixation in 4% phosphate-buffered formaldehyde, the three main coronary arteries were dissected and sectioned at 3-mm intervals. All areas with cross-sectional luminal narrowing were processed for histological study.

The tissue blocks were embedded in paraffin, and 5 μm thickness sections were stained with haematoxylin and eosin, Masson's trichrome, Movat pentachrome and Perls test (to determine iron stores). The percentage of vessel obstruction was estimated according to a -previous equation. (7)

The maximal thickness of the fibrous cap and the macrophage content were determined by morphometric analysis using commercially available software (Image-Pro Plus 4.5 for Windows®, Media Cybernetics, Silver Spring, MD).

Immunohistochemical studies

Tissue sections were rehydrated and incubated with macrophage CD68 (Dako®, Carpinteria, California, USA), smooth muscle anti-actin (Biogenex®, San Ramon, CA, USA) anti-CD34 (Endothelial Cell Marker) (BioGenex®, San Ramon, CA, USA) or anti-von Willebrand factor (Dako) monoclonal antibodies. A commercially available kit containing biotinylated anti-mouse antibody, with streptavidin peroxidase reagents and EAC as chromogen (BioGenex®, San Ramon, CA, USA) was used as secondary detection system.

Morphometry

Microvessels were counted for the whole plaque. Each microvessel was defined as a lumen surrounded by a ring-like structure of endothelial cells immunostained with anti-CD34 or anti-von Willebrand factor antibodies.

The extent of angiogenesis was graded according to a semiquantitative score from 0 to 3. (8) Histopathological changes such as inflammatory cell infiltrate, presence of a lipid core, fibrosis, calcification, hemorrhage and periarteritis were examined in sections stained with haematoxylin and eosin, and quantified according to the involved area -. Changes were graded as absent (0), mild (1), moderate (3) or severe (3) according to the methods described by Cliff and Kumamoto. (9, 10)

Classification of lesions

In this study, plaque vulnerability was categorized according to the American Heart Association (AHA) classification of Stary et al., (11): type IV lesions, type V with subtypes Va, Vb, and Vc lesions; and type VI with subtypes VIa, VIb, and Vic lesions.

Two independent pathologists (C.V. and R.L) blinded to the donor clinical condition performed the histological examination. The percent of luminal narrowing judged by both pathologists was further compared with the following equation: (1 − lumen area/internal elastic lamina area) × 100. (7).

Angiogenesis was analyzed in each coronary vessel and was correlated with the demographic data, the diagnosis of hypertension (HT), presence of advanced types of atherosclerotic lesions according to the AHA classification (11) and the histological features of the plaques.

Statistical Analysis

Continuous variables were expressed as mean (standard deviation) or median (25-75% interquartile range) and categorical variables as percentages. Continuous variables were compared using Student's t test or Mann-Whitney test as applicable. The chi square test or Fisher's exact test was used to compare categorical variables. For the statistical analysis, dichotomous partitioning established the presence (0) or absence (1) of angiogenesis in the plaques. A logistic regression analysis was performed to identify angiogenesis-related risk factors. A p value < 0.05 was considered statistically significant.

Statistical analysis was performed using SPSS ®12.0

statistical package (SPSSInc, Chicago, Illinois).

RESULTS

Table 1 shows population age, gender, history of HT and cause of death and the histopathological variables related to the presence or absence of angiogenesis.

The presence of angiogenesis in at least one of the three coronary vessels was associated with male gender, percentage of vessel occlusion, heart weight and wall thickness. There was no association with age or cause of death.

Forty four of the 121 cases had known HT and 77 had no history of this condition.

The prevalence of HT was greater in men. Heart weight, interventricular septum thickness and left ventricular anterior, lateral and posterior wall thickness were significantly greater in subjects with HT ($p < 0.001$).

The unadjusted analysis demonstrated that the presence of angiogenesis in the coronary vessels was greater in subjects with known HT. However, this finding was only significant in the left anterior descending coronary artery (LAD) ($p = 0.006$) but not in the right (RCA) or the left circumflex (LCx) coronary arteries ($p = 0.760$ and 0.460, respectively).

Table 2 shows the presence of 363 plaques in the 121 studied hearts. One hundred and forty three plaques were advanced types of atherosclerotic lesions (types IV, V and VI of the AHA), 56 (46.3%) located in the LAD, 35 (28.9%) in the LCx and 52 (43%) in the RCA.

Presence of angiogenesis was detected in 50 of the 143 advanced lesions (34.9%) and in 6 of the 220 low-risk plaques (2.7%) ($p < 0.001$).

In 82 hearts there was no evidence of angiogenesis in any of the three analyzed vessels. Angiogenesis was detected in at least one of the three epicardial vessels in 26 hearts, in two vessels in 9 hearts and in the three coronary arteries in 4 hearts.

Figure 1 shows representative images of the AHA type, II and III lesions (11) with absence and presence of intimal neovascularization (Figure 1 A and B, respectively).

Panels C and D of Figure 1, show advanced lesions with neovascularization within the atherosclerotic lesion and Panels E and F demonstrate the presence of angiogenesis in areas with moderate inflammatory infiltrate inside the plaque and in the periadventitial area, respectively. Finally, panels G and H show neovascularization in fibrocalcific plaques (Typs Vb lesions) and in type VI lesions complicated with previous hemorrhage. (11)

The analysis of angiogenesis in each specific vessel and its association with the demographic variables, causes of death and histopathological characteristics are detailed in Table 3.

In Figure 2 A, the y-axis represents the mean semiquantitative score applied to all the histological features of the plaques according to the AHA type of lesion. (11) It can be seen that fibrosis, myofibroblast hyperplasia, lipid content and inflammation precede the development of angiogenesis, while calcification and hemorrhage are more linked with complicated type V and VI lesions of the AHA classification. (11)

Multivariate logistic regression analysis of all the plaques demonstrated that only fibrosis and

	Total	Angiogenesis		p value
		Yes	No	
	n = 121	n = 39	n = 82	n = 82
Mean age, years ± SD	50.9 ± 5.7	51.0 ± 5.8	50.8 ± 5.8	50.8 ± 5.8
Men, n (%)	72	31 (79.5)	41 (50.0)	41 (50.0)
Hypertension, n (%)	44	19 (48.7)	25 (30.5)	25 (30.5)
Cause of death				
Brain trauma, n (%)	16	5 (12.8)	11 (13.4)	11 (13.4)
Ischemic stroke, n (%)	24	3 (7.7)	21 (25.6)	21 (25.6)
Hemorrhagic stroke, n (%)	81	31 (79.5)	50 (61.0)	50 (61.0)
LAD stenosis, median % (percentiles 25-75)	25 (17.5-50)	50 (30-60)	25 (15-37.5)	25 (15-37.5)
LCx stenosis, median % (percentiles 25-75)	25 (15-30)	40 (27.560)	25 (10-30)	25 (10-30)
RCA stenosis, median % (percentiles 25-75)	25 (20-45)	40 (25-50)	25 (20-35)	25 (20-35)
Heart weight, g ± SD	328.8 ± 89.2	369.2 ± 98.6	310.1 ± 78.2	310.1 ± 78.2
RV, mm ± SD	4.2 ± 0.9	4.4 ± 1.0	4.1 ± 0.9	4.1 ± 0.9
IVS, mm ± SD	16.4 ± 3.1	17.2 ± 2.8	16.0 ± 3.2	16.0 ± 3.2
LV anterior wall, mm ± SD	13.9 ± 2.7	14.9 ± 2.8	13.5 ± 2.6	13.5 ± 2.6
VV lateral wall, mm ± SD	14.1 ± 2.7	15.1 ± 2.9	13.6 ± 2.5	13.6 ± 2.5
LVposterior wall, mm ± SD	15.1 ± 2.8	15.8 ± 2.7	14.7 ± 2.8	14.7 ± 2.8

n: Number of patients. mm: Millimeters. g: Grams. SD: Standard deviation. LAD: Left anterior descending coronary artery. LCx: Left circumflex coronary artery. RCA: Right coronary artery. RV: Right ventricular. LV: Left ventricular. IVS: Interventricular septum .

Table 1. Angiogenesis according to demographic data and histopathological features in 121 hearts

Table 2. AHA atherosclerotic plaques and presence of angiogenesis

AHA* atherosclerotic plaques	LAD			LCx			RCA		
	Total	Angiogenesis		Total	Angiogenesis		Total	Angiogenesis	
		Yes n (%)	No n (%)		Yes n (%)	No n (%)		Yes n (%)	No n (%)
Type I lesions	16 (13.2)	0	16	25 (20.7)	0	25	11 (9.1)	0	11
Type II lesions	39 (32.2)	0	39	55 (45.5)	1	54	46 (38.0)	0	46
Type III lesions	10 (8.3)	4	6	6 (5.0)	0	6	12 (9.9)	1	11
Type IV lesions†	23 (19.0)	11	12	24 (19.8)	4	20	17 (14.0)	6	11
Type V lesions†	28 (23.1)	9	19	10 (8.3)	2	8	24 (19.8)	11	13
Type VI lesions†	5 (4.1)	1	4	1 (0.8)	1	0	11 (9.1)	5	6
Total	121	25/121 (20.7)	96/121 (79.3)	121	8/121 (6.6)	113/121 (93.4)	121	23/121 (19.0)	98/121 (81.0)
AHA advanced plaques, total†	56 (46.3)	21/56 (37.5)	35/56 (62.5)	35 (29.0)	7/35 (20.0)	28/35 (80.0)	52 (43.0)	22/52 (42.1)	30/52 (57.7)

*American Heart Association classification. (11) † Corresponding to the American Heart Association type IV, V and VI lesions. LAD: Left anterior descending coronary artery. LCx: Left circumflex coronary artery . RCA. Right coronary artery.

inflammation were associated with the presence of angiogenesis (Figure 2 B). This type of analysis was not performed for each vessel.

DISCUSSION

The results of the present study show signs of active inflammation and angiogenesis in a significant proportion of atherosclerotic plaques in the coronary arteries from apparently healthy subjects. The anomalies were almost exclusively found in vulnerable plaques according to the universally accepted classification of atherosclerotic lesions. (12) These findings confirm previous studies describing atherosclerotic plaques with inflammatory infiltrate in the hearts of subjects with advanced coronary artery or carotid artery disease. (13-15)

They also suggest that inflammation precedes angiogenesis, as infiltration of macrophages and lymphocytes is common in type III lesions while neovessels appear in a greater proportion in type IV lesions. Interestingly, inflammation and angiogenesis develop in the most internal portions of the intima adjacent to the internal elastic lamina while neovessels originate from the vasa vasorum or from the adventitia.

However, it can be speculated that initial angiogenesis and inflammation depends on the development and progression of the atherosclerotic plaque. As the distance between the endothelium and the intima-media junction increases, hypoxia is produced in the region of the arterial wall receiving luminal oxygen supply. Since hypoxia induces angiogenesis by activating hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF), vessel neoformation from the media and the adventitia (4, 16-18) would supply oxygen to that deficient region of the arterial wall.

Morever, insufficient oxygen supply might produce inflammation and fibrosis contributing to reduce the vessel lumen with subsequent progression to thin cap fibroatheroma or vulnerable plaque. (19)

Angiogenesis was present in 15% of 363 analyzed plaques, mainly type IV, V and V lesions. The modified Stary et al. (11) scheme was used to classify lesions into two groups. One group includes early and immature lesions, also called intimal xanthomas by Virmani et al., (20) which probably represent a natural biological evolution of lipid deposits within the vessels. (21) The other group represents larger plaques associated with inflammation which have greater oxygen demands and exhibit potential clinical instability.

Iron content in advanced lesions, an indirect indicator of plaque hemorrhage (22, 23), was uncommon in the studied samples.

Plaque calcification is related with necrosis and is a sign of advanced lesions. (24, 25) In this study, absence of a clear relationship between angiogenesis and calcification might indicate that calcification "conceals" the existence of neovessels which cannot be identified with routine techniques.

In addition, plaque analysis revealed vessels apparently originating from the vascular lumen, though this finding was only seen in advanced lesions (type V and VI).

The processes involved in the progression of the atherosclerotic plaque were originally investigated in animal models as hypercholesterolemic apolipoprotein E-deficient mice, suggesting an evolution similar to

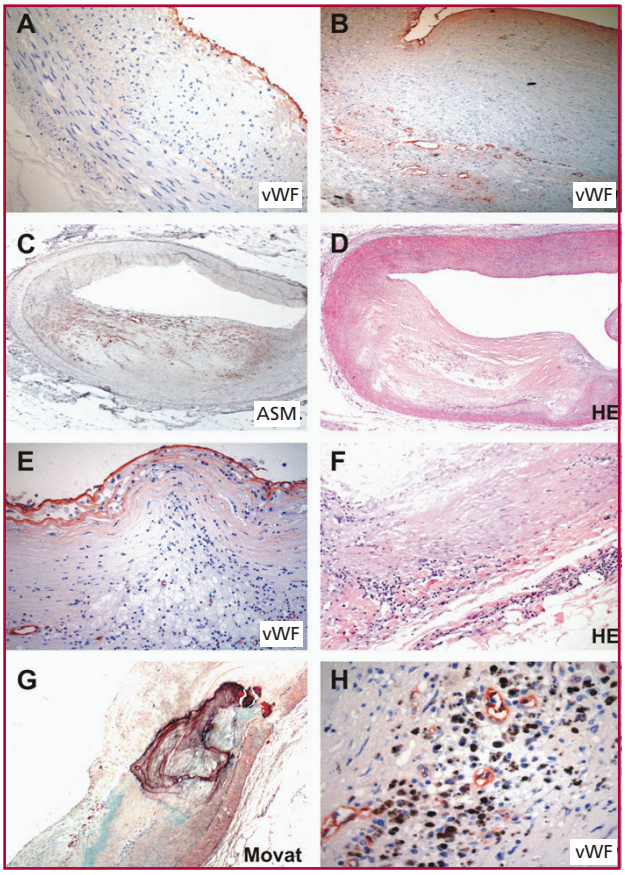
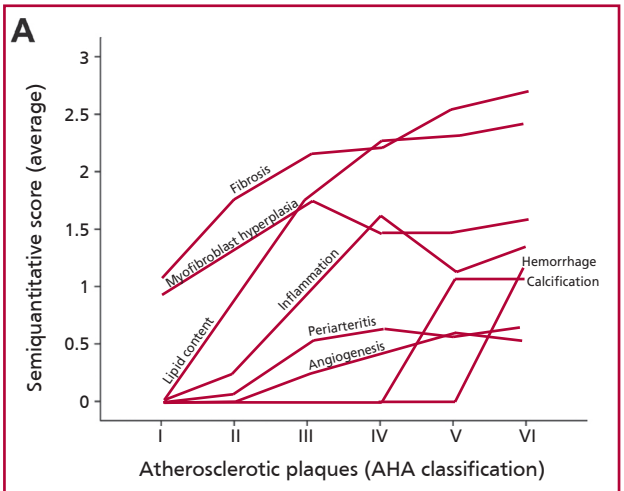


Fig. 1. A. Right coronary artery from a 48 year-old woman who died due to hemorrhagic stroke. Myofibroblast hyperplasia, intimal fibrosis and focal accumulation of lipids (fatty streaks) [type II lesion, AHA classification (11)] are observed. This section was incubated with anti-vWF (von Willebrand factor) monoclonal antibodies. There is no angiogenesis. Magnification x200.
B. Left anterior descending coronary artery from a 55 year-old man who died due to traumatic brain injury. Myofibroblast hyperplasia, intimal fibrosis and focal accumulation of lipids [type III lesion, AHA classification (11)]. Angiogenesis is seen in the depth of the neoitima, adjacent to the media layer. This section was incubated with anti-vWF (von Willebrand factor) monoclonal antibodies. Magnification x100.
C. Left circumflex coronary artery from a 51 year-old man who died due to traumatic brain injury. Type IV lesion corresponding to the AHA classification (11). There is intimal vascularization. This section was incubated with anti-smooth muscle (ASM) actin monoclonal antibodies. Magnification x25.
D. Left circumflex coronary artery from a 68 year-old man who died due to ischemic stroke. Eccentric fibrolipid plaque [type V lesion, AHA classification (11)] with presence of angiogenesis in the intima, inside the plaque and in the periadventitial area. Section stained with haematoxylin and eosin (HE). Magnification x25.
E. Left anterior descending coronary artery from a 47 year-old woman who died due to traumatic brain injury. This section of the atherosclerotic plaque shows macrophage foam cells, chronic lymphocytic infiltrate and neovascularization. This section was incubated with anti-vWF (von Willebrand factor) monoclonal antibodies. Magnification x200.
F. Section from the subadventitial area of the left anterior descending coronary artery from the patient described in E. Mild inflammatory infiltrate in the periadventitial area and consecutive sections of a vessel penetrating from the adventitia are seen. This section was incubated with anti-CD34 monoclonal antibodies. Magnification x200.
G. Left anterior descending coronary artery from a 60 year-old man who died due to traumatic brain injury. Presence of fibrocalcific plaque [type Vb lesion, AHA classification (11)] with angiogenesis in the surface. Movat pentachrome. Magnification x25.
H. Right coronary artery from a 44 year-old man who died due to hemorrhagic stroke. A section incubated with anti-vWF (von Willebrand factor) monoclonal antibodies shows vascularization and many hemosiderin-laden macrophages. Magnification x400.



B

Histological characteristics	Angiogenesis		Logistic regression	
Semiquantitative score (average)	Yes	No	Multivariate analysis	
			Odds ratio (95% CI)	*P*
	N= 56	N= 307		
Fibrosis	2.59(0.50)	1.82(0.62)	6.18(2.94 - 13.0)	<0.001
Myofibroblast hyperplasia	1.59(0.63)	1.32(0.56)	0.87(0.49 - 1.52)	0.617
Lipid content	2.21(0.73)	1.27(0.94)	1.03(0.61 - 1.72)	0.921
Inflammation	1.52(0.74)	0.57(0.79)	2.94(1.65 - 5.23)	<0.001
Periarteritis	0.70(0.71)	0.24(0.53)	0.67(0.36 - 1.25)	0.210
Calcification	0.55(0.81)	0.18(0.53)	1.19(0.72 - 1.97)	0.496
Hemorrhage	0.16(0.50)	0.04(0.26)	0.92(0.43 - 1.98)	0.840

Fig. 2. Angiogenesis according to the histological characteristics of atherosclerotic plaques. A. Histological characteristics of 363 atherosclerotic plaques, graded using a semiquantitative score (from 0 to 3) according to the American Heart Association classification. (11) B. Multivariate logistic regression analysis of angiogenesis in 363 atherosclerotic plaques, according to the histological characteristics.

the one described here. (26)

In this study, increased angiogenesis only in LAD lesions of HT patients might be explained by greater remodeling secondary to increased cardiac mass.

Study Limitations

VEGF identification was not performed. (8). Although this growth factor could have suggested the existence of intraplaque neovessels apart from those originating from the adventitia, previous studies suggest that VEGF plays an inflammatory rather than an angiogenic role in the vessel wall. (2)

CONCLUSIONS

Angiogenesis was significantly associated with the severity of vascular occlusion, inflammatory cell infiltration, and presence of a lipid core. Moreover, only LDA lesions were associated with previous

history of HT. However, different from other studies, there was not a firm association of angiogenesis with plaque hemorrhage or calcification, suggesting that angiogenesis may anticipate plaque rupture.

RESUMEN

Presencia de angiogénesis en placas vulnerables ateroscleróticas en corazones humanos aparentemente sanos

Introducción
La angiogénesis o neovascularización involucra la formación de nuevos conductos en las adyacencias de vasos preexistentes. Esta proliferación vascular es frecuente en varias circunstancias clínicas, como es el caso de la aterosclerosis. Los microvasos de las placas ateroscleróticas coronarias pueden estar vinculados a la inestabilidad de la lesión.

Objetivo
Correlacionar la presencia de angiogénesis en placas ateroscleróticas con los criterios de vulnerabilidad de la clasificación de la American Heart Association (AHA).

Material y métodos
En 121 corazones de donantes no diabéticos aparentemente sanos y mayores de 40 años destinados para homoinjertos se examinaron las arterias coronarias y todas las áreas de estrechamiento luminal se sometieron a estudios histológicos, inmunohistoquímicos y morfométricos. Para el análisis de la angiogénesis se empleó un puntaje semicuantitativo (escala 0-3). Se realizó un análisis de regresión logística univariado y multivariado para identificar factores de riesgo relacionados con la angiogénesis.

Resultados
Se hallaron 143 lesiones de riesgo alto (AHA tipos IV, V y VI) en las arterias descendente anterior (46,3%), circunfleja (28,9%) y coronaria derecha (43%). La angiogénesis se asoció en forma estadísticamente significativa con el grado de oclusión vascular, la infiltración de células inflamatorias, la presencia de centro lipídico, la fibrosis, la periarteritis y, sólo en la descendente anterior, con el antecedente de hipertensión arterial ($p < 0,006$). Se detectó angiogénesis en 1 placa tipo II, en 5 tipo III, en 21 tipo IV, en 22 tipo V y en 7 placas tipo VI (AHA).

Conclusiones
La angiogénesis de placas vulnerables se asoció con el grado de oclusión vascular, la infiltración de células inflamatorias, la fibrosis, la presencia de núcleo lipídico y, sólo en la descendente anterior, con el antecedente de hipertensión arterial. No se encontró asociación con la hemorragia intraplaca o la calcificación, lo cual sugiere que la angiogénesis puede anticipar la rotura de las placas.

Palabras clave > Angiogénesis patológica - Aorta - Placa aterosclerótica - Corazón

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Conflict of interest

None declared.