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Function of s100 Protein in Coronary Atherosclerosis

Función de la Proteína s100 en Aterosclerosis Coronaria

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SUMMARY: Atherosclerosis is a complex disease whose pathogenesis includes endothelial activation, accumulation of lipids in the subendothelium, formation of foam cells, fat bands and formation of atherosclerotic plaque. These complex mechanisms involve different cell populations in the intimate sub-endothelium, and the S-100 protein family plays a role in a number of extracellular and intracellular processes during the development of atherosclerotic lesions. The aim of this study was to determine the phenotypic characteristics of smooth muscle cells and the consequent expression of S100 protein in atherosclerotic altered coronary arteries in advanced stages of atherosclerosis. 19 samples of right atherosclerotic coronary arteries in stages of fibro atheroma (type V lesion) and complicated lesions (type VI lesion) have been analyzed. According to the standard protocol, the following primary antibodies have been used in the immunohistochemical analysis: a-smooth muscle actin (α-SMA), vimentin and S-100 protein. All analyzed samples have been in advanced stages of atherosclerosis, fibro atheroma (stage V lesions) and complicated lesions (type VI lesions). Most of them have had the structure of a complicated lesion with atheroma or fibro atheroma as a basis, subsequently complicated by disruption (subtype VI a), hemorrhage (subtype VI b) or thrombosis (subtype VI c), as well as by the presence of several complications on the same sample. Marked hypocellularity is present in the subendothelium of plaques. Cell population at plaque margins is characterized by immunoreactivity to α-SMA, vimentin, and S100 protein. Some of these cells accumulate lipids and look like foam cells. In the cell population at the margins of the plaques, smooth muscle cells of the synthetic phenotype are present, some of which accumulate lipids and demonstrate S100 immunoreactivity. Summarizing numerous literature data and our results, we could assume that smooth muscle cells, due to their synthetic and proliferative activity in the earlier stages of pathogenesis, as well as the consequent expression of S100 protein, could accumulate lipids in the earlier stages of atherosclerosis which, in advanced stages analyzed in this study, result in immunoreactivity of foam cells of smooth muscle origin to S100 protein.

KEY WORDS: S100 protein; Smooth muscle cells; Coronary artery atherosclerosis.

INTRODUCCIÓN

Atherosclerosis is a complex disease whose pathogenesis includes remodeling of the arterial wall under the conditions of increased pressure, associated with hypercholesterolemia and consequent inflammation. Under the conditions of hypertension, in the first phase, a compensatory dilatation of the vascular wall occurs. During this phase, tissue factors that affect vascular smooth muscle cells, promote their phenotypic modification from contractile

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to synthetic phenotype, and then their migration into the subendothelial intima, where they synthesize large amounts of extracellular matrix, predominantly composed of proteoglycans, are synthesized (Libby, 2007). As a consequence of this process, the second phase of vascular remodeling occurs - hypertrophy. Hypertension associated with hypercholesterolemia leads to lipid accumulation in the subendothelium of the intima, formation of foam cells and forming of atherosclerotic plaque.

Like other muscle-type arteries, the intima of the coronary arteries consists of the endothelium, basement membrane, and subendothelial connective tissue. The endothelium is composed of a single layer of endothelial cells that are connected by tight joints and occluding bonds. Although muscle-type arteries are characterized by the presence of a small number of smooth muscle cells in the subendothelium, coronary arteries, specifically, contain bundles of smooth muscle cells in the subendothelial layer (Thyberg *et al*., 1995; Eefting *et al*., 1997). Intimal smooth muscle cells are longitudinally oriented in the outer part of the subendothelium, so that they form a clearly separated musculoelastic layer (Eefting *et al*., 1997). Although the embryonic origin of this isolated smooth muscle layer is not yet fully elucidated, its presence in the intima of the coronary arteries is considered to cause numerous physiological and morphological specificities of these arteries, and may represent a proaterogenic predisposition.

The tunic of the medium is composed of several layers of smooth muscle cells and a connective tissue composed of elastic fibers, collagen and proteoglycans (Tanaskovic *et al*., 2019). The medium contains more than 40 circumferentially or helically arranged layers of smooth muscle cells. In the media, there is a difference between the inner and outer part with differently directed smooth muscle cells (Bumbasirevic *et al*., 2007).

Adventitia is composed of a fibrous connective tissue (collagen, elastic fibers), in which *vasa vasorum*, nerves and lymph vessels are located.

The embryonic origin of smooth muscle cells, which largely determines their characteristics, is different. It is known that during embryogenesis, arterial smooth muscle cells are formed by the differentiation of mesenchymal cells of the splanchopleural mesoderm. Due to their embryonic origin, during development and in certain pathological changes in adulthood, these cells demonstrate immunoreactivity to vimentin, while in a highly differentiated form they express desmin (Landerholm *et al*., 1999; Nikolic *et al*., 2004; Vukovic *et al*., 2006). On the other hand, in large arteries in the upper part of the body, during embryogenesis, a large number of smooth muscle cells arrive from the nerve crest. These cells, due to their neuroectodermal origin, demonstrate S100 protein expression and consequently high affinity for fatty acid binding (Tanaskovic *et al*., 2019). Coronary arteries that is endothelial, smooth muscle and connective tissue components of their wall, develop from the proepicardial organ, which means that they also originate from the mesenchyme of the splanchopleural mesoderm (Tanaskovic *et al*., 2011; Tanaskovic *et al*., 2013).

S 100 proteins are a family of low molecular weight proteins that bind calcium. So far, 25 different S100 proteins have been discovered (Siengethaler *et al*., 1997; Lackovic *et al*., 2011). They are functionally divided into three main groups: those that only exhibit intracellular regulatory effects, those that have intracellular and extracellular functions, as well as those with extracellular regulatory effects. S100 protein also participates in differentiation, proliferation, migration, as well as in tissue repair (Donato *et al*., 2013; Bertheloot & Latz, 2017). In some cases, especially the C100 protein may be induced under pathological conditions in some cells, in which it is not found under normal physiological conditions (Lacolley *et al*., 2012). Also, S100 protein can be expressed in the cytoplasm of the cell due to cellular stress or damage to cells and tissues. Extracellular S100 proteins are also considered biomarkers for some specific diseases (Xia *et al*., 2018).

The aim of this study was to determine the phenotypic characteristics of plaque smooth muscle cells of atherosclerotic altered coronary arteries in advanced stages of atherosclerosis.

MATERIAL AND METHOD

Ethical concerns. All procedures are done in accordance with ethical standards and approval of local ethical committee (Clinical Center Kragujevac, Serbia, number 01/4988).

Study Protocol. The 19 samples of right atherosclerotic coronary arteries in stages of fibroatheroma (type V lesion) and complicated lesions (type VI lesion) were analyzed. All samples were examined at the Clinic for Pathological Anatomy of the Clinical Center of Kragujevac.

Pathohystological analysis. Samples were fixed in 4 % neutral buffered formalin solution, for 24 h, at room temperature. Upon completion of fixation, the samples were dehydrated

by passing through a series of alcohol of increasing concentration (70 %, 96 % and 100 %), clarified in xylene and molded in paraplast. Serial cross-sections, 5 μm thick, were cut with a Leica SM 2000R microtome. In routine analysis of samples, after dewaxing in xylene and hydration in descending order of alcohol, sections were stained with Haematoxylin according to Mayer, clarified in 2 % eosin solution, then dehydrated, clarified and mounted on plates with Canada balm, according to a standard procedure (Bancroft & Gamble, 2002; Jones, 2002). The classification of atherosclerotic lesions was performed according to a Report from the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association (Stary *et al*., 1995).

For immunohistochemical analysis, 5 μm thick sections were mounted on special highly adherent SuperFrost plates and dried at 56 ºC for 1 hour. The immuno histochemical staining procedure included antigen unmasking procedures, endogenous peroxidase blocking, incubation of the preparation with primary antiserum, and the immunohistochemical method - LSAB + / HRP, in the manner previously described (Vukovic *et al*., 2004, 2006; During immunohistochemical staining, the following primary antisera were used in the given dilutions: α-Smooth Muscle Actin - α-SMA (1:25); vimentin (1: 100) and S-100 protein (1: 200).

RESULTS

All analyzed samples were in advanced stages of atherosclerosis, fibroatheroma (stage V lesions) and complicated lesions (type VI lesions). Most of them had the structure of a complicated lesion that has atheroma or fibroatheroma as a basis, subsequently complicated by disruption (subtype VI a), hemorrhage (subtype VI b) or thrombosis (subtype VI c), as well as the presence of several complications on the same sample.

Endothelial discontinuity is observed in all analyzed samples of atherosclerotic coronary arteries. Intima has thickened and exceeds the thickness of the media. It is the thickest in the area of the atheroma, so it focally, eccentrically narrows the lumen. It is characterized by a stenotic fibrous tissue, eccentrically placed, so that it significantly narrows the lumen.

In fibroatheroma, the endothelium and basement membrane are discontinued. There is a thin fibrous cap over the lipid nucleus. Distinct hypocellularity is observed everywhere in the plaque. Cell population at plaque margins demonstrates an immunohistochemical response to α-SMA, vimentin, and S100 protein. The thinned medium contains α-SMA- and vimentin- immunoreactive smooth muscle cells. Periadventive adipose connective tissue is S100 protein immunoreactive (Fig. 1a-1b).

Hypocellularity is also observed in the subendothelium of the intima of complicated plaques. Preserved foam cells are immunoreactive to vimentin and S100 protein. Neovascular formations whose wall demonstrates a reaction to α-SMA are observed. Smooth muscle cells that are immunoreactive to α-SMA and vimentin are present on the margins of fibrous plaque.

The tunic of the media is completely thin. Smooth muscle cell media are immunoreactive to α-SMA and vimentin. Periadventitial adipose tissue demonstrates immunoreactivity to S100 protein (Fig. 1c-1d).

The analyzed samples in the stage of complicated atherosclerotic lesion are characterized by a stenotic fibrous tissue, eccentrically placed, so that it significantly narrows the lumen (Fig. 2a, 2b).

In the subendothelium of the intima of the analyzed samples, there are complicated plaques that occupy a very wide area. Within the large plaque, a number of smaller plaques can be seen that are separated from each other by elastic membranes. In some places, between the formed smaller plaques, recanalization can be observed, that is a newly formed lumen (Figs. 2c, 2d). The formed organized plaque (often with several lumens) is composed of collagen fibers. There are cracks present everywhere in the plaque, in which there are foam cells, some of which are immunoreactive to α-smooth muscle actin and vimentin. Calcifications and neovascular formations are observed, whose endothelium demonstrates a reaction to vimentin, and the wall to α-smooth muscle actin.

Cellular infiltrations are observed on the margins of the fibrous plaque. In addition to leukocytes, the presence of S100 protein, α-smooth muscle actin and vimentin immunoreactive cells is observed in these cell populations on the margins of plaques. Some of them contain lipid inclusions, they look like foam cells (Fig. 2a, 2b).

The inner elastic membrane is reduplicated on individual samples.

The tunic of the media is completely thin. The cells demonstrate an immunohistochemical reaction to α-smooth muscle actin and vimentin (Figs. 1 and 2). Newly formed blood vessels are present everywhere in the media.

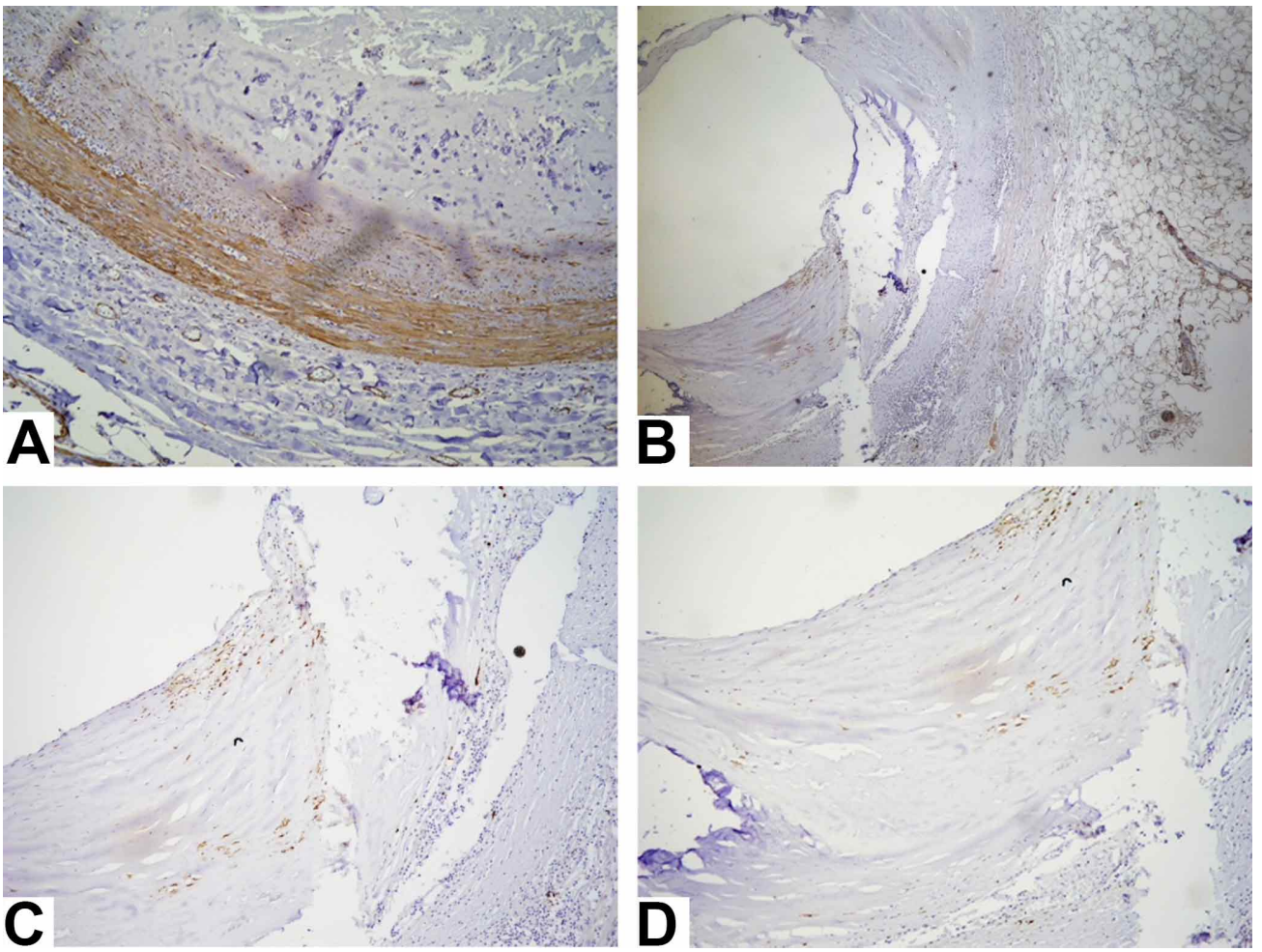

Fig. 1. Atherosclerosis of coronary arteries - fibroatheroma stage and smooth muscle cell media A) immunohistochemical staining for α-SMA, x 128; B) immunohistochemical staining for S100 protein, x 32; C) immunohistochemical staining for S100 protein, x 64; D) immunohistochemical staining for S100 protein, x 64.

The outer elastic membrane is completely thin and difficult to see due to the general disorganization of the medium.

Adventitia is composed of loose connective tissue with a lot of collagen and few elastic fibers. Fibroblasts in adventitia demonstrate immunoreactivity to vimentin. Adventitial blood vessels *(vasa vasorum*) have thickened walls, with an immunohistochemical reaction of the wall to a-smooth muscle actin, as well as of their endothelium to vimentin. Periadventitial adipose connective tissue demonstrates an immunohistochemical response to S100 protein.

DISCUSSION

The results of our study showed hypocellularity of plaques of advanced lesions with cells present at plaque margins. In this cell population, cells that demonstrate an immunohistochemical response to α-SMA, vimentin, and S100 protein are present. According to the literature data, as well as to the results of our previous research, immunoreactivity to α-SMA is a common characteristic of vascular smooth muscle cells, while immunoreactivity to vimentin, in the absence of immunoreactivity to desmin as a marker of highly differentiated contractile phenotype, suggests that smooth muscle cells of the synthetic phenotype are in question (Vukovic *et al*., 2010). It is also observed that these synthetically active smooth muscle cells demonstrate immunoreactivity to S100 protein, which we will consider separately. Parallel with the above mentioned, it is noticed that some of these cells accumulate lipids and look like foam cells (Libby, 1996).

Although muscle-type arteries, according to the

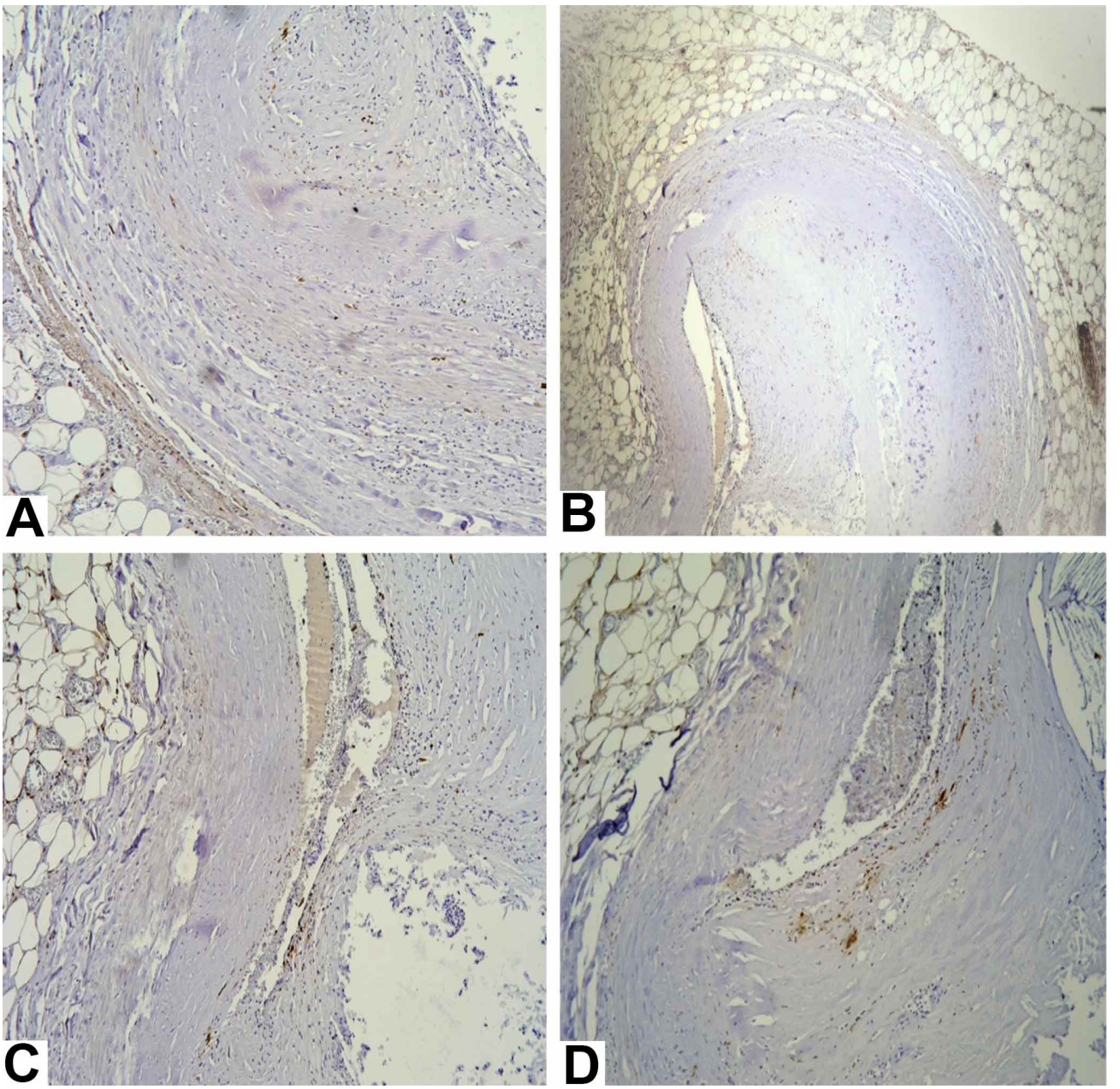

Fig. 2. Atherosclerosis of coronary arteries - stage of fibroatheroma. Rare cells in hypocellular plaque, thinned media, and adventitia vimentin-immunoreactive fibroblasts A) immunohistochemical staining for vimentin, x 64; B) Immunoreactivity of smooth muscle and foam cells in fibrous plaque, as well as periadventitial adipose tissue (immunohistochemical staining for S100 protein, x 16), C) Immunoreactivity of smooth muscle and foam cells in fibrous plaque, as well as periadventitial adipose tissue (immunohistochemical staining for S100 protein, x 64); D) immunohistochemical staining for S100 protein, x 128.

literature data, are characterized by the presence of a small number of smooth muscle cells in the subendothelium, the coronary arteries, specifically, contain bundles of smooth muscle cells in the subendothelial layer (Libby, 1996). According to the same data, the intimate smooth muscle cells of the unaltered coronary artery are longitudinally oriented in the outer part of the subendothelium, so that they form a clearly isolated layer (Libby, 1996). Although the embryonic origin of this isolated smooth muscle layer has not yet been fully elucidated, it is believed that its presence in the intima of the coronary arteries causes their numerous specificities. In light of this belief, the results of our study, which show the immunoreactivity of these cells to S-100 protein, are interesting.

Namely, according to the literature data, S-100 protein is found in cells originating from the neural crest, glial and ependymal cells of the CNS, neurolemmocytes (Schwann cells) of the peripheral nervous system, melanocytes, chondrocytes, adipocytes, cardiomyocytes, smooth muscle and heart cells, histiocytes and dendritic cells (Langerhans cells), as well as interdigital reticular lymph node cells. In these cells, S-100 protein is found in both the nucleus and cytoplasm (Gould *et al*., 1986). In addition, the expression of S-100 protein subtypes is associated with intracellular processes that have Ca ++ mediated regulation, such as proliferation, differentiation, and phosphorylation (Gould *et al*., 1986).

Results of recent studies have also shown that S100 protein plays a role in various intracellular and extracellular processes, to interact with various receptors by participating in the processes of inflammation, cell differentiation, proliferation and calcium homeostasis (Donato, 2003).

Other authors also confirm that S100 family of proteins is a family that includes members of a similar structure, out of which some are more and some are less important for inflammation, proliferation, differentiation and cell migration (Gould *et al*., 1986; Donato).

In light of these data, our results that show the presence of S100 protein- and vimentin-immunoreactive cells in the subendothelium, could suggest the neuroectodermal origin of these cells, but also the presence of these cells in a calcium-mediated process, such as proliferation. According to the literature data and the results of our previous research, after the loss of contractile characteristics, smooth muscle cells proliferate, which is the characteristic of the earlier stages of the pathogenesis of atherosclerotic lesions. As the expression of this protein is expressed in processes mediated by Ca ++, such as migration (in this case cells from the medium) and proliferation in the intima, both resident intimate and cells arriving from the medium, their S100-immunoreactivity is expected. In addition, according to literature data, the complex composed of S100A8 and S100A9 proteins (whose target protein is vimentin) shows a high affinity for binding unsaturated fatty acids in order to modulate their proinflammatory functions (Gould *et al*., 1986; Donato).

Summarizing the above literature data and our results, we could assume that smooth muscle cells, due to their synthetic and proliferative activity in the earlier stages of pathogenesis, as well as the consequent expression of S100 protein, could accumulate lipids in the earlier stages of atherosclerosis, which in advanced phases, analyzed in this study, results in immunoreactivity of foam cells of smooth muscle origin to S100 protein.

Due to the role of S100 protein family in cellular processes during the pathogenesis of atherosclerosis, phenotypic modulation of cells involved in this process and lipid metabolism, recent research has focused on a detailed examination of this role in order to improve therapeutic procedures. Given that atherosclerosis is a complex disease characterized at various stages by dyslipidemia, coronary artery calcification and oxidative stress, and that with the progression of atherosclerosis, acute inflammation in the subendothelium takes the form of a chronic inflammatory response, the role of S100 protein in these processes was examined. It was found that in response to proinflammatory cytokines, S100 protein is expressed both in monocytes and endothelial cells and in vascular smooth muscle cells, which is in accordance with the results of our study. In addition, it was confirmed that during lesion development, some members of the S-100 protein family, S100A8, S100A9 and S100A12 synthesized in plaque, bind to scavenger receptors and thus promote the proinflammatory phenotype of endothelial, smooth muscle cells and leukocytes, contributing to lesion development followed by calcification and plaque rupture (Gould *et al*., 1986; Libby, 1996; Donato *et al*., 2013).

Other authors have also pointed to the functional association of S100 protein and scavenger receptors such as CD36 (Gould *et al*., 1986; Libby, 1996; Donato *et al*.), which indicates the role of this protein in the internalization of lipids during the formation of foam cells of both macrophage and smooth muscle origin and is in compliance with our results, showing the presence of S100 immunoreactive foam cells at plaque margins.

The results of some recent studies have contributed to the partial elucidation of intracellular and extracellular mechanisms and receptors through which the S100 family of proteins performs a regulatory function in the aforementioned processes, although they confirm that the exact mechanisms regulating the release of synthesized S100 protein remain unclear (Bertheloot & Latz, 2017). What is known is that the intracellular expression of S100 protein is regulated by specific growth factors, cytokines and the activation of pattern-recognition receptors on the cell surface, as well as that the cellular potential for expression is derived from neural crest origin (Gould *et al*., 1986; Libby, 1996; Donato *et al*.).

In the initial stages of atherosclerotic lesions, cell activation and consequent cell damage that are the characteristic of the initial stages of atherosclerosis are accompanied by secretion of S100 protein in the subendothelium, and the mechanism of this process, as previously mentioned, is not fully elucidated (Vukovic *et al*., 2004). It is thought that signaling pathways triggered by S100 protein in tissue cause the proinflammatory phenotype

of different cell populations in the subendothelium, but also the expression of matrix metalloproteinases (MMPs) and cell adhesion molecules (CAMs) (Gould *et al*., 1986; Libby, 1996; Donato *et al*.).

When extracellular S100 proteins are secreted in the subendothelium, they bind to their receptors such as RAGE (receptor for advanced glycation endproducts), scavenger receptors (such as CD36), or toll-like receptor 4 (TLR-4) on the cell's surface. After binding to receptors, S100 proteins perform their intracellular functions by binding to target proteins (target molecules) in both the nucleus and cytoplasm (Gould *et al*., 1986; Libby, 1996; Donato *et al*.). We have already mentioned that the target molecule of S100100 / S100A9 protein complex is the intermediate filament vimentin, which would suggest that its expression, occurred due to the loss of contractile characteristics of smooth muscle cells derived from mesenchyme, presents a predisposing factor for protein binding and further promoting cell migration, proliferation and differentiation. This complex also demonstrates marked susceptibility to oxidation, probably having a bifunctional, proinflammatory or anti-inflammatory role, depending on its own redox status - (Gould *et al*., 1986; Libby, 1996; Donato *et al*.; Vukovic *et al*., 2010).

In addition, they are considered mediators of seizures in atherosclerosis, so they can be considered the goal of therapeutic procedures. It can be used as a predictive factor of atherosclerosis (Libby, 1996).

CONCLUSION

The S-100 protein family plays a significant role in a number of extracellular and intracellular processes in the subendothelium of the intima during the pathogenesis of atherosclerosis, which is the reason why they have been the subject of numerous studies in recent years aimed at improving therapeutic procedures. The results of our study also showed that a number of smooth muscle cells and foam cells on the margins of hypocellular plaques of advanced stages of coronary artery atherosclerosis demonstrate S100 immunoreactivity, which opens the field of further research to determine the extent to which histological specifics of coronary arteries contribute to their predisposition for the development of atherosclerosis, that is the extent to which the phenotypic status of smooth muscle cells, their characteristics conditioned by embryonic origin, as well as the possible presence of cells of neuroectodermal origin in the coronary artery wall present predisposing factors for lipid accumulation and atherosclerotic plaque development.

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RESUMEN: La aterosclerosis es una enfermedad compleja cuya patogenia incluye activación endotelial, acumulación de lípidos en el subendotelio, formación de células espumosas, bandas grasas y formación de placa aterosclerótica. Estos complejos mecanismos involucran diferentes poblaciones celulares en el subendotelio íntimo, y la familia de proteínas S-100 juega un papel en varios procesos extracelulares e intracelulares durante el desarrollo de lesiones ateroscleróticas. El objetivo de este estudio fue determinar las características fenotípicas de las células de músculo liso y la consecuente expresión de la proteína S100 en arterias coronarias alteradas ateroscleróticas en estadios avanzados de aterosclerosis. Se analizaron 19 muestras de arterias coronarias ateroscleróticas derechas en estadios de fibroateroma (lesión tipo V) y lesiones complicadas (lesión tipo VI). Según el protocolo estándar, en el análisis inmunohistoquímico se utilizaron los siguientes anticuerpos primarios: α-actina de músculo liso (α-SMA), vimentina y proteína S-100. Todas las muestras analizadas han estado en estadios avanzados de aterosclerosis, fibroateroma (lesiones estadio V) y lesiones complicadas (lesiones tipo VI). La mayoría de ellos han tenido la estructura de una lesión complicada con ateroma o fibroateroma como base, complicada posteriormente por disrupción (subtipo VI a), hemorragia (subtipo VI b) o trombosis (subtipo VI c), así como por la presencia de varias complicaciones en la misma muestra. La hipocelularidad marcada estaba presente en el subendotelio de las placas. La población celular en los márgenes de la placa se caracterizaba por inmunorreactividad a α-SMA, vimentina y proteína S100. Algunas de estas células acumulan lípidos y parecen células espumosas. En la población celular en los márgenes de las placas, estaban presentes las células de músculo liso de fenotipo sintético, algunas de las cuales acumulaban lípidos y mostraban inmunorreactividad S100. Resumiendo numerosos datos de la literatura y nuestros resultados, podríamos suponer que las células del músculo liso, debido a su actividad sintética y proliferativa en las primeras etapas de la patogénesis, así como la consecuente expresión de la proteína S100, podrían acumular lípidos en las primeras etapas de la aterosclerosis que, en estadios avanzados analizados en este estudio, dan como resultado inmunorreactividad de células espumosas de origen muscular liso a la proteína S100.

PALABRAS CLAVE: Proteína S100; Células del músculo liso; Aterosclerosis de las arterias coronarias.

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