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IMMUNOHISTOCHEMICAL EXPRESSION OF INOS AND MATRIX METALLOPROTEINASES MMP-2 AND MMP-9 IN AORTIC ANEURYSMS

[Espressione immunoistochimica della iNOS e delle metalloproteinasi MMP-2 e MMP-9 negli aneurismi aortici]

Giovanni Francesco Spatola

DI.ME.S., Sezione di Istologia ed Embriologia, Facoltà di Medicina, Università degli Studi di Palermo (I)

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Abstract. Aortic aneurysms (AA) is a degenerative vascular disease characterized by localized dilatation of the aortic wall as a result of altered matrix composition (elastin and collagen degradation). Howewer the pathogenesis of the changes is elusive and unclear. Some experimental evidences suggest that iNOS (who synthesize a large amount of NO in inflammatory processes) and the metalloproteinases (MMP) are implicated in the pathogenesis of AA but the relationship between NO and MMP to aneurismal disease is currently unknown. The aim of this study is to investigate the immunohistochemical expression of iNOS and MMP-2 and MMP-9 in human aneurysmal tissues.

Riassunto. Gli aneurismi aortici (AA) sono una patologia vascolare degenerativa caratterizzata da una locale dilatazione della parete aortica derivante da una alterazione della matrice extracellulare (in particolare deriva da una degradazione delle fibre collagene ed elastiche). Tuttavia, a tutt'oggi non si sono del tutto chiariti i meccanismi patogenetici. Diverse evidenze sperimentali suggeriscono che l'iNOS (che sintetizza grandi quantità di NO durnate i processi infiammatori) e le metalloproteinasi (MMP) sono implicate nella patogenesi degli AA anche se la relazione tra NO e MMP nella patologia aneurismatica è attualmente poco chiara. Lo scopo di questo studio è quello di investigare l'espressione immunoistochimica della iNOS e delle MMP-2 e MMP-9 in tessuti aneurismatici umani.

Introduction

The cellular components of blood vessels wall are supported and organized by a complex structure of collagens, elastins, laminins, fibronectins, and proteoglycans known as the extracellular matrix (ECM) [1]. Researchers in nearly every medical discipline are examining the ECM in their quest to arrest disease, with many of the most promising advances taking place in cardiovascular research. Much of this cardiovascular research is focused on the family of ECM-remodeling enzymes collectively termed the matrix metalloproteinases (MMPs). Twenty-three MMPs have been described in humans, although they share a high degree of homology in their structure. Most MMPs are dismissed freely into the extracellular space immediately after synthesis as proenzymes, but some are stored

within cells (eg, MMP-9 in neutrophil granules), and others are bound to cell surface membranes (eg, MT1-MMP) [2,3].

Rigorous regulation of MMP production and activity is a crucial part of ECM homeostasis. MMPs are formed as inactive proenzymes and are activated by proteolysis in the extracellular fluid, a process that is tightly regulated by other proteases and by endogenous MMP inhibitors. Plasma proteins and tissue inhibitors of metalloproteinases (TIMPs) are the primary endogenous inhibitors of MMPs, although they also serve other physiologic functions [2,3].

For example, TIMP-2 inhibits MMP-2, but is also required for MT1-MMP–mediated activation of proMMP-2 [4].

The vascular microenvironment provides several specific modes of MMP regulation. For example, the cyclic strain on endothelial cells created by arterial pulsation has been shown to increase expression and activity of MMP-2 and its activator, MT1-MMP [5,6,7].

Other recent studies clarified a leading role of MMP2 in the angiogenesis and in the hypoxia. The endothelial cells overexpression MMP2 not induced by MT1-MMP reduced during hypoxia on the membrane cell, caused a migration of endothelial cells in accordance with the proangiogenetic role ascribed to MMP2. The involvement of this protease in the hypoxia-related death of endothelial cells support an additional apoptotic role of this protease [8,9,10,11,12]. Furthermore, emerging evidence suggests that nitric oxide inhibits gene expression of MMP-2 from endothelial cells, and increase a production of MMP-9 from endothelial cells and vascular smooth muscle cells. MMPs contribute to many normal and necessary physiologic processes through their modification of the ECM. Inflammation accompanied by increased MMP activity also contributes to many disease processes several are sequestered in inflammatory cells. In fact, increased inflammation and loss of MMP regulation is the hallmark of many pathologic states, including many of the disease processes treated by vascular surgeons [13,14].

The permanent and irreversible expansion of a tract of artery is defined aneurysm. Related of the seat distinguish, thoracic (TAA), abdominal thoracic (TAAA) and abdominal (AAA) aneurysms.

The TAA can be localized to level of the ascending aorta, with eventual interest of the aortic valve, arc or the descending aorta. The TAAA and the AAA imply the involvement, with variable extension, of the descendant thoracic aorta and the abdominal aorta [15,16]. The pathogenesis of several types of aneurysms often differ based of their localization: in particular the TAA are in kind to degenerate, dilatative character or dissecting [17,18] while the AAA recognize a multifactorial genesis and an inflammatory origin (infective processes, aortities type Takayasu etc.) [19,20].

According the anatomo-pathologic point of view, the aneurysms have a degenerative

vascular basis that are caused by altering of the cellular components of ECM with degradation of elastic and collagen fibers.

Recent experimental studies have clarified that MMP are responsible of these alteration particularly the MMP-2 and the MMP-9. The MMP-2 is physiologic formed by tiny quantity in the muscle cells and in the fibroblast [21,22] and only a little quantity is formed by macrophages. Furthermore, the MMP-2 as we have seen before, it is perceptible in the hipoxia and it makes up one case of the apoptotic endothelial processes because it is produced also by these cells in particulary pathologic situation [8,9,10,11,12]. This production, localized, in the first time, in the aneurysm wall it will be exalted because it is the "primum movens" of degradative processes of the collagens fibres and causes the display of elastic fibres.

The MMP-9 are formed by a huge quantity by macrophages [21,22] and only in one little side by the fibroblasts, act, on the contrary, degrading mostly elastic fibres. In addition, the inflammatory processes, that are involved in the first step of the pathological aneurysm process, provoke in the vessel wall forms an infiltrate consisting of, in the main way, macrophages that are producing a huge quantity of MMP-9.

MMP-9, working at the same time with MMP-2, reduces in a full way the extracellular matrix by causing the dilatation by deterioration of the vessel [22].

The aim of our study is to investigate and compare the immunohistochemical expression of MMP-2 and MMP-9 and iNOS in human probable inflammatory abdominal aneurysmal tissues and in the dilatative and dissecting thoracic aneurysm.

Materials and Methods

Fragments of human 10 AAA, 10 dilatative TAA and 10 dissecting TAA were obtained during surgical procedure. However, during surgical aorto-coronaric bypass were obtained 10 punches of normal aorta in the side of joint of bypass, to use like normal control (the sample came from GENURTO O.U. of Cardiac surgery and O.U. of Vascular surgery, University of Palermo). All the specimens were fixed in Bouin's mixture and embedded in paraffin; obtained sections were processed with anti MMP-9 (Chemicon International), monoclonal anti MMP-2 (Chemicon International), anti iNOS (Transduction laboratories) by EnVision+System HRP (AEC) (Dako Cytomation). In the same time, the negative controls have been realized on sections that are near by and processed on the base of the same protocol but without the passage of the primary antibody. Other sections near by those processed have been submitted at the histological stain using the method of Mallory Azan, and this to look forward to seeing the fibrous component and the miocytes**.** All the samples have been studied with microscope Leica DM1000 and Nikon OPTIPHOT 2.

Results

Morphological results

The microscopic observation of the normal and pathologic fragments that are coloured with the Mallory-Azan's method, who has pointed out the total subversion of the structure of arterial media and intima layers in aneurysmatic fragments compared to the controls. Furthermore, in the specimens of AAA aneurysm, was emphasized a plentyfull inflammation infiltrate in the media and adventitial tonaca. This infiltrate shows to be less evident in the dilatative aneurysm TAA and totally absent in those dissecant. However, the structure of the layers shows to be adulteraded in the aneurysm inflammatory**,** in those dilatative and in some points it becomes really difficult to distinguish it (Fig. 1,2,3,4).

However, in the dissecant aneurysm, we emphasize the presence of the lesion at the level of the muscular layer that seem to be fractured in different points (Fig.5).

Immunohistochemical results

iNOS: In the fragments of the normal aorta hasn't been emphasized any activity of iNOS (fig. 6). In the fragments of the AAA has been emphasized a widespread immunoreactivity more intense at the level of the inflammatory cells that are in the context of the vascular wall (fig. 7). There is a weak reactivity in the dilatative TAA aneurysm endothelial cells (Fig.8) and not have reactivity in the specimens of the aortic dissection (Fig.9).

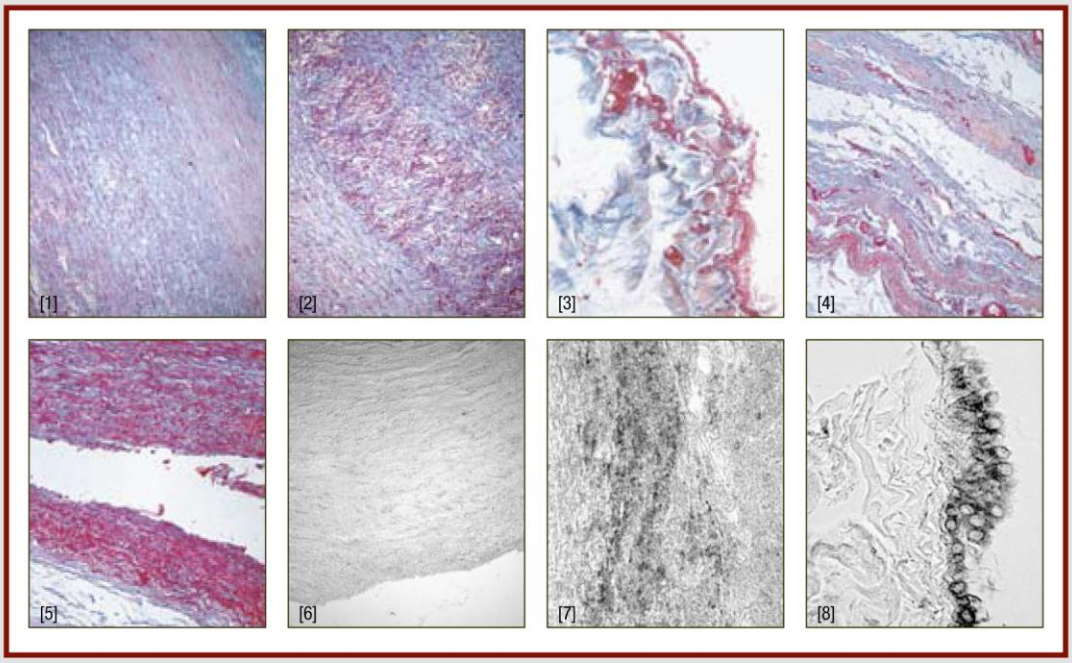
MMP-2: There is a discrete reactivity of MMP-2 in the normal aortic wall localized in the tonaca media, where there are positive some miocytes, as in the tonaca adventitia, where some fibroblasts are immunopositive (Fig.10). In the aneurysm samples of inflammatory nature (AAA), we emphasize an intense MMP-2 immunoreactivity, that is located both in musculary cells and in fibroblasts. (Fig. 11). In the TAA of dilatative nature, we emphasize a strong granular reactivity in the cytoplasm of the endothelial cells (Fig.12,13). However, this reactivity shows to be absent in the dissecant TAA, while we emphasize a reactivity in the capillars and in a few fibroblasts (Fig.14).

MMP-9: The fragments of the normal aorta show a weak immunoreactivity located in the tonaca media (Fig. 15). In the AAA there is an intense reactivity widespread in all components of the wall, more evident in the areas where there is much more presence of the inflammatory infiltrate (Fig. 16). In the dilatative TAA there is the presence of an intense immunoreactivity at the level of the endothelial cells (Fig.17) and moreover there is a modest immunoreactivity in some cell members of probable fibroblastic nature. This distribution is the same also in the dissecant aneurysm.

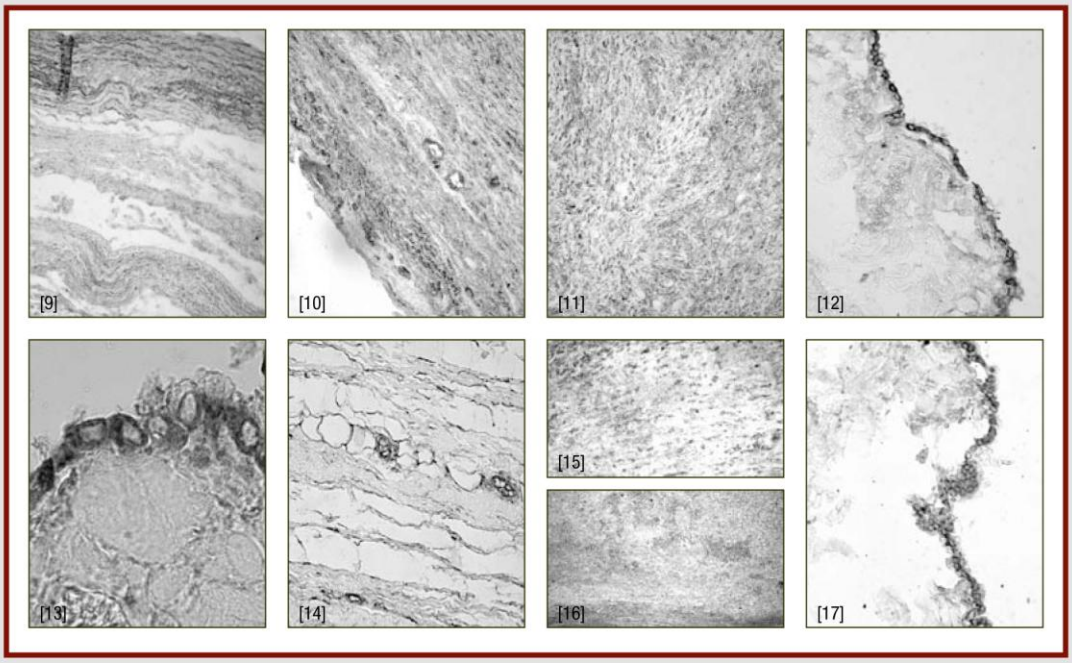
Conclusions

Our results document that the iNOS is present only in the aneurysm wall of AAA, in fact according the recent literature, they are considered as a inflammatory genesis, while it proves to be absent or present in small amount, in the TAA dilatative or dissecant and totaly absent in the normal vessels used as control. The reactivity of MMP are significantly increased in the pathologic specimens compared to those of the normal aorta. MMP-2, in AAA is more present at the level of the muscle cells and in the fibroblast, while in TAA of dilatative nature, that are present at in the endothelial cells according with the recent letterature that invest those cytotipes of angiogenetic role (the intima to be absolutely disarranged with an obvious and followed tissutal hypoxia), and, in second time, of apoptotic functions.

MMP-9 in the AAA is produced in a big quantity in the inflammatory cells, while in the TAA it presents a reactivity like to MMP-2. This research gives the immunohistochemical basis to support the role of the NO and the metalloproteinases in the AAA's pathogenesis and suggests the hypothesis that the iNOS is produced in huge quantity below the inflammatory cytochines induction and it stimulates the activity of the MMP during the formation of the aneurysm. Moreover it confirm other biochemical data that give to the MMP-2 a fundamental role in the angiogenetic and apoptotic processes in the TAA.



Figures 1-8: [1] **Normal Aorta Mallory-Azan 10x** aortic media and adventitia with the normal parallel distribution of myocites and the presence of collagen and elastic fibers, fibroblast and vasa vasorum - [2] **AAA Mallory-Azan 10x** inflammatory infiltrate in the disarranged media and adventitia - [3] **Dilatative TAA Mallory-Azan 20X** endothelial cells and the totally disrupted intima layer - [4] **Dilatative TAA Mallory-Azan 20X** alteration of aortic wall - [5] **Dissecant TAA Mallory Azan 20X** the muscular layer of aorta that seem to be fractured - [6] **Aorta iNOS 10x** iNOS reactivity is totally absent - [7] **AAA iNOS 40x** immunoreactivity iNOS more intense in the inflammatory cells - [8] **Dilatative TAA iNOS 63X** iNOS immunopositive endothelial cells.



Figures 9-17: [9] **Dissecant TAA iNOS 20X** poor and aspecific immunoreactivity - [10] **Aorta MMP-2 40x** presence of MMP-2 reactivity in the myocites and in adventitia layer - [11] **AAA MMP-2 20x** strong immunoreactivity in the muscular layer - [12] **Dilatative TAA MMP-2 20x** positive endothelial cells - [13] **Dilatative TAA MMP-2 100x oil** positive endothelial cells - [14] **Dissecant TAA MMP-2 40x** reactivity in the capillars and in a few fibroblasts - [15] **Aorta MMP-9 20x** immunoreactivity located in the tonaca media - [16] **AAA MMP-9 20x** strongly immunoreactivity in the media and the adventitia (macrophages positive) - [17] **Dilatative TAA MMP-9 20x** positive endothelial cells.

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