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Transforming growth factor-β1 SMAD effectors and medial cell number in ascending aorta diseases

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ABSTRACT

In ascending aorta aneurysms and dissections, the extracellular matrix is degraded. Transforming growth factor (TGF)-β1 modulates its synthesis. The production and presence of SMADs, intracellular effectors of TGF-β1 signaling, were analyzed in patients with these diseases. To verify whether medial cells are lost, their total numbers were computed. Ascending aorta samples from 19 patients and 18 controls underwent immunoperoxidase reactions to SMADs 2, 3, 4, and 7. Positive and negative cells were counted, and total numbers of cells and positive/total ratios were calculated. Samples from other 14 patients and 7 normal controls were used for the quantification of SMAD3, SMAD4, and SMAD7 mRNA. For SMAD4, both mRNA (2.36 vs. 0.37, P = .03) and ratio of positive cells (0.94 vs. 0.73, P = .02) are increased in patients with ascending aortic diseases. SMAD3 mRNA was also increased (1.19 vs. 0.20, P = .05). The cell ratios of this and the other SMADs, SMAD7 mRNA, and the total cell count did not differ between groups. In conclusion, in ascending aortic aneurysms and dissections, there is an increase in SMAD4, implicated in extracellular matrix production, possibly as an attempt to compensate for extracellular matrix deficiency. Lost medial cells are replaced, since their number is not diminished.

1. Introduction

Fragility of the arterial wall is thought to be involved in the pathogenesis of both aortic dissections, characterized by a separation of the arterial wall into two sheets along its longitudinal axis [1], and aneurysms, dilatations of the vessel including the three wall layers [2], but the factors underlying such fragility remain unclear. In spite of the differences between them, ascending aortic aneurysms and dissections (AscAADs) might be investigated altogether because they share not only a frequent relationship with underlying conditions [such as systemic arterial hypertension (SAH), Marfan syndrome, bicuspid aortic valve, and others], but also their histopathological features, situated at the medial layer of the artery: delicate fragmentation of elastic fibers, in places where mucoid material (that corresponds to proteoglycans) accumulates, and an apparent diminution in the cell population [3]. There is also a decrease in collagen content [4], in the whole extension of the media in aneurysms and at the outer half of this layer — where the tearing process always occur — in dissections. Inflammation is usually inconspicuous or appears after the tearing in dissections.

Transforming growth factor (TGF)-β is a major regulator of the production of extracellular matrix (ECM) components, mostly collagen. Interestingly, some molecules that are part of ECM have also been reported to modulate TGF-β1 action [5,6]. In some of the conditions associated with AscAAD, there is a recognized defect at any location on the TGF-β axis: fibrillin in Marfan syndrome [7] and TGF-β receptor I or II in Loeys–Dietz syndrome [8]. TGF-β may also mediate apoptosis in dilatative diseases of the abdominal aorta [9,10].

SMADs are a family of proteins that are pivotal elements among the intracellular effectors of TGF-β1 [11]. TGF-β1 ligand binds to TGF-βRII/TGF-βRI receptors leading to phosphorylation of SMAD2/3. Phosphorylated SMAD2/3 binds to SMAD4 to form a protein complex that undergoes nuclear translocation and regulates the expression of the components of ECM. On the other hand, SMAD7 acts as negative feedback. Alterations in all these molecules in AscAAD have been detected by nonmorphological methods [12], and we have shown an increase in phosphorylated SMAD2 in aneurysms [13], but a more complete approach including the comparison between aortic media halves is still lacking.

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In this study, we analyzed the production and expression of SMADs 2, 3, 4, and 7 in patients with AscAAD, focusing on those that correspond to the majority of the cases and without a plausible explanation for the underlying wall fragility — namely, those without Marfan or other recognized syndrome. To evaluate if an increase in SMADs occurs or only an increase in their phosphorylation, we quantified the presence of cells with nonphosphorylated SMADs 2 and 3 in the cytoplasm (since phosphorylated, active SMAD2 has already been analyzed [13]). We counted the number of cells in which SMAD4 is present at the nuclei, to verify any potential action in ECM expression, and in which SMAD7 was positive at the cytoplasm. Considering the possible role of TGF-β in apoptosis [9,10], we used the material to confirm if the number of medial cells is decreased in AscAAD.

2. Patients and methods

This study was approved by the ethics committees of the Heart Institute, University of São Paulo School of Medicine, São Paulo, Brazil, and of the Centre Hospitalier Ambroise Paré, Boulogne, France.

2.1. Histopathological evaluation

For an illustrative overview of the main histopathological changes of the ECM in the aortic diseases, selected cases from our archives were stained with Picrosirius solution and observed under fluorescence microscopy [14] for the visualization of collagen and the elastic system fibers.

2.2. Sample collection

To focus on the majority of AscAAD patients, who are middle-aged or old hypertensive patients without any recognized syndrome, Marfan syndrome patients were excluded from the main study groups. Considering that in these diseases, inflammation is usually not prominent, which is important to understand their pathogenesis, eventual specimens with aortitis were also excluded.

We used two methods: immunohistochemistry followed by morphometry and real-time reverse transcription polymerase chain reaction (RT-PCR). The patients who entered each of such studies were not the same: immunohistochemistry was performed in patients from the Heart Institute, in Brazil, and RT-PCR in patients from the Centre Hospitalier Ambroise Paré, in France. Taking into account that there are not only similarities but also differences between aneurysms and dissections, for both methods we made two separate statistical analyses: (1) considering together cases of aneurysms and cases of dissections on one side and on the other side controls as a whole, and (2) a further analysis considering the same patient samples, but separating cases of aneurysms, dissections, hypertensive controls, and normotensive controls (to verify if hypertension itself caused any change in SMADs or number of cells; however, for the RT-PCR tests, we had no information about pressure status; thus, controls — possibly normotensive — remained as only one group). Only for these secondary statistical analyses, for both immunohistochemistry and RT-PCR, we included, for comparison, an additional group constituted by patients with Marfan syndrome and either aneurysm or dissection. Thus, in this secondary analysis, there were four groups for RT-PCR (aneurysms, dissections, controls, and Marfan syndrome) from a French cohort and five groups for immunohistochemistry plus morphometry (since control cases were split into hypertensive and normotensive) from a Brazilian cohort. The results of cell counting in this last method were used to verify the total number of medial cells, again through a main analysis considering AscAAD as a whole vs. controls and in a secondary analysis of five groups.

2.2.1. Sample collection for immunohistochemistry (Brazilian cases)

Samples of the ascending aorta were taken during surgery at the Heart Institute in 19 patients, 9 with aneurysms and 10 with acute dissections (12 male, with ages ranging from 45 to 79 years, mean 58.5 years, median 57 years). All but one patient (94.7%) had SAH, and 2 had bicuspid aortic valve. Only patients operated on within 4 days after the beginning of symptoms (8 with 1 day or less, mean 1.1 day, median 1 day) were included in this study. The additional group of patients with Marfan syndrome and AscAAD (for the secondary analysis) was constituted by 10 patients (6 male, with ages ranging from 19 to 51 years, mean 32.7 years, median 33.5 years). For the controls, fragments taken during coronary artery bypass surgery at the same hospital were used as controls. Aortas with mild atherosclerosis were accepted, but specimens exhibiting any other lesions were not. Due to the importance of SAH in AscAAD, the control group included 10 patients with and 8 without hypertension (12 male, with ages ranging from 35 to 82 years, mean 59.9 years, median 58 years).

2.2.2. Sample collection for PCR studies (French cases)

Samples from patients submitted to surgery at the Centre Hospitalier Ambroise Paré, 8 with non-Marfan ascending aorta aneurysms (7 male, with ages ranging from 42 to 79 years, mean 61.0 years, median 62 years; 5 of them with bicuspid aortic valve), 6 with aortic dissections (5 male, with ages ranging from 46 to 75 years, mean 59.5 years, median 58 years), also without this syndrome. The additional group with Marfan syndrome patients (for the secondary statistical analysis) had 5 male patients with ages ranging from 22 to 48 years. For the controls, 7 normal controls from heart donors (5 male, with ages ranging from 22 to 68 years, mean 51.0 years, median 51 years; we have no information concerning SAH; mean 33.6, median 29).

2.3. Immunohistochemistry

After 24–72 h of fixation in normal buffered formalin, the samples underwent standard histological processing and were embedded in paraffin. Immunoperoxidase reactions were performed in 4-μm-thick sections according to laboratory standards. Primary antibodies for SMAD-2 (Ab33875, monoclonal, 1:200 dilution, citrate buffer pH 7.2, 1-min digestion in a Pascal pressure cooker (Dako, Denmark)], SMAD-3 (Ab28379, polyclonal, 1:50 dilution, treatment with TRIS/EDTA pH 9.0), and SMAD-4 (Ab40759, monoclonal, 1:50 dilution, citrate buffer pH 7.2, 1-min digestion in a Pascal pressure cooker) were produced in rabbits by Abcam (Cambridge, UK); for SMAD-7, we used a mouse monoclonal antibody (HO 00004092-M09) produced by Abnova (Jhongli, Taiwan; 1:100 dilution, treatment with Tris/EDTA, pH 9.0).

The slides were analyzed with a 20× objective, and the numbers of cells with nuclear positivity for SMAD4 and with cytoplasmic positivity for the other three SMADs were counted in a microscope coupled to an image analysis system (Axiovision; Zeiss, Germany) in 10 consecutive adequate fields (avoiding those with an eventual cutting or staining artifact), near the intima, starting from the area close to the slide tag, and in 10 similar fields near the adventitia. The evaluations were made in each half to verify if there were differences between the internal half and the external half of the artery (where the dissections occur). The percentages of positivity of each half in each case were computed.

Table 1
List of q-PCR primers used for mRNA quantification in tissue

SMAD3	Forward	5′-GCAGAGTGCCTCAGTGACAG-3′
	Reverse	5′-CACATTCGGGTCAACTGGTA-3′
SMAD4	Forward	5′-GCCCCATCTGAGTCTAATGC-3′
	Reverse	5′-ATCCATTCTGCTGCTGTCCT-3′
SMAD7	Forward	5′-TACCGTGCAGATCAGCTTTG-3′
	Reverse	5′-AGTTTGAAGTGTGGCCTGCT-3′
β-Actin	Forward	5′-TCCCTGGAGAAGAGCTACGA-3′
	Reverse	5′-TTTCGTGGATGCCACAGGAC-3′