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ADAMTS-4 and ADAMTS-5 Expression in Human Temporomandibular Joint Discs with Internal Derangement, Correlates with Degeneration

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Abstract

Temporomandibular joint (TMJ) internal derangement (ID) is one of the most common form of temporomandibular disorders. There is evidence showing the increased expression of matrix metalloproteinases (MMPs) in the cells from degenerated TMJ disc. ADAMTS are a large family of metalloproteases which are responsible for proteoglycans degradation. The present study aimed to evaluate ADAMTS-4 and ADAMTS-5 immunohistochemical expression in human TMJ discs from patients affected by ID, and to find out if there is any correlation with the degree of histopathological changes. Eighteen temporomandibular displaced disc specimens and sixteen TMJ disc control were used for the present study. Specimens were immunohistochemically processed and ADAMTS-4 and ADAMTS-5 expression were obtained respectively for the anterior (AB), intermediate (IB) and posterior (PB) bands and compared to the histopathological degeneration score (HDS). Immunoreactivity for ADAMTS-4 and -5, was observed in both not degenerated and degenerated human TMJ discs. Both the percentage of ADAMTS-4 and -5 immunostained cells (ES) and the intensity of staining (IS) were significantly greater in affected specimens compared with those in control discs. The ADAMTS-5 ES and IS of the 3 bands of the disc correlated to the TMJ disc HDS ($0.001 < P < 0.05$), on the other hand only AB and IB, ADAMTS-4 immunostaining scores correlated to HDS. According to these findings it can be assumed in that the more histopathological changes in the disc are detected, the higher levels of ADAMTS are produced. This in turn can lead to ECM breakdown and in turn to a more advanced disc displacement.

Introduction

Temporomandibular joint (TMJ) internal derangement (ID) is one of the most common form of temporomandibular disorders (TMD), and it is characterized by an abnormal positional relationship of the articular disc to the mandibular condyle and the articular eminence [1]. Depending on the stage of derangement, the disc either regains its normal position on top of the condyle when the mouth is opened, or remains anterior to it, thus interfering with condylar translation [2].

These mechanical disturbances produce an imbalance between anabolic and catabolic processes, which progressive alter TMJ disc tissue [3-10]. Damage of the TMJ disc is associated with catabolic activity that results from cytokines and proteases [11]. This cascade alters the extracellular matrix (ECM), by depleting matrix substances, such as collagen [3, 7].

There is evidence showing the increased expression of matrix metalloproteinases (MMPs) in the cells from degenerated TMJ disc [5, 9], which is accompanied with an increase in collagen degradation products.

However, the degradation of proteoglycans, which are another main ECM component of the TMJ disc, has not been extensively investigated. Proteoglycans play an important role in determining the viscoelastic properties of the disc and, therefore, give the disc a greater capacity for distributing and reducing stresses [12].

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) are a large family of metalloproteases different from MMPs, which have received increasing attention in recent years [13-15]. They have

been suggested to play a major role in the pathogenesis of intervertebral disc degeneration because they responsible for proteoglycans degradation [14, 16, 17].

Several members of the ADAMTS family of proteins have been shown to cleave aggrecan *in vitro* at the aggrecanase cleavage site. Of these, ADAMTS-4 and ADAMTS-5 are the most efficient aggrecanases [16, 17]. Loss of aggrecan is considered to be a critical early event in the destruction of articular cartilage, followed by degradation of collagen fibrils and irreversible mechanical failure of tissue [18].

As far TMJ is concerned, just one study has investigated the expression of ADAMTS-5 in discs from patients affected by ID and osteoarthritis (OA); findings from this investigation demonstrated, all in all, an over-expression of ADAMTS-5 in diseased discs [19]. On the basis of these preliminary results, we aimed in our investigation to evaluate ADAMTS-4 and ADAMTS-5 immunohistochemical expression in human TMJ discs from patients affected by ID, and to find out if there is any correlation with the degree of histopathological changes.

Materials and methods

Patient and tissues

Our study sample comprised eighteen TMJ-displaced disc specimens, obtained from patients treated between 2002 and 2009 at the University of Pontifical Catholic University of Paraná, Brazil. Healthy control samples were obtained from sixteen virtually unaffected human TMJ discs. After project approval by the ethic committee of the same University, each patient signed an informed consent form before tissue collection.

The study group (10 discs from the right TMJ and 8 discs from the left) were acquired from 14 female and four male patients, affected by ADDwoR. Their clinical history, examination and magnetic resonance imaging studies confirmed the diagnosis. The mean age of all patients was 41.2 ± 6.5 years. Surgery therapy was proposed to treat the disc derangement with associated pain and impairment of function. These patients had been unsuccessfully non-surgical treated before surgery and had TMJ pain or interference with mandibular movements. The duration of ID before surgery was and 7.9 ± 0.9 (range 7–9 months). Exclusion criteria were TMJ discs from patients with other TMJ diseases, major jaw trauma, previous TMJ surgery, previous steroid injections in the TMJ and dentofacial deformity.

None of the surgically removed patients' discs presented a normal biconcave shape and they were macroscopically altered.

The control group made of sixteen autopsy TMJ disc specimens was obtained bilaterally from eight cadavers (five males and three females). The mean period (and standard deviation) between the death of the donor and tissue harvest was 11 ± 2 h. The cadaver donors' mean age was 41 ± 7 years, at the time of death. The causes of death of the donors were cardiovascular disease and cancer.

None of the donors' clinical history revealed generalized joint disease or TMJ arthropathy. Macroscopic examination of the discs showed no signs of degenerative or inflammatory joint disease nor disc displacement and no pathological changes at microscopic examination.

Both study and control specimens included the anterior band (AB), intermediate band (IB) and the posterior band (PB) of the disc.

Immunohistochemistry

The discs were fixed overnight in 10% neutral-buffered formalin (Bio-Optica, Milan, Italy). After fixation and overnight washing, each disc was sectioned through its centre along a parasagittal plane, perpendicular to its long axis. Each tissue block was dehydrated in graded ethanol and embedded in paraffin, preserving the

longitudinal anatomical orientation. Specimens were sectioned at a thickness of 5 μm using a microtome, placed on silanized glass slides (the anterior band being always on the right side of the slides), and warmed at 60°C for a minimum of 2 h to ensure proper tissue adhesion to the slides, prior to immunohistochemical staining. The TMJ disc sections were deparaffinized in xylene and rehydrated in reagent alcohol and then were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity and then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica). The sections were incubated (5 min × 3) in capped polypropylene slide holders with citrate buffer (pH6), using a microwave oven (750 W) to unmask antigenic sites.

Primary antibodies diluted in 1% bovine serum albumin were as follows: goat polyclonal antibody against ADAMTS-4 (1:25 dilution) (catalog no. SC-16533; Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit polyclonal antibody against ADAMTS-5 (1:100 dilution) (catalog no. Ab13976; Abcam, Cambridge, UK). The secondary antibody, biotinylated anti-mouse⁄anti-rabbit IgG was applied (for 30 min, at RT), followed by the avidin–biotin–peroxidase complex (Vector Elite Kit Abbott, Chicago, IL, USA) for 30 min, at room temperature. The immunoreaction was visualized by incubating the sections for 4 min in a 0.1% 3,3′-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit; Vector Laboratories, Burlingame, CA, USA). The sections were lightly counterstained with Mayer's haematoxylin (Histolab Products AB, Goteborg, Sweden) and finally mounted in GVA mount (Zymed Laboratories Inc., San Francisco, CA, USA), observed with Axioplan Zeiss light microscope (Germany) and photographed with a digital camera (Canon, Tokyo, Japan).

Evaluation of immunohistochemistry

ADAMTS-4 and ADAMTS-5 staining status were identified as either negative or positive. Positive staining was defined as the presence of a brown chromogen detection, distributed within the cytoplasm or in the immediate lacunar/pericellular space. The percentage of ADAMTS-4 and ADAMTS-5 immunopositive cells (extent score = ES) was independently evaluated by three expert investigators (two anatomical morphologist and one histologist) and scored as a percentage of the final number of 100 cells in four categories: 0 = <5%; 1 = 6–30%; 2 = 31–50%; 3 = >51–75% and 4 = >76–100%. Counting was performed at 200 magnification. Also, the intensity of staining (IS) was evaluated and it was graded on a scale of 0–4, according to the following assessment: 0 = no detectable staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining, 4 = very strong staining.

Positive and negative controls

Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at a protein level. Sections from oesophagus and from lung were, respectively, used for positive control testing. For negative control testing, sections of TMJ displaced discs were randomly drawn from degenerated disc samples. These latter were then treated with normal rabbit serum instead of specific antibodies.

Histopathological degeneration grading score

One of three sections for each disc was stained with haematoxylin & eosin (H&E), to assign a histopathological degeneration score (HDS) [4, 20] (Table **1**). Briefly, this score takes into account pathological disc tissue transformation, that is collagen bundles, non-specific degenerative changes and the presence of blood vessels. This grading system results in a score ranging from 0 up to 8 for heavily degenerated disc tissue.

Table 1. Scheme for the histopathological assessment of the features of degenerative TMJ disc changes

	Collagen bundles
0 points	Preservation of multidirectional collagen bundles
1 point	Altered collagen bundles architecture and/or fragmentation of collagen bundles
2 points	Tears
3 points	Splitting
	Non-specific degenerative changes

0 points	No degeneration
1 point	Fatty degeneration or calcified areas or hyalinization (fibrosis) or chondroid metaplasia
2 points	Two kind of the following: fatty degeneration or calcified areas or hyalinization (fibrosis) or chondroid metaplasia
3 points	3 or more kinds of degenerations
	Degree of vascularization
0 points	Absence
1 point	Capillaries
2 points	Arterioles and venules
Sum 0 or 1	No TMJ disc degeneration
Sum 2–3	Moderate grade of degeneration
Sum 4–8	Severe grade of degeneration.

Statistical analysis

Mean values and standard deviations were evaluated for the ES and IS staining in AB, IB and PB, respectively for each sample and for each antibody. A Shapiro–Wilk test was performed to test normality. Data results had a nonparametric distribution so Mann–Whitney *U*-tests was assessed to evaluate any significant statistical difference, both for pathological and for normal discs. One-way analysis of variance (ANOVA) was carried out to evaluate in there were any statistical significant difference between control and diseased discs.

The nonparametric Spearman rank correlation test was used to obtain the correlation coefficient between the ADAMTS-4 and ADAMTS-5 staining and the histopathological score of each sample. *P*-values of <0.05 were considered statistically significant. All data were analysed with the SPSS program (SPSS release 16.0; SPSS, Chicago, IL, USA).

Results

The control discs showed no signs of cellular and tissue alteration, with the absence of clefts and/or fraying; fibroblast-like cells with few fibrochondrocytes and chondrocyte-like cells aggregates were the cell population. In control sample the histopathological score ranged from 0 to 2 points.

In contrast, diseased TMJ disc sections, showed altered collagen bundles architecture, fragmentation of collagen bundles, tears and clefts and a decrease in cellularity in the more injured discs. In this instance the histopathological score was 3.94 ± 0.7.

Immunoreactivity for ADAMTS 4 and 5 was observed in both non-degenerated and degenerated human TMJ discs. Both for ADAMTS 4 and 5, cell showed a cytoplasmic staining. ECM, was also immunostained although to a much less extent and just on diseased discs. In the non-degenerated samples (grades 0–2), low proportions of cells were immunostained by ADAMTS-4 (ES = 0.5 ± 0.5) and ADAMTS-5 (ES = 0.5 ± 0.5) antibodies (the percentage was within 10% for each antibody), respectively in the three bands of the disc. Furthermore, the intensity of staining was faint, both for ADAMT-4 (IS = 1.0 ± 0.7) and for ADAMTS-5 (IS = 1.12 ± 0.6), (Fig. **1**).

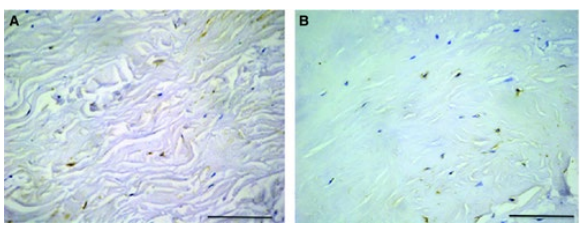

Figure 1 (A) ADAMTS-4 immunostaining in the intermediate band of not degenerated TMJ disc. Magnification 40×. Bar 100 µm. (B) ADAMTS-5 immunostaining in the anterior band of not degenerated TMJ disc. Magnification 40×. Bar 100 µm.

On the other hand, both the intensity of staining (IS) and the numbers of immunopositive cells (ES) were increased in the degenerated discs, respect to the control sample, both for ADAMTS-4 (Fig. **2**) and for ADAMTS-5 (Fig. **2**), and these differences were statistically significant ($P < 0.001$).However, not all disc specimens and band of the disc showed the same ES and IS of staining, therefore, mean values and standard deviations were calculated and referred to each band of the disc (Table **2**).

Table 2. Mean values and standard deviations for ADAMTS-4 and ADAMTS-5 extent score (ES) and intensity of staining (IS), for the study group in the three bands of the discs. Anterior band (AB), intermediate band (IB) and the posterior band (PB) of the disc

	ADAMT-4	***P*-value**	**ADAMT-5**	***P*-value**
IS AB	3.11 ± 0.67	**	3.11 ± 0.67	**
IS IZ	3.16 ± 0.61	*	3.11 ± 0.69	**
IS PB	3.33 ± 0.59	N.S.	3.27 ± 0.57	***
ES AB	3.61 ± 0.60	*	3.16 ± 0.61	*
ES IZ	3.61 ± 0.50	**	3.22 ± 0.64	**
ES PB	3.72 ± 0.52	N.S.	3.22 ± 0.68	*

P-value for Spearman's rho (rank correlation coefficient) $P > 0.05$ (N.S.); $P < 0.05$(*); $P < 0.01$(**); $P < 0.001$(***).

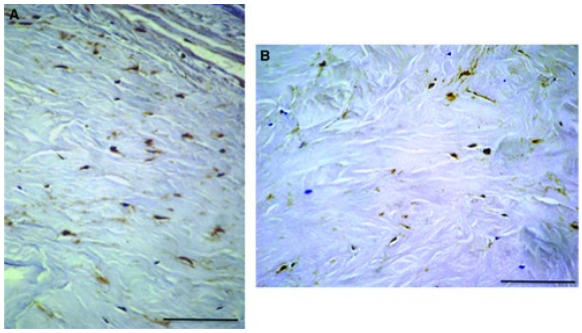

Figure 2 (A) ADAMTS-4 immunostaining in the posterior band of degenerated TMJ disc. Magnification 40×. Bar 100 µm. (B) ADAMTS-5 immunostaining in the intermediate band of degenerated TMJ disc. Magnification 40×. Bar 100 µm.

Furthermore, most of the ES and the IS both for ADAMTS-4 and ADAMTS-5 obtained, respectively, in each band of the disc were positively correlated to the TMJ HDS to the Spearman's rank correlation coefficient. However, ADAMTS-5 showed a higher correlation to histopathological score (HDS) ($0.001 < P < 0.05$) respect to ADAMTS-4 ($0.01 < P < 0.05$). Among the three bands of the disc, the IS and ES of the AB and IB were always positively correlated to HDS, in that the higher was the expression of ADAMTS-4 and ADAMTS-5 in the IB the more severe were the histopathological changes (Table **2**).

No immunoreaction was observed in the negative control treated with PBS without the primary antibodies. Interobserver agreement, measured using the Kappa coefficient, was 0.94 (almost perfect).

Discussion

The TMJ disc is subjected to intermittent loading during mastication and to sustained loading during clenching. In response to dynamic force conditions, the disc behaves in a viscoelastic fashion, which is different from the response to static conditions [7, 12]. Proteoglycans play an important role in determining the viscoelastic properties of the disc, and therefore, give the disc a greater capacity for distributing and reducing stresses [12].

In cartilage, aggrecan forms aggregates with link protein and hyaluronan. The aggregates, with their high negative charge, attract water molecules and endow cartilage with resistibility to compression and deformation. Therefore, aggregate formation is of much significance, as it ensures the retention of aggrecan within the collagen network [13]. Loss of aggrecan is considered to be a critical early event in the destruction of articular disc and cartilage, followed by degradation of collagen fibrils and irreversible mechanical failure of tissue. ADAMTS-4 and ADAMTS-5 are considered the primary enzymes responsible for the cleavage of aggrecan.

Several studies have been carried out, in joints other than TMJ, and their pivotal roles in disc and articular cartilage diseases, have been pointed out [13-16, 21]. As far as, TMJ disc is concerned except for one study which demonstrated immunohistochemically an up-regulation of ADAMTS-5 (but not ADAMTS-4) in TMJ discs [19] and another one, which detected aggrenases by Western blot, in synovial fluid from diseased TMJ [22], literature is lacking. However, in both studies the sample consisted of patients affected by ID and OA, and any difference as far as degree of TMJ disease was reported. In this study, immunohistochemical staining was employed to determine ADAMTS-4 and ADAMTS-5 expression in human TMJ diseased disc and correlated the immunostaining pattern to the degree of disc tissue degeneration.

Findings from our investigation demonstrated that ADAMTS-4 and ADAMTS-5 are constitutively expressed in normal TMJ disc, although to a very low extent. The fact that expression is seen in non-degenerated discs could indicate a possible role for the ADAMTS enzymes in the normal turnover of aggrecan and other matrix molecules in the healthy disc matrix.

On the other hand, there was an up-regulation of ADAMTS-4 and ADAMTS-5 expression in degenerated discs and there was also a positive correlation between the severity of disc degeneration and the increase of immunoreactions products in specimens.

As far as, the three bands of the disc are concerned, every band showed a close relationship between ADAMTS-5 expression and degree of disc degeneration. In fact, both the ES and IS were statistically correlated to the HDS.

Furthermore, results are consistent also to previous data obtained from TMJ disc tissue which reported a stronger expression of ADAMTS-5 in severe diseased discs [19], especially around tears and clefts. All in all, these data could explain the reason why after surgical creation of an anterior disc displacement, there is a decrease of PG in disc tissue [23, 24]. The fact that ADAMTS-4 expression and HDS were correlated just for the AB and IB and not for the PB deserves further investigation in order to understand if this finding could correspond to an altered loading in the joint or should be correlated to ADAMTS-4 function.

According to our data and to previous findings [22, 25], demonstrating that compressive load leads to the increase in ADAMTS-1, 4 and 5 which in turn contribute to the decrease of aggrecan in joint disc [21], it would seem that the more a disc is displaced the higher levels of ADAMTS are produced, which lead to ECM breakdown and in turn to a more advanced disc displacement. Therefore, ADAMTS-4 and ADAMTS-5 could be important biomarkers for diagnosis of joint destruction diseases and they could represent potential targets for therapeutic intervention of these pathologies [16, 18].

Based on these findings, it is not unreasonable to conclude that an early therapeutic blocking of ADAMTS-4 and -5 activity of even one of these proteases would provide protection against the disease without affecting normal physiology, by limiting breakdown of the aggrecan-rich matrix and possibly mitigate degenerative disc disease.

Furthermore, our results might be helpful for further understanding the pathogenesis of TMJ disc degeneration, in order to prompt the design of new drugs or other therapeutic strategies to specifically interfere only with the proteolytic events, which are destructive to the tmj disc tissue.

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