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LETTER TO THE EDITOR

P53 AND VEGF EXPRESSION IN HUMAN TEMPOROMANDIBULAR JOINT DISCS WITH INTERNAL DERANGEMENT CORRELATE WITH DEGENERATION

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To the Editor,

Temporomandibular joint (TMJ) disorders are one of the most relevant causes of chronic facial pain and disability, internal derangement (ID) is the most common TMJ arthropathy and is often related to disc damage (1). ID could be considered as an earlier stage of histopathologic changes of the TMJ disc tissue including breakup of dense collagen fiber bundles, new vessel generation and innervation of the central zone, cell proliferation and an increased number of cell types.

Anterior disc displacement with (ADDwR) and without reduction (ADDwoR) are the most common types of ID. In ADDwR, the disc slides into and out of its normal functional position when the jaw opens and closes; in ADDwoR the disc slides anteriorly to a lower rest position, remaining stuck in the anterior joint recess and failing to revert to its normal position with condylar movement. When the disc slips out of place or is displaced, it can determine improper condyle movement, causing dysfunction.

Human tumor protein p53, is known as a “guardian of the genome”, and its target genes are involved in cell-cycle control and induction of apoptosis. As tumor suppressor protein, p53 can act as transcription factor for genes of pro-apoptotic effector proteins and it is also implied in transcription-independent cellular signaling that leads to cell death via pathways localized in the mitochondria or the cytosol. Furthermore, p53 determines transcription of DNA repair enzymes that promote cell survival. This highlights the functional double role of p53, namely apoptosis or promoting cell survival. In physiological conditions, cellular p53 level expression is low and the protein has a short half-life of 20 min. Upon DNA damage, p53 levels increase primarily through stabilization of the protein (2-3).

Vascular endothelial growth factor (VEGF) has a pivotal role in increasing capillary vessel permeability and acts in angiogenesis of inflamed TMJ synovial tissue (4).

The aim of the present research was to analyzed p53 and VEGF immunoexpression patterns in disc patients with ID, with ADDwR and ADDwoR and to

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correlate the results with the degeneration grade. The purpose is to gain insight into the pathophysiological mechanisms underlying the morphological changes of the different areas of the articular disc.

MATERIALS AND METHODS

Tissue samples

TMJ disc specimens, surgically obtained from patients with TMJ ID, were provided by the Pontifical Catholic University of Paraná, Brazil (5). After project approval by the Ethics Committee of the same University, each patient signed an informed consent form before tissue collection. The discs had been obtained from 11 females and 7 males aged 24 to 41 years with TMJ ID, 8 with ADDwR and 10 with ADDwoR diagnosed on the basis of the following parameters: history, clinical examination, and magnetic resonance imaging data. Mean patient age was 34.2±5.4 years; mean disease duration from ID symptom onset to surgery was 8.7±1.2 months.

Unassisted maximum mouth opening (MMO) and a visual analog scale (VAS) for pain were used to evaluate the disease severity. MMO was calculated with a millimeter ruler as interincisor distance; pain intensity in the preceding week was rated on a 100 mm VAS from 0 (no pain) to 100 (the worst pain imaginable). The diagnosis that led to the surgical procedure was painful ID with functional impairment. The inclusion criteria for disc excision were: i) unsuccessful conservative management; ii) tenderness to TMJ palpation; and iii) TMJ pain or interference with jaw movement. Exclusion criteria were: i) other TMDs; ii) dentofacial deformity; iii) major jaw trauma; iv) previous TMJ surgery; and v) prior TMJ treatment with steroid injections. The discs appeared macroscopically deformed and none had preserved a normal biconcave shape. The anterior, intermediate and posterior band were preserved in all specimens.

Four TMJ discs from the collection of the Anatomy Institute of Catania University were also included in the study as controls. They were autopsy specimens from one male and three female donors (mean age 49.7±4.4 years) that were selected, as previously reported (5), for their virtually normal shape and condition, since none had macroscopic signs of joint degeneration and/or inflammatory outlook on dissection and were not displaced; in addition the donors’ clinical histories were negative for general joint disease or TMJ arthropathy.

Immunohistochemistry

Sections were processed as described previously (5). Briefly, they were incubated for 30 min in 0.3% H2O2/methanol and rinsed with phosphate-buffered saline (PBS; BioOptica, Milano, Italy). The antigen retrieval was performed using a microwave oven, as already described (5). Then slides were incubated with the following primary antibodies: a rabbit polyclonal anti-p53 antibody (FLN-393:sc-6243; Santa Cruz Biotechnology, Inc., Dallas, USA) and an anti-VEGF antibody (A-20:sc-152; Santa Cruz Biotechnology, Inc., Dallas, USA) diluted 1:100 in PBS, 0.1 % bovine serum albumin, and incubated overnight at 4°C. The secondary antibody, biotinylated anti-mouse/anti-rabbit IgG, was applied for 30 min at room temperature followed by the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The immunoreaction was demonstrated by 4 min incubation in 0.1 % 3,3’-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector). Finally, sections were counterstained with Mayer’s hematoxylin (Histolab Products AB, Goteborg, Sweden), mounted on GVA mount (Zymed Laboratories, San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRC5, Carl Zeiss, Oberkochen, Germany).

Positive and negative controls

In order to test the specificity of the primary antibodies, we used esophagus and lung tissue as positive control and some of the disc sections that were tested with normal rabbit serum, without primary antibody.

Computerized image analysis

The antibodies-staining (p53 and VEGF) status was identified as either negative or positive. Immunohistochemical positive staining was demonstrated by the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation by light microscopy. Fifteen fields, the area of which was about 600,000 µm², randomly selected from each section, were
analyzed and the percentage areas stained with antibodies (p53 and VEGF) were calculated using a software for image acquisition and histomorphometric analysis (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the area of positive immunolabelling in each field, expressed as % positive, dark brown pixels of the analyzed fields, as described previously (6). Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany). Evaluations of immunohistochemical localizations were made by three blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the case was reconsidered to reach a unanimous agreement.

**Histopathological degeneration score**

Some paraffin sections were stained with H&E in order to identify histopathological degeneration and evaluate them by a histopathological degeneration score (HDS). The score considers the changes and the degree of modification observed in pathological samples, i.e. collagen bundles, non-specific degenerative changes, and the presence of blood vessels, and ranged in a score from 0 (normal tissue) to 8 (severe tissue degeneration). The HDS was estimated by three observers, two anatomists and a histologist (7) who used the mean value of scores as the value of each sample.

**Statistical analysis**

P53 and VEGF expression of all discs was compared among them and with the HDS. All experiments were made in triplicate. Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. The anterior, intermediate, and posterior bands in sections from patients with ADDwR and ADDwoR were considered for comparisons. Comparison between immunoexpression (p53- and VEGF-) and HDS was performed through Spearman’s test. Immunohistochemical comparisons between means from the different bands were tested with One-way ANOVA post-test: Tukey-Kramer Multiple Comparisons Test; a p level < 0.05 was considered significant, p level < 0.01 was considered very significant. All data were analyzed with SPSS software (SPSS release 16.0, Chicago, IL, USA). Data are presented as the mean±SD.

**RESULTS**

**Immunohistochemistry**

To investigate the expression of p53 and VEGF, we analyzed discs of patients with ID ADDwR and

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**Fig. 1.** P53 immunoexpression in sections from patients with ADDwR and ADDwoR. **A** posterior band - ADDwR. **B** intermediate band - ADDwR. **C** anterior band - ADDwR. **D** Graph. A bar chart representing a comparison of the % p53 immunostained area in the posterior, intermediate and anterior bands of ADDwR samples. **E** posterior band - ADDwoR. **F** intermediate band - ADDwoR. **G** anterior band - ADDwoR. **H** Graph. A bar chart representing a comparison of the % p53 immunostained area in the posterior, intermediate and anterior bands of ADDwoR samples. Thick arrows: chondrocyte-like cells; thin arrows: fibroblast-like cells. Data are presented as mean±SD. *p<0.05, **p<0.01. Scale bar: 50 µm.
ADDwoR. In particular, p53 immunoexpression was detected in fibroblast- and chondrocyte-like cells. In ADDwR, the histomorphometric analysis demonstrated an expression of p53 principally in the anterior and the intermediate disc areas when compared to the posterior band, in fact the percentage of immunostained area was significantly higher in the anterior and the intermediate bands compared with the posterior one (p<0.0) as shown in Fig. 1, A-D. On the contrary, in ADDwoR, a very low (not detectable) expression of p53 was noticed in the anterior and the intermediate bands and a high expression intensity in the posterior band; the percentage of immunostained areas was much higher in the posterior band when compared to the anterior and the middle ones (p<0.01) (Fig. 1, E-H). The expression of VEGF was detected in endothelium of newly formed vessels, in fibro- and chondrocyte-like cells. ADDwR histomorphometric analysis demonstrated a higher percentage of immunostained area in the anterior and intermediate bands compared to the posterior band (p<0.05) (Fig. 2, A-D). Instead, in ADDwoR, a lower percentage of immunostained area was detected in the anterior and the intermediate areas when compared to the posterior band (p<0.01) (Fig. 2, E-H). Moreover, increased immunoexpression of p53 and VEGF was correlated with the increase of the HDS (p<0.05).

TMJ discs from cadavers (control) showed a not detectable expression of both p53 and VEGF. In the negative control sections immunoexpression was not detected.

**Histopathological degeneration score**

In H&E-stained pathological discs, a reduced number of cells was shown, a decreased number of fibroblast-like cells and a number of chondrocyte-like cells increased in relation to the severity of the morphological damage with abnormal collagen fiber arrangement, collagen bundle fragmentation and tearing. The histopathological score was 3.93±0.6. Differently, control discs showed no signs of cellular or tissue alterations and histopathological score ranged from 0 to 1 points (7-8).

**DISCUSSION**

The present study gave insights of this synergy of p53 and VEGF expression showing the different patterns of immunodetection in the three areas of TMJ discs in patients with ID ADDwR and correlated these results with the histopathological grading score.
In vivo studies on rodents have underlined the pivotal role of apoptosis in articular cells of displaced rabbit discs and rat TMJ tissues after acute inflammation (9). Also Fas ligand (FasL), an apoptosis-inducing factor activated by distinct signal pathways, was shown to be highly immunostained in ADDwR (10). In this context p53 plays an important role as it is activated by cellular stress and may act promoting DNA repair when the DNA damage is limited or induces apoptosis, in relation to high DNA damage, to re-establish the tissue integrity (11). Leonardi et al. (7) proved an overexpression of death receptor-5 (DR5) and ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which triggers cell death in ID TMJ discs of patients with both ADDwR and ADDwoR. Our previous investigations have already demonstrated an increased expression of VEGF in human dysfunctional TMJ discs. In particular, VEGF immunolocalization was found related with angiogenesis, morphogenesis and differentiation of chondrocytes. The present study showed VEGF immunolocalization is related not only with the histopathological grading score, but also with the different insults exercised in the three bands in patients with ADDwR and ADDwoR (12). Liu et al. (11) demonstrated, on rat degenerated intervertebral disc tissues, that both p53 and VEGF are involved in the process of neovascularization and capillary infiltration, in particular p53 and VEGF immunoexpressions were significantly higher in tissue areas of increased load, indicating that p53 is likely involved in neovascularization and degenerated disc areas (12). According to our results, it can be assumed that when more histopathological changes in the disc are relieved, major levels of p53 and VEGF are produced. In conclusion, the present study may be useful for further investigations, despite the limitation determined by the relatively small number of samples, in the pathogenesis of TMJ disc degeneration and to stimulate the design of new therapeutic pharmacological strategies.

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