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Chondrocyte-like apoptosis in temporomandibular joint disc internal derangement as a repair-limiting mechanism. An *in vivo* study

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Summary. Temporomandibular joint internal derangement (TMJ ID) is characterised by disc displacement and degenerative tissue changes involving an active cellular response, with cell phenotype transformation from fibroblast-like to fibrochondrocyte and, eventually, to chondrocyte-like, possibly as a response to abnormal loading. However, only small patches of chondral tissue are detected in TMJ discs with ID. We decided to explore the reasons for such incomplete tissue change, postulating an involvement of the apoptosis process.

Twenty-one discs removed from 19 patients with TMJ ID were processed for TRAIL and DR5 immunohistochemical localisation, and subjected to the TUNEL assay.

Overexpression of DR5 receptor and its ligand (TRAIL) in chondrocyte-like cells suggested activation of programmed cell death, as also demonstrated by TUNEL-positive cells.

The data suggest a failed adaptive response to disc displacement through chondroid metaplasia. The apoptotic death of chondrocyte-like cells, which is at least partly regulated by TRAIL and its death receptor, appears to underpin the failed disc repair, eventually leading to its perforation.

Key words: TMJ, internal derangement, DR5, TRAIL, TUNEL

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Introduction

The most common temporomandibular joint (TMJ) arthropathy is internal derangement (ID), which involves an anatomical disturbance in the relationship of the disc-condyle complex. ID is likely to be a precursor to histopathological changes in TMJ disc tissue. Disc displacement is associated with degenerative tissue changes (Carlsson et al., 1967; Scapino, 1983; Hall et al., 1984; Castelli et al., 1985; McCoy et al., 1986; Helmy et al., 1990; De Bont and Stengenga, 1993; Marchetti et al., 1995; Milam et al., 1998; Jibiki et al., 1999), although the underlying mechanisms remain unclear.

According to several studies, apoptotic cells and apoptosis-related factors are associated with articular tissue destruction in joints other than TMJ in patients with rheumatoid arthritis (Gavrieli et al., 1992; Hashimoto et al., 1998). Recently, a strong correlation has been documented between TMJ ID and programmed cell death studied by in situ cell detection, tumour necrosis factor α (TNFα), Fas and caspase activities (Matsuda et al., 1997; Gu et al., 2002; Nagai et al., 2003; Spears et al., 2003; Huang et al., 2004). Most available data come from animal models; there are no studies exploring the activation of the apoptotic mechanism in a human in vivo model using TMJ discs.

In previous investigations we demonstrated histological differences between normal and pathological TMJ discs, in line with the literature (Leonardi et al., 2007). Degenerative aspects in pathological discs included an active cellular response, with a change in cell phenotype from fibroblast-like to fibrochondrocyte, and eventually to chondrocyte-like, possibly as a response to abnormal loading. However only small areas of chondral tissue were detected. These findings prompted us to investigate the reasons for such

incomplete tissue change and to postulate the involvement of apoptotic processes.

Apoptosis is an integral part of development; it is involved in embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, normal tissue turnover, and a variety of pathological conditions resulting from its dysregulation (Huppertz and Kaufmann, 1999). The two major mechanisms regulating apoptosis are the intrinsic pathway, mediated by mitochondria, and the extrinsic pathway, induced by death-signalling ligands, like TNFα and FasL, which bind their receptor and induce caspase 8 activation (Charriaut-Marlangue and Ben-Ari, 1995; Adams and Cory, 1998; Green and Reed, 1998; Ferri and Kroemer, 2001). Crosstalk has been documented between the upstream component of the extrinsic and the intrinsic pathway (Zhaoyu and Wafik, 2005).

TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) is a member of the TNF family of ligands which is capable of initiating apoptosis through engagement of its death receptors (DR4 and DR5) (Wang and El-Deiry, 2003); it selectively induces apoptosis in a variety of tumour and transformed cells (Wang and El-Deiry, 2003), and has been found to play an important role in cell regulation and inflammation processes (Hasel et al., 2003; Robertson et al., 2004).

In this study we explored the hypothesis that activation of DR5 by TRAIL may have an important role in enhancing chondrocyte-like cell susceptibility to apoptosis, and that it may at least partially account for the incomplete transformation of disc tissue into cartilage. Activation of the apoptotic process was investigated via immunohistochemical detection of TRAIL and its death receptor DR5; DNA strand breaks were evaluated using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) method.

Materials and methods

Tissue

We studied 21 discs removed from 19 patients, 16 females and 3 males aged 24 to 52 years (median: 43), affected by TMJ ID without reduction. The diagnosis leading to surgery was disc derangement associated with pain and functional impairment without reduction. All patients had already unsuccessfully been treated non-surgically.

Three virtually unaffected human TMJ discs from the University's collection were also studied; they were autopsy specimens from two men and one woman whose clinical histories were negative for TMJ conditions.

The discs were fixed in 10% neutral buffered formalin (Bio-Optica, Italy) and washed overnight. Each specimen was sectioned through its centre along a parasagittal plane, perpendicular to the long axis. Tissue blocks were dehydrated in graded ethanols, embedded in paraffin with their anatomical orientation preserved, cut to obtain 3-4 µm thick sections according to routine procedures, mounted on slides and air-dried.

Immunohistochemistry

For immunohistochemistry, sections were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity, then rinsed for 20 min with 0.01 M phosphate-buffered saline (PBS), pH 7.4 (Bio-Optica). Sections were irradiated (3x5 min) in capped polypropylene slide-holders with citrate buffer (pH 6.0; Bio-Optica) using a microwave oven (750 W) to unmask antigenic sites.

Rabbit polyclonal anti-TRAIL (Santa Cruz Biotecnology, Inc., USA) and anti-DR5 (Novus Biologicals, Inc., USA) antibodies were used at 1:20 working dilution.

After overnight incubation (4°C) in a humidified chamber, sections were incubated with the secondary antibody; detection was performed with the streptavidin-biotin method (LSAB 2 System-HRP, Dako, Denmark) using 3,3'-diaminobenzidine (DAB) as the chromogen (Dako). Sections were counterstained with haematoxylin and observed with an Axioplan light microscope (Zeiss, Italy).

Positive controls consisted of tissue specimens with known antigenic positivity. Negative control sections were processed like the experimental slides, except that they were incubated with PBS instead of the primary antibody.

Disc cells were considered immunopositive if definite cellular staining was observed at a distance from the edge of the section. In each specimen, immunostaining was scored by three independent observers (two anatomical morphologists and a histologist) based on the number of stained cells, as follows: >50% positive cells, ++; 10-50% positive cells, +; and <10% positive cells or no staining.

In situ detection and measurement of apoptotic cells

For in situ detection of apoptosis at the single cell level we used the TUNEL method (In Situ Cell Death Detection Kit, POD, Roche Applied Science, Germany). The assay involves the addition of deoxyuridine triphosphate (dUTP) labelled with fluorescein to the ends of DNA fragments by the catalytic action of TdT. All end-labelling experiments were performed in triplicate so that the results for various tissue samples, including rat prostate, could be standardised. Paraffin-embedded lung sections 5 µm in thickness were dewaxed as previously described. Slides were rinsed twice in 0.01 M PBS (pH 7.4), transferred to 0.07 M citrate buffer (pH 6.0) and subjected to 750 W microwave irradiation for 1 min for permeabilisation. Sections were then immersed in Tris-HCl 0.1 M, pH 7.5, containing 3% bovine serum albumin (both from Roche) and 20% normal bovine serum (Sigma-Aldrich, USA) for 30 min at 20°C, rinsed twice in PBS and immersed in TdT buffer (Roche). Sections were covered with TdT and fluorescein-labelled dUTP in TdT buffer and incubated in a dark humid chamber at 37°C for 60 min.

All sections were incubated with an antibody

specific for fluorescein conjugated to peroxidase for 30 min at 37°C. Staining was visualised with DAB, which stained brown nuclei with DNA fragmentation. Sections were counterstained with the nuclear dye methyl green, 0.5%.

For negative controls, TdT was omitted from the reaction. Positive controls consisted of rat prostate gland after castration.

Ten fields from randomly selected slides were observed under a light microscope. Each field was photographed with a digital camera (Canon, Japan) at x60 magnification. On each photomicrograph three observers blinded, to sample identity, counted the total number of cells as well as the number of these cells exhibiting a positive TUNEL reaction. The proportion of positive cells out of the total number of cells was calculated for each photomicrograph and a mean value was obtained for each sample. The results were expressed as a percentage.

Statistical analysis

Data analysis was performed using the SPSS-PC software (SPSS Inc., USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between means were tested with Student's t test. A value of $P \leq 0.05$ was considered significant. Cohen's kappa was applied to measure interobserver agreement and averaged to evaluate overall agreement using the following grading: 0-0.2 (slight), 0.21-0.40 (fair), 0.41-0.60 (moderate), 0.61-0.80 (substantial), and 0.81-1.0 (almost perfect).

Results

Immunohistochemistry

The three control discs were composed of densely woven fibrous tissue. Numerous tapered or oval cells (fibrocyte-like) and some round-tapered cells (fibrochondrocytes) were seen parallel to collagen bundles. Cells with rounded nuclei surrounded by a large cytoplasm halo (chondrocyte-like) were occasionally observed in collagen bundle interfibrillary spaces.

Different cell population ratios were found in the two groups of discs, the pathological discs exhibiting a larger number of fibrochondrocytes and chondrocyte-like cells, while fibroblast-like cells were the most prevalent cell type detected in normal discs.

Scarce immunoreaction product and, rarely, faint TRAIL and DR5 staining were detected in control discs (-) (Fig. 1e). In patients' discs immunolabelling was mainly observed in chondrocyte-like cells.

Patients' discs were abnormal in shape and deformed. Histopathological changes were seen both in the extracellular matrix and in cell populations. Abnormal collagen fibre arrangement and collagen bundle fragmentation and tearing were the most frequent findings. An increased number of fibrochondrocytes and chondrocyte-like cells was also noted. In general, the more severely damaged the disc, the greater the number of TRAIL- and DR5-immunolabelled cells (++) (Fig. 1a, 1d).

Nearly all chondrocyte-like cells but only about half of all fibrochondrocytes and a few scattered fibroblast-like cells were TRAIL-positive.

Positivity for DR5 was detected in plasma membranes. Most chondrocyte-like cells (++) (Fig. 1c) and some fibrochondrocytes (+) (Fig. 1d) were immunostained, whereas a very small number of fibroblast-like cells displayed immunostaining (-).

The less damaged discs showed fibroblast-like cell TRAIL and DR5 immunostaining (+) (Fig. 1b).

Immunohistochemical controls for reaction specificity produced no labelling and revealed no endogenous peroxidase activity.

The proportion of immunopositive cells was significantly greater ($P \leq 0.01$) in patients' discs.

Interobserver agreement, measured as kappa coefficient, was 0.84 (almost perfect).

In situ detection of DNA fragmentation

A large number of apoptotic TUNEL+ cells were observed in patients' discs. These cells displayed both non-apoptotic (type I) and apoptotic (type II) morphologies.

Type I cells (necrotic cells) exhibited diffuse and non-uniform cytoplasmic staining (black arrow), while type II cells displayed intensely TUNEL+ nuclei as well as round and shrunken nuclei and surface blebbing (white arrow) (Fig. 2a). The proportion of positive cells was significantly greater ($P<0.01$) in TMJ ID than in control discs.

Interobserver agreement, measured as kappa coefficient, was 0.82 (almost perfect).

Discussion

Discs removed from patients with TMJ ID show histological features (Scampino, 1983; Hall et al., 1984; Isacson et al., 1986; McCoy et al., 1986; Kurita et al., 1989) that reflect a general remodelling response caused by abnormal loading (Scampino, 1983). One such modification is a change in cell phenotype from fibroblast-like to fibrochondrocyte and eventually to chondrocyte-like; however, only small areas of changed tissue are observed in these discs, reflecting incomplete transformation (Leonardi et al., 2007).

Intense mechanical insult can reduce cell viability in unstable joints and in injured cartilage (Repo and Finlay, 1977; Jeffrey et al., 1995). Some studies suggest that in situ apoptosis is involved in the loss of cellularity (Blanco et al., 1998). Preservation of cell viability in joint cartilage after mechanical damage therefore seems to be very important for matrix metabolism and tissue healing (Chen et al., 2001).

Chondrocyte necrosis and apoptosis have been described following load application in an in vitro study

of articular cartilage (Chen et al., 2001). In our in vivo study, a large number of TRAIL- and DR5-immunolabelled cells (++) were seen in several discs from patients with TMJ ID, lending support to the concept of chondrocyte apoptosis following mechanical injury. Control discs, which came from individuals with no TMJ conditions and had therefore not been subjected to mechanical overloading, did not display activation of the apoptotic process.

Chondrocyte death, revealed by in situ cell detection, is suspected to be involved in cartilage repair in response to disc displacement (Gu et al., 2002). Apoptotic cells and apoptosis-related factors have also been reported to be associated with joint tissue destruction in patients with rheumatoid arthritis (Nakajima et al., 1995; Asahara et al., 1996; Nishioka et al., 1998). Activation of apoptotic signalling pathways in TMJ in a CFA-induced inflammation rat model has suggested an activation of death domains on TNF· receptor, demonstrating activation of the death receptor/death receptor ligand system in synovial lining cells of TMJ disorders, and suggesting that Fas mediates the apoptotic reaction in these tissues (Nagai et al., 2003; Spears et al., 2003).

In situ TUNEL and electron microscopy studies have shown increased chondrocyte apoptosis in human osteoarthritis, correlating with the severity of cartilage destruction (Blanco et al., 1998; Kim et al., 2000), although chondrocyte apoptosis was also demonstrated in early-stage TMJ disorder with disc displacement,

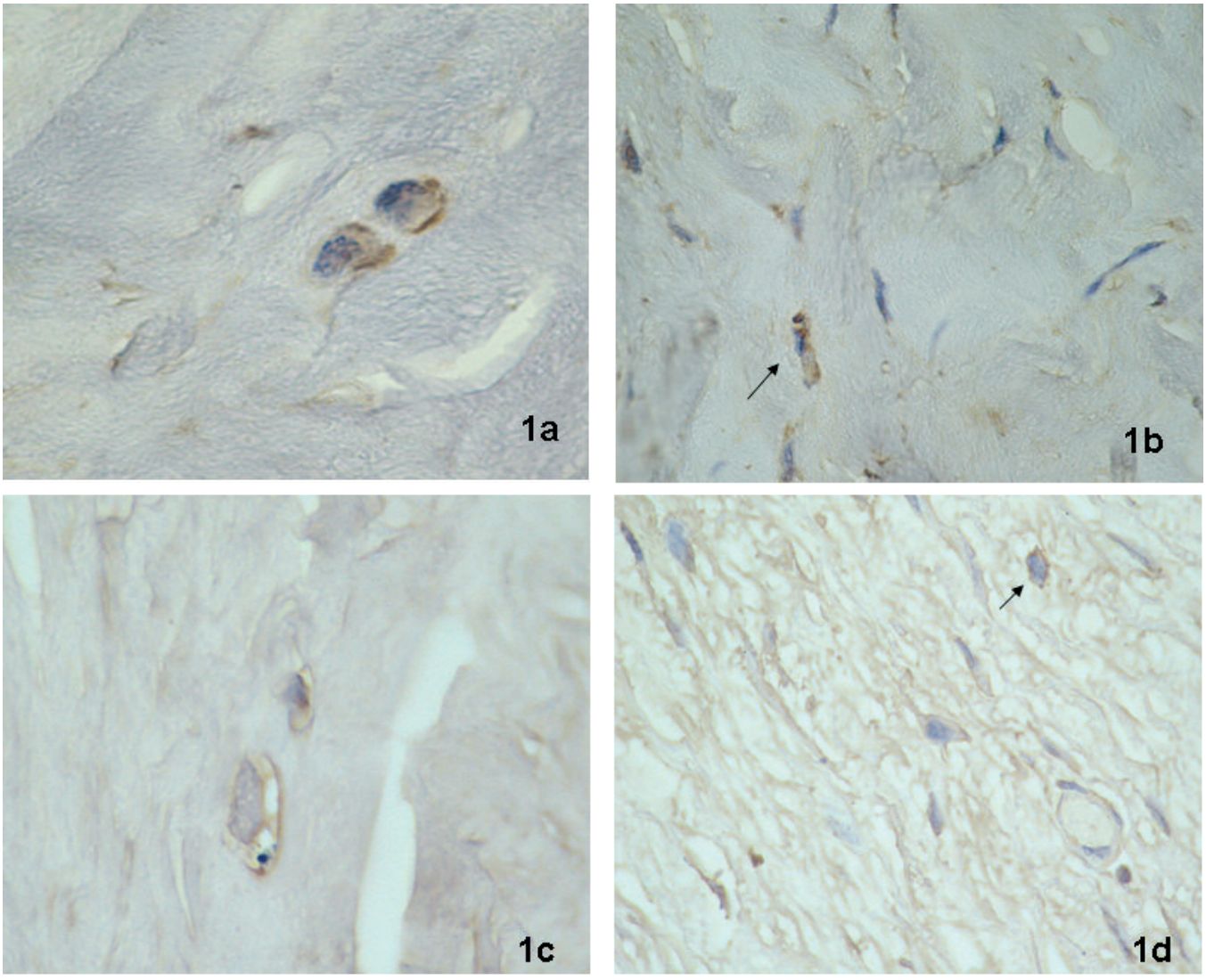

Fig. 1. TRAIL immunolocalisation in pathological disc. Chondrocyte-like cells were consistently labelled **(a)** whereas only a small number of fibroblasts exhibited TRAIL immunostaining (arrow) **(b)**. DR5 immunolocalisation in pathological disc: labelled chondrocyte-like cells **(c)** and labelled fibrochondrocyte (arrow) **(d)**. x 100

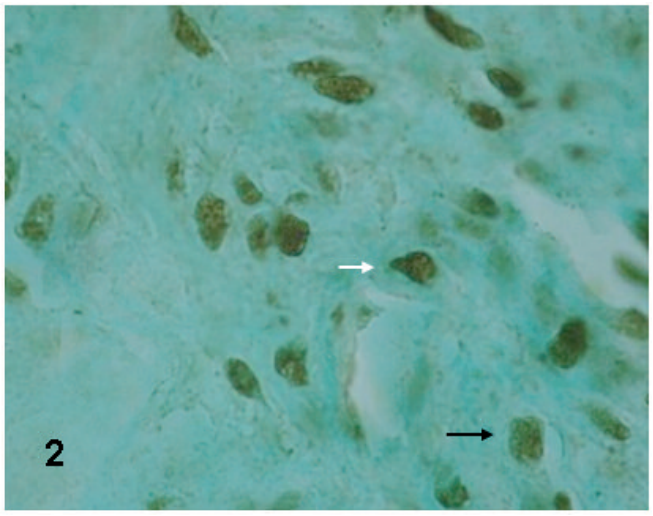

Fig. 2. TUNEL reaction in pathological disc, high-magnification photomicrograph. In the pathological disc most cells (chondrocyte-like cells and fibrochondrocytes) are TUNEL+. Type I cells (black arrow), type II cells (white arrow). x 100

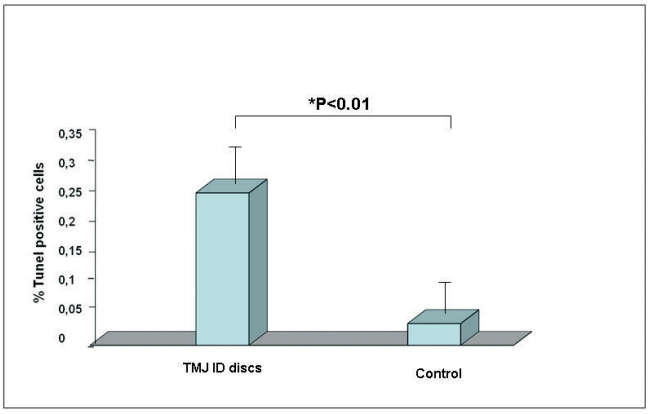

Fig. 3. Percentage of TUNEL positive cell, in pathological and control discs.

predominantly in proliferative and hypertrophic areas (Gu et al., 2002). It is also well known that meniscal degeneration may contribute to the development of TMJ disorders, both through mechanical means and by secretion of pro-apoptotic mediators, such as Fas and Fas ligand into synovial fluid, resulting in chondrocyte apoptosis (Renoux et al., 1996; Hashimoto et al., 1997; Robertson et al., 2006).

Chondrocyte apoptosis may disrupt the cartilage repair process, eventually resulting in bone–cartilage deformation, such as osteophyte formation or cartilage thinning (Duke et al., 1993; Lots et al., 1999; Ghivizzani et al., 2000; Lane Smith et al., 2000; Yao et al., 2003). Aizawa and co-workers (2001) demonstrated that chondrocyte apoptosis can be induced both through Fas and TNFα receptor-mediated signalling, and suggested that chondrocytes are more sensitive to the apoptotic effects of TNFα. Yao et al. (2003) showed that adenovirus-mediated gene transfer of TRAIL to rabbit knee could induce apoptosis through an interaction with receptors containing death domains (Hirota et al., 2006).

Consistent with these data, our findings show overexpression of DR5 receptor and its ligand (TRAIL) in chondrocyte-like cells, suggesting an activation of programmed cell death that was also demonstrated by TUNEL-positive cells. In particular, nearly all chondrocyte-like cells but only about half of all fibrochondrocytes and a small number of scattered fibroblast-like cells were TRAIL-positive, while fibroblast-like cells displayed weak or negative DR5 immunolabelling. These findings suggest an incomplete activation of the apoptotic process in fibroblast-like cells, resulting in failure to undergo apoptosis.

In conclusion, our data are consistent with a failed adaptive response to disc displacement through chondroid metaplasia. The apoptotic death of chondrocyte-like cells, which is at least partly regulated by TRAIL and its death receptor, appears to underlie the failed disc repair and its eventual perforation.

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