Eur Spine J (2002) 11 : 308–320
DOI 10.1007/s00586-002-0472-0

C. Weiler
A. G. Nerlich
J. Zipperer
B. E. Bachmeier
N. Boos

2002 SSE Award Competition in Basic Science: Expression of major matrix metalloproteinases is associated with intervertebral disc degradation and resorption

Received: 12 June 2002
Accepted: 12 June 2002
Published online: 9 July 2002

C. Weiler
Institute of Pathology,
University of Munich, Germany

A.G. Nerlich · J. Zipperer
Institute of Pathology, Academic Teaching Hospital Munich-Bogenhausen, Germany

B.E. Bachmeier
Department of Clinical Chemistry,
University of Munich, Germany

N. Boos (✉)
Orthopedic University Hospital Balgrist,
Forchstrasse 340, 8008 Zürich, Switzerland
e-mail: Norbert.Boos@balgrist.ch,
Tel.: +41-1-3861111,
Fax: +41-1-3861609

Abstract During the process of degeneration, the intervertebral disc (IVD) shows a progressive and significant reduction in height due to tissue resorption. Intradiscal clefts and tears are major hallmarks of disc degeneration. Matrix-degrading enzymes such as matrix metalloproteinases (MMPs) are assumed to play a pivotal role in disc tissue degradation and resorption. The objective of this study was therefore to investigate the potential role of MMPs in extracellular matrix degradation leading to disc degeneration. This study was conducted on 30 formalin-fixed and EDTA-decalcified complete cross-sections of lumbar IVDs from cadavers of individuals aged between 0 and 86 years. Tissue sections were used for the immunolocalization of MMPs-1, -2, -3 and -9. The number of labeled cells was assessed by morphometric analyses, and was statistically correlated with the formation of clefts and tears, cellular proliferation, granular matrix changes and mucous degeneration. Furthermore, 30 disc specimens obtained during spinal surgery were used for in situ hybridization of MMP-2 and -3-mRNA. In addition, the enzymatic gelatinolytic activity was determined by in situ zymography in autopsy material. Immunohistochemistry showed the intradiscal expression of all four MMPs, which was confirmed by in situ hybridization, providing clear evidence for the synthesis of the enzymes within nucleus pulposus and annulus fibrosus cells. Gelatinolytic enzymatic activity was verified by in situ zymography. IVDs from infants and young adolescents remained almost completely unlabeled for all MMPs tested, while more MMPs-1 and -3 were seen in disc cells of younger adults than in those of a more advanced age; MMP-2 remained unchanged over the adult age periods, and MMP-9 was expressed in only relatively few cells. This pattern significantly correlated with the occurrence of clefts and tears. This correlation was strongest for MMP-1 ($P<0.0001$), MMP-2 ($P<0.0017$) and MMP-3 ($P<0.0005$) in the nucleus, and MMP-1 ($P<0.0001$) and MMP-2 ($P<0.038$) in the annulus. In parallel, the proliferation of disc cells and matrix degeneration (granular changes and mucous degeneration) were related to MMP expression. Likewise, enzymatic activity was seen in association with cleft formation. Our data suggest that major MMPs play an important role in the degradation of the IVD. This is evidenced by the high correlation of MMP expression with the formation of clefts and tears. These findings implicate a leading function for MMPs in IVD degeneration resulting in the loss of normal disc function, eventually leading to low-back pain.

Keywords Intervertebral disc · Disc degeneration · Histology · Immunohistochemistry · In situ hybridization · Matrix metalloproteinases

Introduction

The integrity of the intervertebral disc (IVD) depends largely on the equilibrium between matrix degradation and synthesis. Any imbalance might lead to impaired functional properties. From a histologic point of view, the occurrence of clefts and tears are the major hallmarks of disc degeneration, [5, 6, 7, 9, 10, 11, 15, 18, 36, 40, 41, 42, 43], but there is very little (almost no) evidence that invading cells might contribute to this process of matrix degradation. Therefore, it can be assumed that local cells are involved in any degradation process. During the process of degeneration, the IVD exhibits a progressive and significant reduction in height due to tissue resorption. Since collagen is the most abundant disc matrix protein, and since collagen molecules can be cleaved by only very few proteases, the group of collagen-degrading enzymes – the matrix metalloproteinases (MMPs) – have recently been attracting more and more attention [12, 13, 20, 21, 25, 26, 27, 31, 35]. MMP-1 is unique in degrading native, intact interstitial collagen (such as collagen I, II and III), while the two gelatinases (MMPs-2 and -9) cleave denatured collagen molecules and basement membrane collagen type IV. Stromelysin-1 (MMP-3) degrades non-collagenous matrix proteins, but also denatured collagen.

Recent research has focused on the role of MMPs in tissue degradation in the process of disc aging and/or degeneration. Preliminary studies [12, 20, 21, 25, 26, 27, 31] suggested that MMP expression and activity is up-regulated in disc tissue with degenerative lesions, but little is known about any association between the expression and activity of MMPs and the histologic evidence for tissue degradation in the various anatomic regions.

In analyzing human lumbar intervertebral discs obtained during routine autopsy and spinal surgery by histochemical and immunohistochemical methods, in situ hybridization and in situ zymography, the objective was to:

1. Study the temporo-spatial distribution and enzyme activity of major MMPs in normal and degenerated lumbar intervertebral discs of various ages
2. Investigate the potential role of MMPs in the extracellular matrix degradation leading to disc degeneration
3. Correlate the expression of major MMPs with the occurrence of cells with phagocytic activity in relation to tissue resorption

Materials and methods

Study populations and tissue preparation

Two different study populations were included to investigate the aforementioned study objectives.

Study group I

The first group consists of 30 intervertebral disc specimens covering the whole age range (fetal to 86 years) obtained from 20 cadavers (9 female, 11 male). None of the individuals had died of a consuming illness (i.e. tumor, infection) or had known spinal problems. Complete lumbar motion segments were harvested at routine autopsy, as described in a previous study [33]. Mid- and parasagittal sections through the motion segment were prepared for subsequent histology and immunohistochemistry to allow for a concise correlation of any observation with the respective anatomic regions (i.e. nucleus vs. annulus). For the present analysis, these cases were selected to be representative with respect to age, sex, and histomorphologic features.

Thin sagittal slices (5 mm) of the complete motion segments were fixed in 4–6% buffered formaldehyde (pH 7.4), subsequently decalcified (0.1 M EDTA, pH 7.4) for a 1- to 4-months period and, after completion of decalcification, finally embedded in paraffin wax as routinely performed.

The rationale for this study group was to obtain specimens allowing for a detailed correlation between MMP expression and histomorphologic alterations in the different histoanatomic regions of the disc.

Study group II

The second group encompassed 30 lumbar specimens obtained during surgical procedures (i.e. discectomies, anterior lumbar interbody fusion and scoliosis surgery) from 23 individuals (age range 11–68 years, 14 females and 9 males). Eight patients had undergone surgery for degenerative disc disease; ten patients for disc protrusion; and five patients for scoliosis correction. The distribution of the 30 samples obtained from these 23 patients across the disc levels was as follows: T12/L1 (n=6), L1/2 (n=2), L2/3 (n=2), L4/5 (n=8), L5/S1 (n=12). Clinical data on low-back pain history, related disability and imaging findings (radiographs, magnetic resonance images) were available for all cases.

The material was immediately fixed in buffered 4–6% formaldehyde (pH 7.4) in order to ensure conservation of mRNA. All manipulations on this material were carried out under RNase-free conditions (gloves, RNase-free reagents etc.). The formalin-fixed tissue samples were treated as in group I, i.e. cases with obvious calcifications or residual bone material had to be smoothly decalcified before embedding, which was performed in a significantly shorter time period than in group I. All specimens were cut into slices (2–4 μm) and placed on silanized glass slides both for routine and histochemical stainings (H&E, Masson-Goldner or Elastica-van Gieson's connective tissue stain, Alcian blue-PAS), as performed in a previous study [33], and additionally for immunohistochemistry.

The rationale for a second study population was to obtain fresh tissue samples, because autopsy material is unsuitable for in situ hybridization analysis due to the well-known rapid postmortem degradation of the mRNA.

Immunohistochemical staining procedures

For this study, we used antibodies specifically reacting with MMP-1 (Oncogene, Boston, USA), MMP-2 and -3 (Chemicon International, Ltd., Hofheim, Germany) and MMP-9 (Dr. Lichtinghagen, Hannover, Germany). The specificity of the antibodies had been tested extensively prior to the study by various tests including Western blots [2, 3, 28]. In addition, we localized cells with phagocytic cellular features by immunostaining for CD 68. This lysosomal enzyme is usually expressed in granulocytes and macrophages/histiocytes, and indicates the cell's capability for phagocytic (digestive) activity. Therefore, we applied the commercially available monoclonal

antibody against the epitope KP-1 of CD 68 (DAKO, Hamburg, Germany) [32, 38].

Appropriate tissue sections from groups I and II were deparaffinized and subsequently pretreated with microwave radiation, as indicated by the manufacturer, to enhance immunoreactivity. The pretreated sections were then incubated with the monospecific MMP antibodies, which were finally visualized either by the APAAP method (DAKO, Hamburg, Germany) or the ABC technique (Vector, Burlingame, USA) [22]. The chromogenes were fast red for the APAAP reaction or diaminobenzidine for the ABC reaction (Sigma Chem., Deisenhofen, Germany). For negative controls, we used parallel sections treated with normal serum instead of the specific antibodies.

In situ hybridization analysis

The rationale for in situ hybridization investigations was to provide additional evidence for the synthesis of the MMPs within resident disc cells that had been localized in any given disc tissue by immunohistochemistry.

For the in situ analysis of the mRNA expression of MMP-2 and -3, we applied non-radioactive in situ hybridization, as recently described more extensively in a different context [28]. Therefore, specific gene probes recognizing the MMP-2 and -3 gene, respectively, were subcloned in pBluescript KS II plasmids (Stratagene, La Jolla, USA). The specificity of the probes had previously been tested by Northern blot. After linearization of the plasmids, single-stranded RNA probes complementary (anti-sense) or anti-complementary (sense probes, negative controls) were obtained by running off transcription using T3 or T7 polymerase. The probes were labeled by the incorporation of digoxigenin (Roche Chem., Basel, Switzerland) according to the manufacturer's instructions.

The fixed and embedded sections were deparaffinized, rehydrated and subjected to microwave pretreatment and proteinase-K digestion (Qiagen, Hilden, Germany). In a first set of experiments, the reaction criteria were determined for each set of samples, i.e. the optimal pretreatment and reaction conditions. Hybridization with the specific probes was carried out overnight at 49°C. Hybridized probes were detected by an anti-digoxigenin antibody (Fab-fragment, Roche Chem.) labeled with alkaline phosphatase and stained as indicated before.

In situ zymography

In addition to the histologic and immunohistologic tissue processing on the autopsy material, disc material (ten randomly selected specimens covering the age range of study group I) was obtained to demonstrate that the immunolocalized MMPs with gelatinolytic properties (i.e. MMPs-2, -3, and -9) actually exhibit biologic activity. In this context, the in situ zymography data served as an additional control for the data obtained by immunohistochemistry and in situ hybridization.

In order to localize the gelatinolytic activity within disc tissue, we applied in situ zymography using a modified method described by Nemori and co-workers [23]. This technique uses denatured collagen ("gelatin"), which is covered by a frozen section of the target tissue. In those areas with collagenolytic activity (i.e. mainly by MMPs-2, -3 and -9), the underlying gelatin is digested and thus removed from the slide by diffusion during subsequent washing. Thereby, any reduction of the staining intensity of the gelatin pad indicates enzymatic activity at that site.

This in situ zymography was performed using gelatin-covered slides that had been prepared by a mixture of 1.5% gelatin with 12% polyacrylamide (Fluka, Buchs, Switzerland). After polymerization of the gelatin/polyacrylamide, a frozen section of native IVD tissue was placed upon the gelatin pad and incubated in a wet chamber for 16 h. Subsequently, the slide was stained with ponceau red (Merck, Darmstadt, Germany), which stains the remaining gelatin. Thereby, gelatinolytic activity can be localized by showing a reduction of the ponceau red staining in areas of gelatinolysis beneath structures of the tissue section.

Data assessment and analyses

Histologic analyses

Histomorphologic analyses of the disc specimens were performed separately for the nucleus pulposus and the annulus fibrosus using a recently developed grading system [4]. Briefly, this grading system takes into account the following parameters that are graded at varying scores:

1. Cell density (chondrocyte proliferation)
2. Structural alterations (tears and clefts)
3. Mucoid degeneration
4. Granular matrix changes

The scores for each criterion are summarized in a histologic degeneration score (HDS) ranking of between 0 and 18 points (Table 1). Reproducibility assessment of the HDS has demonstrated a satisfactory level of reliability, with kappa values ranging from 0.71 to 0.80 [4].

All histomorphologic assessments for the purpose of this study were done by two pathologists, and disagreement was resolved in conference. The consensus assessment was used for further statistical analyses. The evaluation of the histomorphology was performed independently of the immunohistochemical observations, to exclude any bias.

Immunohistochemical analyses

The assessment of the abundance of MMP- and CD 68-labeled cells was accomplished by a quantitative morphometric analysis. Twenty fields for each anatomic region (i.e. nucleus pulposus, annulus fibrosus) were randomly selected, and the averaged proportion of labeled cells was calculated in relation to the total number of cells. Thus, quantitative values were obtained for the expression of MMP-1, -2, -3 and -9 as well as for CD 68-positive cells (i.e. cells with phagocytic activity).

In situ hybridization

The results of the in situ hybridization (MMP-2 and -3) were recorded on a qualitative basis (presence/absence), and compared with the results obtained by immunolocalization of the respective MMPs in study group II.

In situ zymography

Similarly, the results of in situ zymography were recorded in a qualitative manner (presence/absence) and compared with the results obtained by immunolocalization of the respective MMPs with gelatinolytic activity (i.e. MMP-2, -3 and -9) in study group I.

Correlative analyses

Statistical data analysis was accomplished in several steps. First, the influence of age, disc level and anatomic regions (i.e. nucleus pulposus vs annulus fibrosus) on the expression of MMPs was explored using a Pearson correlation, one way ANOVA and paired *t*-test, respectively. Second, simple relationships between MMP-1, -2, -3, and -9 and histologic features of disc degeneration were ex-

Table 1 Parameters collected for the histologic assessment of disc degeneration and scoring

Criteria	Grading
Cell density (chondrocyte proliferation): multiple chondrocytes growing in small rounded groups or clusters, sharply demarcated by a rim of territorial matrix	0=no proliferation 1=increased cell density 2=connection of two chondrocytes 3=small size clones (several chondrocytes grouped together, 3–7 cells) 4=moderate size clones (8–15 cells) 5=huge clones (>15 cells) 6=scar/tissue defects
Structural alterations (tears and clefts): concentric tears following the collagen fiber bundles' orientation in the annulus fibrosus or radiating defects extending from the nucleus pulposus to the outer annulus lamellae parallel or oblique to the end plate (clefts)	0=absent 1=rarely present 2=present in intermediate amounts between 1 and 3 3=abundantly present 4=scar/tissue defects
Granular changes: eosinophilic-staining, amorphous granules within the fibrocartilage matrix	0=absent 1=rarely present 2=present in intermediate amounts, between 1 and 3 3=abundantly present 4=scar/tissue defects
Mucous degeneration: cystic, oval or irregular areas with an intense deposition of acid mucopolysaccharides (i.e. sulfated glycosaminoglycans) staining dark blue with Alc-PAS	0=absent 1=rarely present 2=present in intermediate amounts, between 1 and 3 3=abundantly present 4=scar/tissue defects
Histologic Degeneration Score	0–18 points

plored using the Pearson correlation. Finally, the relationship between expression of MMPs and CD 68-positive cells was explored with a partial correlation procedure controlling for histologic features of disc degeneration (i.e. cell density, structural alterations, mucous degeneration, granular changes) and age. The level of significance was set at $P<0.05$, two tailed.

Results

Histomorphologic alterations

The histomorphologic alterations were analyzed in study group I. While in the fetal and infantile discs almost no signs of degenerative lesions were seen in either the annulus fibrosus or nucleus pulposus, slight focal chondrocyte proliferation and minimal granular changes were seen in adolescents aged between 10 and 15 years. In contrast, the adolescent/young adult group aged between 16 and 30 years revealed significant degenerative alterations as seen by extensive formation of clefts and tears, focal chondrocyte proliferation and significant granular matrix degeneration (Fig. 1a–d). In addition, focal minor mucoid degeneration was noted. This was seen mostly in the nucleus pulposus, but not – or to a much lower extent – in annular tissue. The highest frequency of histopathologic abnormalities was seen in the IVDs of the adult group (31–60 years) and the group of individuals of advanced age (>60 years). In addition to a pronounced development of tears and clefts, huge clones of hypertrophic chondrocytes indicating cell proliferation were found, frequently close to the clefts. Furthermore, granular changes and mucoid matrix degeneration were present to significant extent. These changes affected both the annulus fibrosus and the nucleus pulposus.

Temporo-spatial distribution of MMP expression by immunohistochemistry

In the fetal, infantile and adolescent age group, there was almost no staining for MMP-1 (Fig. 2a), MMP-2, MMP-3 and MMP-9, in either the nucleus or the annulus. In contrast, adolescents aged more than 15 years and young adults revealed a significant level of cellular MMP expression for MMP-1 (Fig. 2b), MMP-2 (Fig. 2c) and MMP-3 (Fig. 2d), but not for MMP-9, which was seen only very occasionally and at low levels in disc cells in both the annulus and the nucleus (Fig. 2e). MMP-1, -2 and -3 positive cells were frequently, but not exclusively, located close to tissue clefts, and cells of chondrocyte clones were also often labeled. The group of adults aged between 31 and 60 years also revealed an extensive

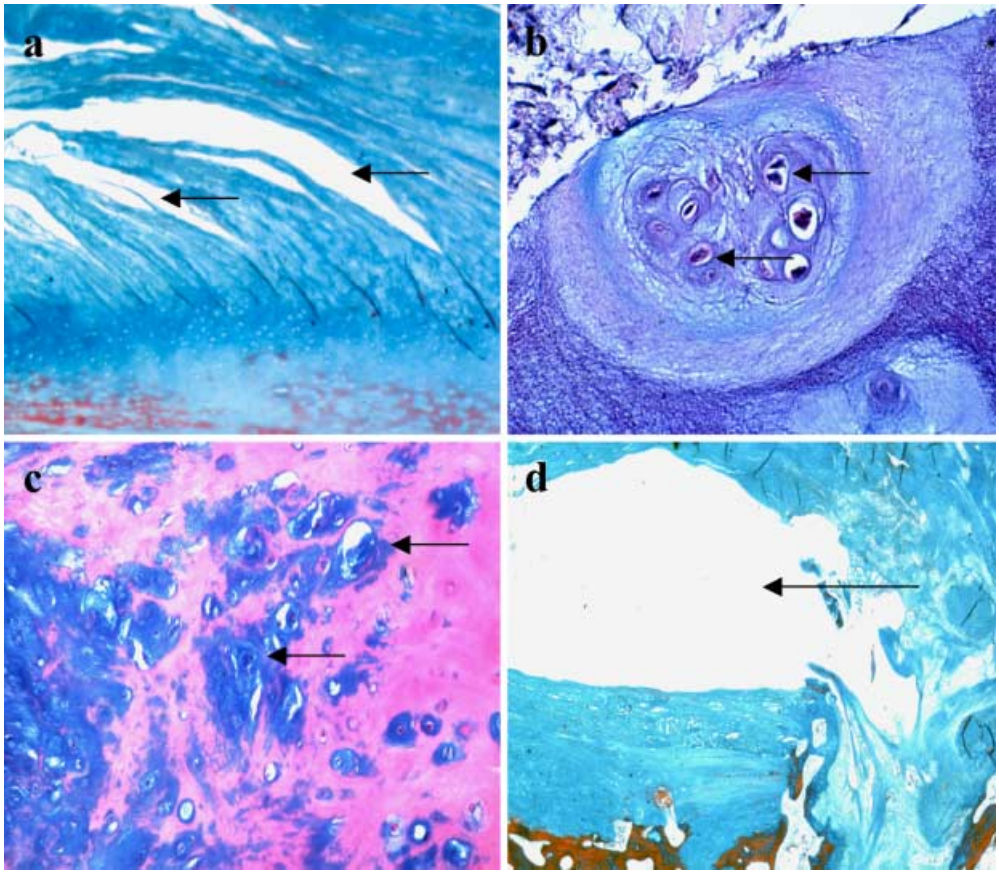

Fig. 1a–d Histomorphologic signs for disc degeneration. **a** The appearance of clefts (*arrows*) within the nucleus pulposus (here at the transition zone to the cartilaginous end plate) suggest tissue disruption (adult, aged 22 years). **b** Focal cell proliferations can manifest as chondrocyte clones (*arrows*) and are an important parameter for disc degeneration (adult, aged 28 years). **c** The occurrence of mucoid material (*arrows*) indicates matrix disarrangement (adult, aged 62 years). **d** More advanced signs reveal significant tear formation (*arrow*), extending from the inner to the outer annulus fibrosus, potentially leading to disc protrusion (adult, aged 28 years). (Original magnification: **a**, **c** ×250; **b** ×400; **d** ×100)

staining of disc cells for these three MMP isotypes. Again, the MMP-9 level was low. In the advanced age group (>60 years), the number of labeled cells was reduced for MMP-1, -2 and -3 (with the exception of MMP-2 in the nucleus pulposus) when compared to young and mature adults. In general, the amount of those MMPs was slightly greater in the nucleus pulposus than in the annulus fibrosus.

Localization of phagocytic cells by immunohistochemistry

In order to localize cells with phagocytic activity (as evidenced by the lysosomal protein CD 68), we immunolocalized a specific CD 68 epitope [32] in the tissue material from study group I. Using this method, we observed no CD 68-positive cells in fetal, infantile or young adolescent discs, but these cells were observed to a considerable extent in older individuals with degenerative disc morphology (those aged between 15 and 86 years). There were no major differences in the number of CD 68-positive cells according to age for those falling within this age group. The positively labeled cells were morphologically not different from disc cells of the nucleus and/or annulus.

In situ expression of MMP-2 and -3-mRNA by in situ hybridization

Due to technical reasons, the expression of mRNA could only be evaluated in sample material from study group II. Almost all surgical specimens in this group exhibited pronounced histologic signs of IVD degeneration with extensive tissue defects caused by large tears or clefts, mucoid degeneration and extensive chondrocyte proliferation. As expected, the scoliosis specimen exhibited substantially fewer alterations, but in none of the cases was a normal disc found. Although the degenerative changes affected both compartments, it seems that the alterations were more pronounced in the nucleus pulposus.

Using non-radioactive in situ hybridization, we detected specifically labeled cells in all samples analyzed, providing evidence for active synthesis of MMP-2 and -3 by various disc cells. The labeling was only within the cytoplasm of the cells (Fig. 3a,c), and all control experiments, in particular the corresponding sense controls, were negative (Fig. 3b,d).

Cells showing positive for MMP-2 and -3-mRNA were essentially the same as those labeled by the respective antibodies (see above). This was verified, in particular, by semi-serial sections stained for MMP-3-mRNA and the

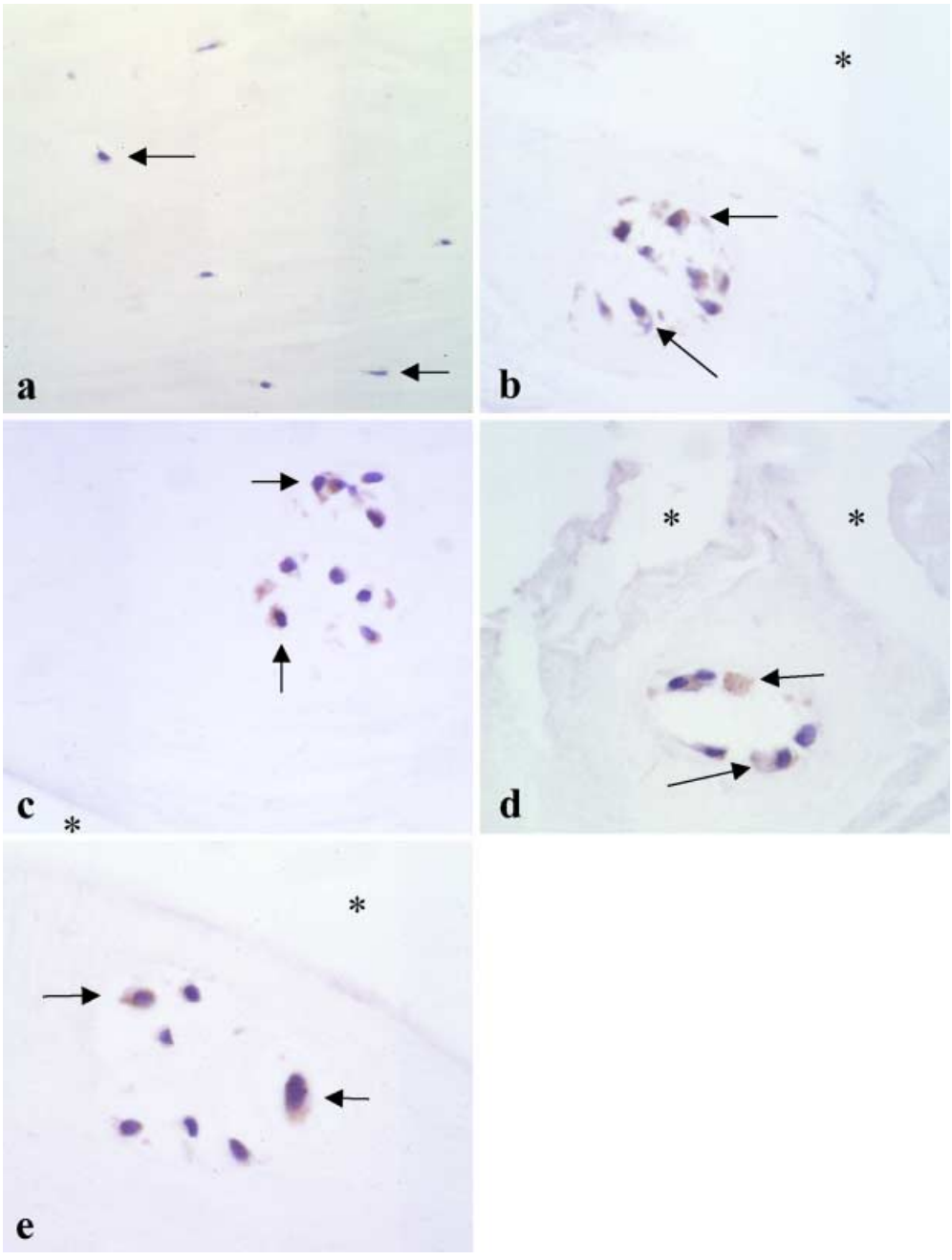

Fig. 2a–e Immunohistochemical features of MMP expression. **a** In this juvenile sample (10 years old) no expression is seen for MMP-1 (*arrows*). **b** In an adult (24 years old) MMP-1 is expressed in numerous cells (*arrows*), there is no extracellular staining. Note the proximity to the clefting of this material (*asterisk*). **c** Similarly, MMP-2 is preferentially, but not exclusively, expressed (*arrows*) close to tissue clefts (*asterisk*) (adult, aged 24 years). **d** An intracellular MMP-3-staining is also seen in a significant proportion of disc cells (*arrows*), frequently close to tissue clefts (*asterisk*) (adult, aged 21 years). **e** In contrast, MMP-9-expressing cells (*arrows*) are seen in only few areas (adult, aged 21 years) (**a**,**b** anti-MMP-1, **c** anti-MMP-2, **d** anti-MMP-3, **e** anti-MMP-9; original magnification **a–e**: ×400)

corresponding protein. These showed a significant number of cells expressing the MMPs close to tissue clefts, and also showed positive reactions among some isolated cells of chondrocyte clusters. The number of nuclear cells with a positive signal was somewhat higher than that of clearly annular cells. There was no significant difference between the two MMPs.

In situ zymography

In the ten samples of autopsy cases, in situ zymography revealed a focal significant gelatinolytic activity, indicating active degrading of the collagenous network by enzymes in those areas. These were mainly seen adjacent, or at least close, to areas with tissue disruption and cleft formation (Fig. 4a). However, similar gelatinolytic activity in areas with cell proliferation without cleft formation was also occasionally seen in young and older adult disc tissue, but not in material from juvenile individuals (Fig. 4b).

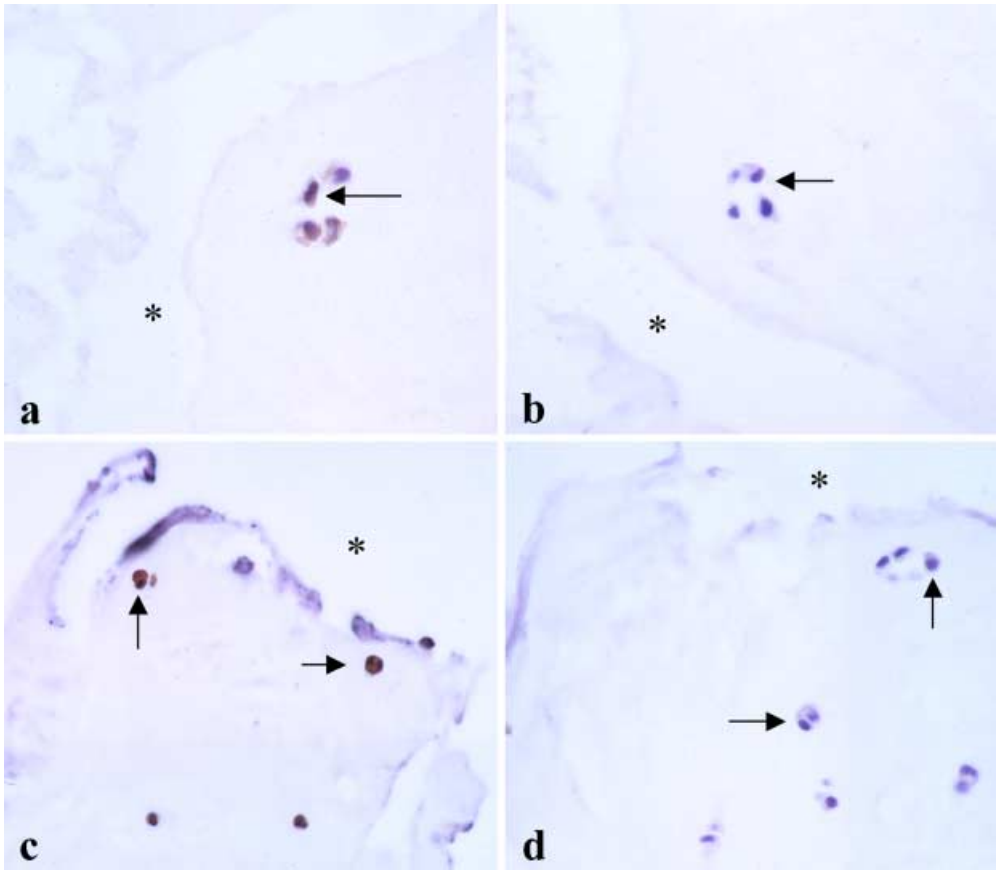

Fig. 3a–d In situ hybridization results in disc biopsy material. **a** Typical cytoplasmic expression of MMP-2-mRNA in disc tissue, with signs of disc degeneration (adult, aged 21 years). The antisense staining shows a specific staining (*arrow*) in cell groups resembling those seen in the immunohistochemistry (MMP-2-antisense). **b** The corresponding sense control reveals a negative result, indicating specificity of the antisense staining (MMP-2-sense control). **c** The localization of MMP-3-mRNA shows a very similar tissue distribution as for MMP-2, with a significant labeling of disc cells (*arrows*) close to clefts (*asterisk*) (adult, aged 21 years). **d** The corresponding sense control again reveals a negative result. (Original magnification **a–d**: ×500)

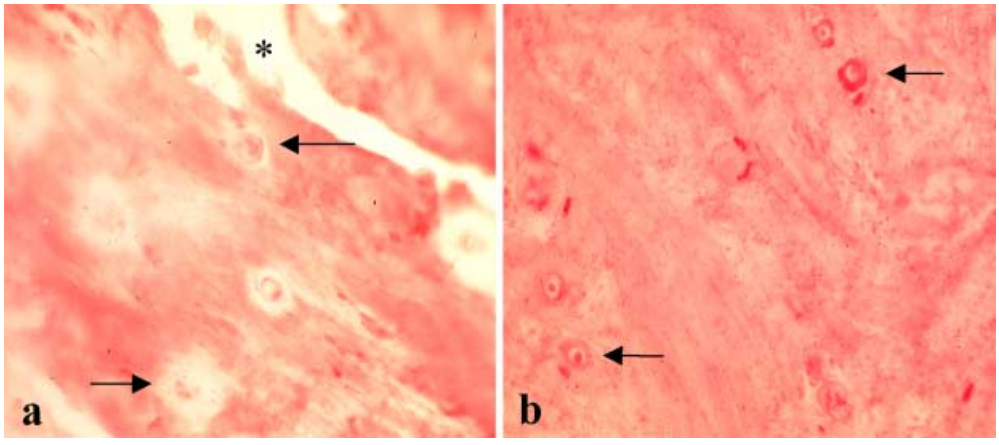

Fig. 4a,b Localization of gelatinolytic activity by in situ zymography. **a** In areas close to nuclear tissue defects and clefts (*asterisk*), the reduction of the ponceau red staining (*arrows*) indicates gelatinolytic activity. Note the large cell clones within the lytic area (adult, aged 52 years). **b** A comparable section adjacent to the nucleus pulposus in a juvenile (aged 12 years) reveals no significant gelatinolytic activity (homogeneous red staining in ponceau red staining) (*arrows*). (Original magnification **a,b**: ×200)

These observations clearly indicate gelatinolytic activity within disc tissue with pathological destruction, particularly cleft formation.

Correlative analyses

The statistical evaluation of the relation between MMP expression and age revealed a highly significant correlation between age and the immunohistochemical expression of MMP-1 ($P<0.002$), MMP-2 ($P<0.0001$) and MMP-9 ($P<0.03$) for nucleus pulposus and of MMP-2 ($P<0.007$) for the annulus fibrosus (Fig. 5a,b). While histologic alterations (HDS) were significantly ($P<0.0001$) more pronounced in the nucleus pulposus, there were no significant differences with regard to the expression of MMP-1 and MMP-9. In contrast, MMP-2 ($P<0.002$) and MMP-3 ($P<0.0001$) exhibited significant differences between nu-

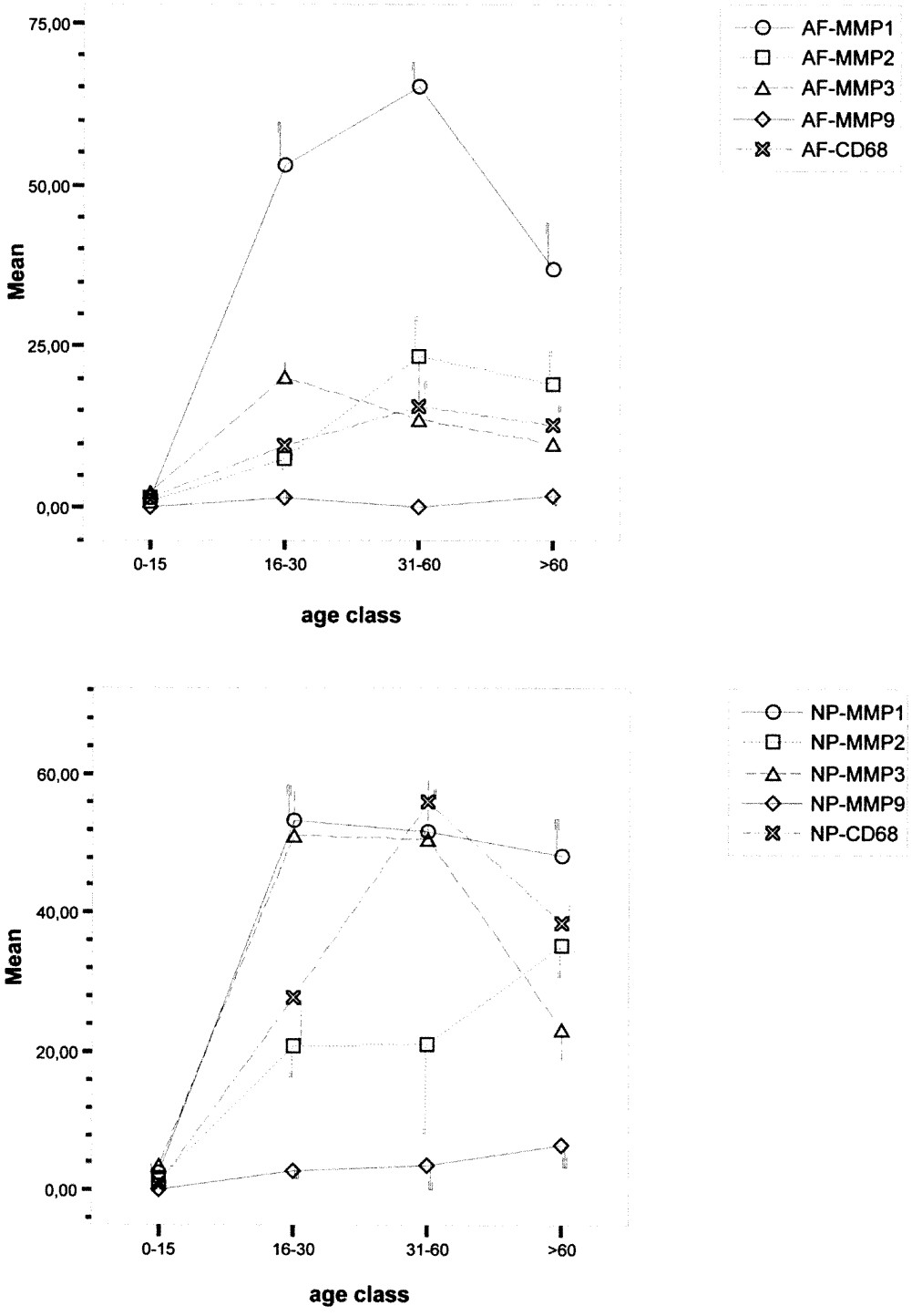

Fig. 5 Graphic demonstration of the association between age and MMP expression (morphometric analysis) in the annulus fibrosus (**a**) and nucleus pulposus (**b**)

cleus and annulus. The disc level did not have any significant influence on the expression of the analyzed MMPs.

The correlation between MMP expression and labeling for CD 68-positive cells with histomorphologic alterations is reported in Table 2. Most obvious is the strong correlation of MMP-1 ($P<0.0001$), MMP-2 ($P<0.002$) and MMP-3 ($P<0.0005$) with the formation of clefts (nucleus pulposus) and MMP-1 ($P<0.0001$) and MMP-2 ($P<0.038$) with the formation of tears (annulus fibrosus). The correlation of MMP expression and CD 68-positive cells with the extent of histomorphologic tissue degradation is illustrated in Fig. 6a,b.

Exploring the relationship of CD 68-positive cells with the expression of MMPs-1, -2, -3, and -9, significant cor-

Table 2 Pearson correlation coefficients and significance levels for the correlation of histologic criteria versus MMP expression and cells with phagozytic activity (CD 68) in study group I (*HDS* histologic degeneration score)

Criteria	MMP-1	MMP-2	MMP-3	MMP-9	CD 68
Nucleus pulposus					
Cleft formation	0.8169 *P*<0.0001	0.5484 *P*<0.0017	0.5933 *P*<0.0005	0.1163 *P*<0.5404	0.6875 *P*<0.0001
Chondrocyte proliferation	0.6756 *P*<0.0001	0.5445 *P*<0.0018	0.3560 *P*<0.0535	0.2494 *P*<0.1838	0.6572 *P*<0.0001
Granular changes	0.6132 *P*<0.0003	0.3287 *P*<0.0760	0.5830 *P*<0.0007	0.2228 *P*<0.2365	0.4479 *P*<0.0130
Mucoid degeneration	0.3626 *P*<0.0488	0.3875 *P*<0.0343	0.1210 *P*<0.5240	0.1263 *P*<0.5059	0.5610 *P*<0.0012
HDS (0–18 pts)	0.7671 *P*<0.0001	0.5750 *P*<0.0008	0.4358 *P*<0.0160	0.2990 *P*<0.1083	0.6732 *P*<0.0001
Annulus fibrosus					
Tears formation	0.6479 *P*<0.0001	0.3818 *P*<0.0373	0.1937 *P*<0.3048	0.3010 *P*<0.1059	0.5114 *P*<0.0038
Chondrocyte proliferation	0.2714 *P*<0.1467	0.3051 *P*<0.1010	–0.0197 *P*<0.9175	0.1640 *P*<0.4722	0.3590 *P*<0.0513
Granular changes	0.6728 *P*<0.0001	0.2683 *P*<0.1515	0.4062 *P*<0.0258	0.2445 *P*<0.1928	0.2991 *P*<0.0499
Mucoid degeneration	0.3276 *P*<0.0771	0.3681 *P*<0.0453	0.1229 *P*<0.5173	0.1814 *P*<0.3372	0.29918 *P*<0.1082
HDS (0–18 pts)	0.5524 *P*<0.0015	0.4268 *P*<0.0186	0.1324 *P*<0.4853	0.2302 *P*<0.2209	0.5168 *P*<0.0034

relations with all MMPs in the nucleus and MMPs-1,-2, and -3 in the annulus were found. However, the correlation between CD 68 and MMP-9 did not quite achieve statistical significance ($P<0.06$) in the annulus. When the correlation was controlled for histologic alterations (i.e. cell density, structural alterations, mucoid degeneration, granular changes) and age, a significant correlation was demonstrated for MMP-1 ($P<0.002$) and MMP-3 ($P<0.0001$) for the nucleus and MMP-1 ($P<0.02$), MMP-3 ($P<0.04$) and MMP-9 ($P<0.001$) for the annulus. This indicates a direct effect of the respective MMPs on the cells, independent of histomorphologic degeneration and age.

Discussion

While the macroscopic and histologic features of disc degeneration are well described in a phenomenological manner [9, 10, 11], the understanding of the underlying molecular mechanisms of disc aging and/or degeneration is still limited. Previous studies demonstrated a loss of the collagenous meshwork, an increasing replacement by hyaline collagen fibers and the occurrence of fissures within the disc tissue as major histopathologic features associated with disc degeneration. These features increase with age. Similar observations have been made by others [5, 7]. In a previous study, we demonstrated that these changes occur as early as in adolescence [33]. We provide circumstantial evidence that the proliferation of discal chondrocytes, the formation of clefts and tears and both mucoid and granular matrix degeneration are statistically associated with aging and/or disc degeneration. These data served as a basis in order to establish a novel grading system semiquantitatively assessing the extent of histologic tissue degradation [4].

Within the IVD, the normal nucleus pulposus is mainly composed of collagen type II (together with collagen types VI, IX and XI), while the annulus fibrosus contains large amounts of collagen type I with other “minor” collagen types intermingled (mainly collagens III, V, VI) [33]. The interaction of these collagens and their integrity appear essential for the mechanical function of the disc. These collagen molecules can be degraded only by specific enzymes, particularly the various matrix metalloproteinases (MMPs), which are the main players in the catabolism of collagen molecules. Within the various MMPs, there exist significant differences in substrate specificity, so that collagen molecules are usually degraded by a concerted action of distinct MMPs.

The MMPs can be divided into four groups of enzymes [13] on the basis of the substrate specificity:

- The *collagenases* (MMPs-1, -8, -13 and -18) are the only enzymes that can cleave intact interstitial collagen molecules.

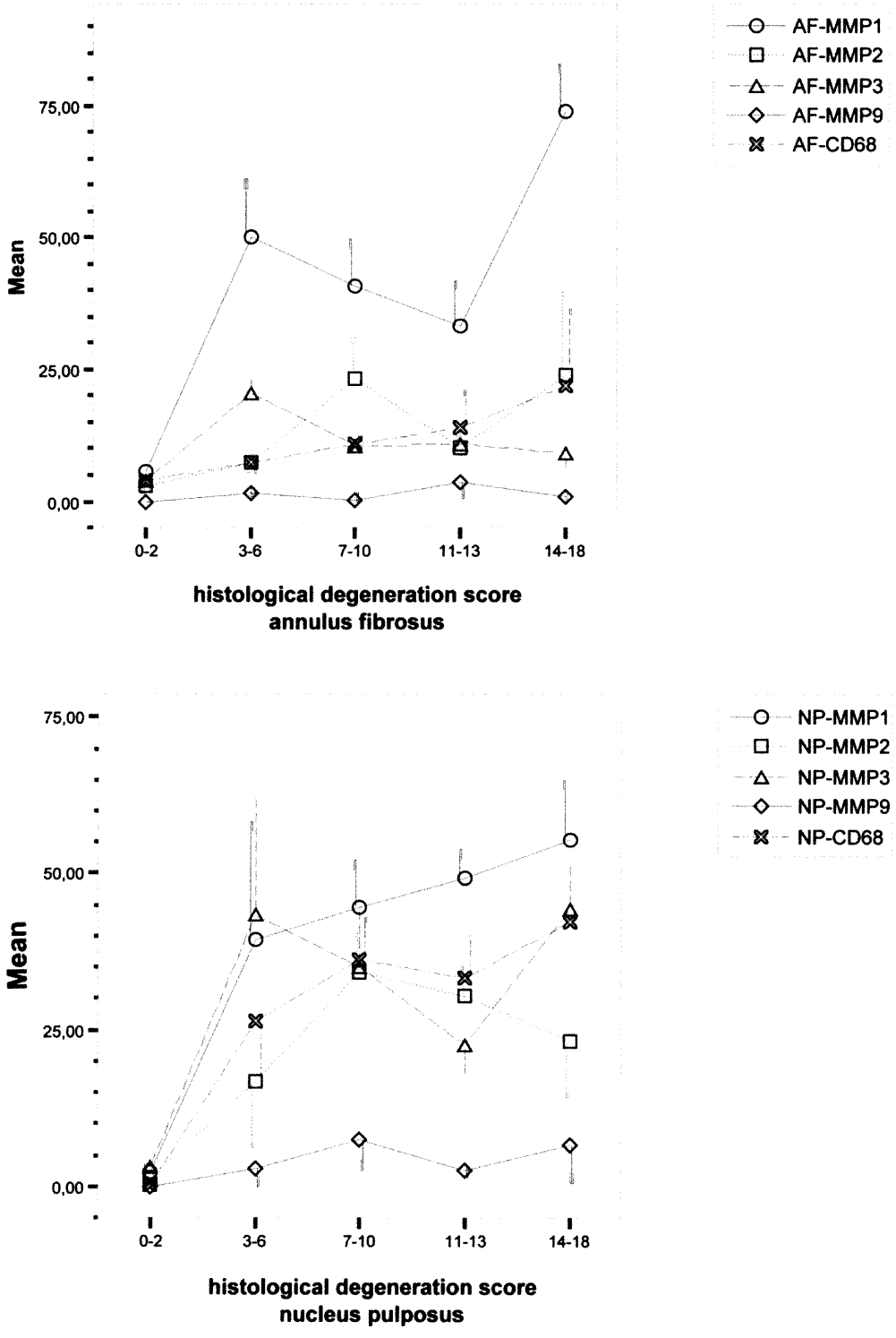

Fig. 6 Graphic demonstration of the correlation between the histologic grade of disc degeneration and MMP expression in the annulus fibrosus (**a**) and nucleus pulposus (**b**)

- The *gelatinases* (MMPs-2 and -9) degrade denatured collagen molecules and basement membrane collagens.
- The *stromelysins* (MMPs-3, -10 and -11) digest non-collagenous matrix proteins and also denatured collagen molecules.
- The MMPs-14, -16, -17 and -18 are known as the *membrane-type MMPs*. They are responsible for the activation of other MMPs, but only play a secondary role in direct matrix degradation.

MMP-1, as the most abundant enzyme in the first group, has been identified mainly in fibroblasts, chondrocytes, histiocytes and macrophages. The other MMPs of the collagenases group also cleave intact interstitial collagen molecules; however, these enzymes are restricted to a few, specialized cell types. For instance, MMP-8 ("neutrophil collagenase") is present only in neutrophil granulocytes. Both gelatinases, MMP-2 and -9, are mainly involved in the destruction of basement membrane collagen, but they can also cleave denatured collagen molecules, such as the fragments of interstitial collagen following MMP-1 digestion. MMP-3, as the main stromelysin, is engaged in the destruction of a multitude of non-collagenous proteins, including proteoglycans, but can also degrade denatured collagen molecules. Therefore, this enzyme is of significance for the destruction of the intermolecular network between collagen fibrils.

These data indicate that any change in the occurrence and/or activity of those enzymes may significantly alter the composition and integrity of connective tissues. Up to now, only a few studies have investigated the presence and activity of collagenolytic enzymes in IVD, mostly by biochemical methods. Crean et al. [12], Goupille et al. [13], Kang et al. [25, 26, 27], Liu et al. [29, 30], and Matsui et al. [31] reported on an increase of MMP activity in disc material of various clinical settings, mainly on herniated disc material. These studies used biochemical techniques on tissue homogenates, so that no information was available on the tissue localization of enzymatic activity, and, in particular, no data indicated which cells were involved in proteolysis. Furthermore, the MMP levels in these observations may have been influenced by inflammatory cells, which are frequently present at least in herniated discs [14, 16, 17, 18, 19, 40]. None of these studies localized the MMP-producing cells. In addition, only isolated groups of the enzymes, such as the gelatinases or single stromelysins, were analyzed in most of the reports. Therefore, these studies provided only limited information on the role of MMPs during disc degeneration.

So far, the only study on this issue using immunohistochemical techniques is one that was recently presented by Roberts et al. [39]. They also found an increase in the presence of various MMPs, including MMP-isoforms tested in our study. In addition, Roberts et al. [39] used in situ zymography, which also showed enhanced enzymatic activity within disc material. In this respect, we can confirm that major MMPs are expressed at elevated levels in disc material in conjunction with tissue degradation. Our analyses, however, go far beyond these previous studies, because we localized the MMP pattern within the various histoanatomic regions. Our study is also the first to correlate the expression pattern for MMPs with the histomorphologic signs for disc degeneration. Furthermore, we also first demonstrate a correlation between discal MMP expression and age. In this way, we provide clear evidence that the aging disc contains more MMP-producing cells and may therefore show a higher breakdown of collagenous matrix molecules. This accords well with the loss of disc tissue with advancing age.

Our results have been obtained mainly by use of immunohistochemistry. However, they were confirmed by in situ hybridization, which was performed on biopsy material (study group II). This is necessary due to technical reasons, because mRNA is not stable in autopsy samples, where it can rapidly be degraded. By two completely different and independent techniques, our approach clearly confirms that local discal cells are involved in the synthesis of MMPs. In general, this rules out any potential influx of enzymes into the disc, such as from foci of peridiscal inflammation.

Furthermore, the presence of gelatinolytic activity is clearly confirmed by in situ zymography. This technique [23] uses gelatin as a substrate to localize matrix degrading tissue components. The staining of the gelatin after the incubation period highlights those areas where the enzymatic activity has reduced the gelatin underneath the tissue section. In our study, we clearly observed the reduction of gelatin staining in areas close to tissue clefts, confirming gelatinolytic activity in those zones.

In addition to these multiple pieces of evidence that disc tissue contains enzymatically active MMPs, we present here circumstantial evidence that the investigated MMPs are involved in the formation of tissue clefts and in the resorption of disc material. This is deduced from the close (and statistically highly significant) association between the occurrence of MMPs and the presence of histomorphologic signs for disc resorption. This correlation is seen best when the different age groups are compared. The infantile, juvenile and young adolescent discs show neither significant MMP expression nor histologic disc degeneration. In this regard, we recently identified a major association between any loss of disc vascularization and the appearance of signs of degeneration [4]. Our present study is a clear extension of these observations, since it links the onset of disc degeneration with the up-regulation of major MMPs.

In addition, we provide evidence that the cells synthesizing those MMPs undergo significant phenotypic changes. Thus, we were able to identify discal cells expressing the lysosomal marker CD 68 [34]. Since both cellular morphology and tissue distribution argue for local but not for invaded, cells, we suggest that local cells undergo a phenotypic switch leading to expression and activation of MMPs [1, 8, 14, 16, 17, 19, 24, 31, 37, 39]. This is possibly induced by a reduction of nutrients, such as oxygen, when the diffusion paths become too long to allow easy support for local cells [29]. This seems to occur early in adolescence or even during childhood, and may be enhanced by other factors, such as mechanical overload. The liberation and activation of MMPs finally leads to a destruction of the discal collagenous framework, resulting in tissue destruction.

Interestingly, not all MMPs analyzed seem to be involved in the process of tissue destruction. Our analysis reveals a close association between the expression of MMPs-1 and MMP-2 and discal changes both in the nucleus and annulus. The extent of MMP-3-expression correlates with nuclear changes, but not with those of the annulus, while MMP-9 is expressed only at a low level, and therefore does not seem to play a significant and immediate role in disc matrix degradation. These differences between the enzymes are also reflected in comparisons of the changes in the various age groups, where MMPs-1 and -2 also seem to be more relevant than MMPs-3 and -9. Our study presents the first data on temporo-spatial variations in MMP expression and their potential role in disc destruction. These associations are further corroborated by a parallel increase in the number of CD 68-positive cells representing phagocytic cells.

While the association between MMP expression and histomorphologic degeneration is evident from our data, we may also speculate on further effects of the MMP induction and tissue destruction. Recently, Haro et al. [20, 21] reported on the enhanced release of MMP-3 and -7 from herniated disc material when co-cultured with macrophages. Interestingly, the induction of MMP-3 led to the attraction of macrophages, and that of MMP-7 to liberation of TNF-α, which is a potent proinflammatory cytokine and which may be associated with inflammation and potential pain induction. Thereby, a close interaction between MMP expression and cellular differentiation may also be effective in non-herniated disc material, as suggested by our observations. The presence of local disc cells with phagocytic properties (CD 68) and enhanced MMP expression in our study indicates that not only the generation of clefts and tears, but also changes in the discal cell differentiation may occur as a consequence of enhanced MMP expression. Furthermore, the formation of clefts and tears may significantly enhance the transport of cytokines (or other transmitter substances) that may be involved in pain induction.

Clinical relevance

This is the first study linking the expression of major MMPs to histomorphologic features of disc matrix degradation. Well in line with our previous findings of an obvious association between the age-related imbalance between vascular supply and the beginning of disc degeneration in early adolescence, this study links these features to enhanced production and activation of MMPs. Therefore, our study supports the notion that disc degeneration starts in childhood and early adolescence.

The occurrence of extensive intradiscal clefts and tears renders the spread of transmitter substances within the disc much easier and more rapid, so that potentially pain-inducing cytokines may become effective in peridiscal pain perception. The knowledge of the close association between MMP expression and cleft/tear formation therefore does not simply further our understanding of biomechanical disc alterations, but may offer new insight into the generation and perpetuation of discogenic pain. The increasing knowledge about the molecular mechanisms of tissue degradation may simultaneously offer new treatment avenues, such as intradiscal pain therapy targeting the release of nociceptive cytokines. However, further investigations are required to uncover the molecular events that precede MMP synthesis and activation and the release of pain-related cytokines.

Acknowledgements The present study was supported by a grant from the SynOs-Foundation, Switzerland.

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