Herpes virus infection can cause intervertebral disc degeneration
A CAUSAL RELATIONSHIP?

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It has been proposed that intervertebral disc degeneration might be caused by low-grade infection. The purpose of the present study was to assess the incidence of herpes viruses in intervertebral disc specimens from patients with lumbar disc herniation. A polymerase chain reaction based assay was applied to screen for the DNA of eight different herpes viruses in 16 patients and two controls. DNA of at least one herpes virus was detected in 13 specimens (81.25%). Herpes Simplex Virus type-1 (HSV-1) was the most frequently detected virus (56.25%), followed by Cytomegalovirus (CMV) (37.5%). In two patients, co-infection by both HSV-1 and CMV was detected. All samples, including the control specimens, were negative for Herpes Simplex Virus type-2, Varicella Zoster Virus, Epstein Barr Virus, Human Herpes Viruses 6, 7 and 8. The absence of an acute infection was confirmed both at the serological and mRNA level.

To our knowledge this is the first unequivocal evidence of the presence of herpes virus DNA in intervertebral disc specimens of patients with lumbar disc herniation suggesting the potential role of herpes viruses as a contributing factor to the pathogenesis of degenerative disc disease.

Disc degeneration is a common condition with a complex multifactorial aetiology. Low-grade infection has been proposed as a cause of disc degeneration with Propionibacterium acne identified in 84% of patients with disc herniation and sciatica using serology and cultures. Molecular changes have been also observed such as the increased production of catabolic enzymes (cathepsin, lysozyme and several matrix metalloproteinases) and inflammatory cytokines. Young patients with lumbar disc herniation showed poorer recovery after surgery when they had displayed elevated serum concentrations of high-sensitivity C-reactive protein (hs-CRP) pre-operatively. However, it is not clear whether high levels of CRP and inflammatory cytokines indicate a local infection or an inflammatory response to disc herniation.

Pathogens such as Parvo virus B19 or Epstein Barr Virus (EBV) play an important role in the pathogenesis of various forms of arthritis. Herpes viruses are a diverse family of large DNA viruses, all of which can establish lifelong latent infections. Primary infection with many of these viruses is common during childhood.

This study aimed to investigate the potential role of herpes viruses, Herpes Simplex Virus type-1 (HSV-1), Herpes Simplex Virus type-2 (HSV-2) and Cytomegalovirus (CMV), in the pathogenesis of intervertebral disc degeneration. A molecular analysis of intervertebral disc specimens obtained during discectomy showed a considerable frequency of CMV and HSV-1 DNA in degenerated discs, suggesting that viral infections might be an important factor in the aetiology of this disease.

Patients and Methods
A total of 16 consecutive patients who underwent discectomy within six months of lumbar disc herniation were studied. There were eight men and eight women with a mean age of 40.38 years (17 to 65). The study had ethical approval. The diagnosis of lumbar disc herniation was established by physical examination and MRI. In order to provide a control group, discs samples were obtained percutaneously from two patients with thoracolumbar burst fractures who were undergoing operation. These patients also underwent MRI examination pre-operatively to exclude concomitant degeneration. The nature of the samples tested in this study did not allow the inclusion of more intervertebral discs from healthy individuals for ethical reasons, while the alternative of cadaveric specimens as controls, was abandoned as the majority of the adult population has a history of low back pain and disc degeneration.
In addition to the material from the herniated disk obtained during discectomy a peripheral blood sample was taken from each patient. Tissue samples were placed in sterilised polypropylene 1.5 ml tubes and stored at -80°C, until DNA/RNA extraction and Polymerase Chain Reaction/quantitative Polymerase Chain Reaction (PCR/qRT-PCR) were performed. The detection of HSV-1 and CMV, IgM+ and IgG+ antibodies from the plasma was carried out using a standard enzyme immunoassay.

**DNA/RNA extraction.** Genomic DNA and total RNA were extracted from the tissue samples using the NucleoSpin Tissue XS kit and NucleoSpin RNA XS kit (Macherey-Nagel GmbH and Co., Duren, Germany), respectively, according to the manufacturer’s instructions. The purified RNA was further treated with DnaseI to avoid any DNA contamination. DNA and RNA sample concentrations were determined using a spectrophotometer (260 nm) prior to PCR amplification or qRT-PCR.

**Detection of herpes viruses DNA.** We tested eight different herpes viruses (HSV-1/-2, Varicella Zoster Virus (VZV), EBV, CMV, Human Herpes Viruses 6, 7 and 8 (HHV6, HHV7 and HHV8)) using RhyMA Test-Herpes Screening according to the manufacturer’s instructions (Euroclone, Pavia, Italy). The RhyMA test was based on the Reverse Hybridisation technology involving multiplex DNA amplification by PCR with specific biotinylated primers, followed by the hybridisation of the amplified and biotinylated DNA fragments with strips on probes, specific for each one of the herpes virus. A final developing reaction using a biotin-streptavidine system was required for the evaluation of the results. Positive and amplification controls were also included in each strip. For the interpretation of the results, the developed strip of each sample was aligned with a standard table. The RhyMA Test-Herpes Screening is licensed for *in vitro* diagnostic use, certifying the high specificity and sensitivity of the assay. In order to verify the results obtained using the RhyMA Test-Herpes screening assay, the PCR products were also run in a 3% low-melting agarose gel producing the expected electrophoretic pattern for each virus. Finally, the PCR-positive samples were subjected to direct sequencing analysis, confirming the results obtained using the RhyMA Test-Herpes screening assay.

**HSV-1 and CMV qRT-PCR.** Total RNA was reverse transcribed using the RETRO-script kit (Ambion Inc., Austin, Texas, USA) with random hexamer primers. cDNAs were tested for template quality by constructing standard curves using serial dilutions of each cDNA as templates in qRT-PCRs. The cDNA templates gave an assay efficiency of 90% to 105%, and the R² of all standard curves was > 0.98. Following reverse transcription, cDNAs were amplified using ICP0- and IE1-specific primers for the quantification of HSV-1 and HCMV mRNAs, respectively, as previously described.9,10 All qRT-PCRs were performed in triplicate using Maxima SYBR Green qPCR master mix (Fermentas Inc., Glen Burnie, Maryland) in an ABI PRISM 7500 Sequence Detector (PE Biosystems, Foster City, Canada), including the extracted RNA samples as well as positive and negative controls. SYBR Green fluorescence was measured over the course of 40 amplification cycles. For each template quantified, the mean cycle number at which product accumulation entered the linear range (C_T) was calculated. Replicate C_T values were within 0.4 cycles of each other. The 18S rRNA C_T value for a given template was subtracted from the C_T obtained for each mRNA of interest (ICP0 and IE1) from the same template to obtain the normalised C_T value (ΔC_T) for each template.

**Tumour Necrosis Factor-α and Interleukin-6 gene expression.** For quantification of TNF-α or IL-6 mRNA, 1 μl of cDNA was used together with the primers shown above in a 20 μl reaction, using SYBR green as a marker for DNA content. The primers used were: beta actin: Forward: 5’-TCAGAAGAAGCT CTA TGT GG-3’, Reverse: 5’-TCT CTT TGA TGT CAC GCA CG-3’; TNF-a: Forward: 5’-CAC GCT CTT CTC TCT ACT GAA CTT CG-3’, Reverse: 5’-GCC TGG GTA GAT GAA TGA TGA ACA CC-3’; IL-6: Forward: 5’-ACA GCC ACT CAC CTC TTC AG -3’, Reverse: 5’-GTG CCT TTT GTC TGC TTT CAC -3’. No byproducts were present in the reaction as indicated by the dissociation pattern provided at the end of the reaction and by agarose gel electrophoresis (data not shown). The amplification efficiency of the TNF-α and the IL-6 products were the same as that of beta actin as indicated by the standard curves of amplification, allowing us to use the formula: fold increase = 2^Δ(C_T_A-ΔC_T_B). Reactions were performed in triplicate to allow statistical evaluation.

**Results**

A PCR–reverse hybridisation assay was employed to screen for the DNA of eight different herpes viruses in the intervertebral disc specimens of patients with disc herniation (Fig. 1). Herpesvirus DNA was detected in 13 out of 16 specimens (81.25%). HSV-1 presented the highest prevalence in nine patients (56.25%), whereas CMV was detected in six patients (37.5%) (Table I). Two patients were co-infected with both HSV-1 and CMV. DNA from HSV-2, VZV, EBV, HHV6, HHV7 and HHV8 was not detected in the disc samples of any of the patients. Neither of the control patients (C1 and C2) tested positive for the presence of herpesvirus DNA (Fig. 1).

The status of HSV-1 and CMV infection, in the intervertebral disc specimens was examined by investigating the potential expression of viral transcripts by Real-Time qRT-PCR. Herpesvirus gene expression was not detected in any of the samples tested, demonstrating the absence of an active viral infection, thereby suggesting the establishment of latent herpesvirus infection in these specimens.

The sero-positivity of patients was also examined. All HSV-1 or CMV PCR–positive patients presented IgG+ antibodies for the same virus, respectively (Table I). In three cases for HSV-1 and in six cases for CMV, IgG+ antibodies...
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were detected with no simultaneous prevalence of viral DNA. Furthermore, the HSV-1 and CMV IgM+ antibodies from the peripheral blood were negative in all patients, indicating that none of the patients had experienced an acute herpes virus infection at the time of surgical excision.

Apart from the macroscopic indication of inflammation surrounding the herniated disc, we also obtained experimental evidence of inflammation in the intervertebral disc specimens by examining the mRNA levels of TNF-α and IL-6, in all the samples using qRT-PCR. The analysis showed approximately two- to threefold increase of both inflammatory markers in the samples compared with the controls (Fig. 2). Interestingly, the patients who were co-infected by both HSV-1 and CMV displayed the highest levels of TNF-α and IL-6. Furthermore, two out of three patients (patients 2 and 8) with no evidence of viral infection exhibited lower levels of TNF-α. This negative correlation was not found for IL-6 levels in these patients.

Discussion

The present study suggests the concept that herpes virus infection might promote disc degeneration. We were able to detect HSV-1 and CMV DNA in a large number of human intervertebral disc samples harvested during discectomy. Earlier low-grade infection has been suggested as a possible cause of disc degeneration. Several authors have emphasised the relationship between Modic type 1 signals (increased marrow signal intensity in T2-weighted MR images and decreased signal intensity in T1-weighted MR images and fibrovascular tissue in the endplates) and infection. If the patients do not have severe back pain, temperature, or an abnormal blood profile it is difficult to distinguish between Modic 1 changes and low-grade infection.

The relationship between viral infection and apoptosis has been widely investigated, clarifying the mechanisms of apoptosis induction by viruses. It is now known that Parvovirus B19 and particularly its NS-1 protein, has a potent cytotoxic effect on the host cells and causes apoptosis in the infected cells. Viruses play a role in the pathogenesis of various forms of arthritis. Stahl et al detected viral DNA from multiple viruses in synovial tissue taken from patients with early arthritis. A recent study suggested that the advanced stages of osteoarthritis may be related to increased inflammation and damage to articular cartilage due to Parvovirus B19. In another study, it was proposed that human Parvovirus B19 is involved in the initiation and perpetuation of synovitis in rheumatoid arthritis, leading to

Table I. Clinical characteristics of the patients with lumbar disc herniation and prevalence of Herpes Simplex Virus type-1 (HSV-1) and Cytomegalovirus (CMV) in intervertebral disc specimens

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
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<th>CMV</th>
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Joint lesions in the course of excessive synthesis of inflammatory cytokines and proteolytic enzymes.\(^{19}\)

HSV-1 infections are endemic throughout the world. Over 90% of adults have antibodies to HSV-1 by the fifth decade. Herpes viruses are ubiquitous pathogens in children, remaining latent after an active infection.\(^{20,21}\) During the initial phase of the infection, viral replication occurs in ganglia with survival of some cells which maintain viral genome compatible with normal cellular function. This fine balance between host cells and the virus can be disturbed by various stimuli, leading to reactivation.\(^{22}\)

In our study, none of the patients had experienced acute herpes infection. This was evident both at the serological level, due to absence of IgM+ antibodies from the peripheral blood, as well as at the molecular level due to the lack of viral mRNA transcripts from the disc samples. Thus, the high prevalence of HSV-1 infection in childhood, as well as its retention in the nervous system, might offer a satisfactory explanation of the presence of both HSV-1 and CMV in the intervertebral disc.

The question arises how the viruses enter the intervertebral disc space. It is well established that large vascular channels cross the end plates during early foetal life, which subsequently decrease with birth and entirely disappear by the age of four to six years old, making the disc one of the largest avascular tissue types in the body.\(^{23}\) One might speculate that the presence of viral DNA in the intervertebral disc is secondary to the migration of macrophages or other cells that contain viral DNA into the disc during childhood while the disc environment is still rich in blood vessels. Other mechanisms might also play a part as it is well known that the herpes simplex virus can be activated in neuronal axons by inflammatory cytokines, such as IL-1 and TNF-\(\alpha\)\(^{24,25}\) and through antidromal migration might involve the disc space.

Thus, the inflamed or injured disc, which produces a variety of pro-inflammatory cytokines, activates latent viruses. These mechanisms could also explain the presence of DNA from two or more viruses in the disc samples. Even if the presence of viruses is a secondary phenomenon, their

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**Fig. 2**

Histograms showing mRNA-expression levels of TNF-\(\alpha\) and IL-6 in intervertebral disc specimens of patients with lumbar disc herniation (patients 1 to 16) and control cases (C1 and C2). The transcription levels of TNF-\(\alpha\) and IL-6 were normalised using beta actin as a reference gene. The bars represent mean values and the error bars show the standard error of each sample.
presence may contribute to the pathogenesis of intervertebral disc degeneration since viral antigens further enhance disc deterioration by activation of the inflammatory milieu which facilitates activated macrophages and other cells to cause tissue damage.

In our study, intra-operative assessment of the surgical field revealed macroscopic evidence of inflammation surrounding the herniated disc. The evidence of the inflammation in the disc specimens and the contribution of viral infection to the enhancement of the inflammatory process came from the analysis of the mRNA levels of well-known genes, which are associated with inflammation, such as TNF-α and IL-6. Both inflammatory markers demonstrated approximately two to three fold higher levels in the patients’ samples as compared with the controls. Interestingly the samples that were co-infected by both HSV-1 and CMV exhibited the highest levels of TNF-α and IL-6, suggesting a synergistic effect of the viruses. Additionally, the levels of TNF-α were as low as the control cases among the patients where we could not detect viral DNA.

A recent review article states that disc degeneration is not a diagnosis but an expression of the state of the disc, which is the result of several factors acting, individually or collectively.1 Rather than being the result of a single process, disc degeneration can have a number of possible causes including mechanical, ageing, genetic, systemic, toxic and/or infection. The absence of viral detection in all of our 16 patients is in accord with the multifactorial causation of disc prolapse. It is known that genetic factors produce abnormal components of the matrix,26-28 which compromise the structure and function of the disc and increase the susceptibility of the disc tissue to mechanical stress.29 Epidemiological studies have shown that genetic factors increase the risk of degeneration,30-33 but they do not account for all cases,28,36 with variations across different ethnic populations37 such that large multi-ethnic population studies are required.38

The presence of the viruses in the disc might create a setting where the disc is vulnerable to damage by mechanical stress and trauma. It is likely that viral DNA would be present at intervertebral disc levels but the mechanical stresses that are postulated to contribute to the degeneration take place at the lumbosacral level where problems are far more common.1 Low-grade infection, with or without genetic influence, might increase the susceptibility of the intervertebral disc to environmental factors, which secondarily drive the biological events resulting in disc degeneration. Possibly herpes DNA acts as a factor that alters the structural characteristics of the matrix in the disc by modulating apoptosis and local inflammatory response, suggesting that viral infection or reactivation is associated with the disease.

The discovery by Marshall and Warren39 that infection by Helicobacter pylori was responsible for the development of gastric ulcers lead to a major shift in medical understanding. The possibility that an infectious process may contribute to disc degeneration should be considered seriously.

We believe this is the first study documenting the presence of herpes viruses in the intervertebral disc of patients with disc herniation. Further studies are necessary to determine the underlying mechanisms and support the potential role of herpes viruses in the pathogenesis of degenerative disc disease.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


