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Herpes simplex virus type-1 infection affects the expression of extracellular matrix components in human nucleus pulposus cells

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ABSTRACT

Intervertebral disc (IVD) degeneration has a complex multifactorial origin and it is tightly associated with changes in the secretion of proteoglycans and collagen of the Nucleus Pulposus (NP) extracellular matrix. Chronic infection by Herpes virus has been previously associated with disc degeneration after detection of Herpes Simplex Virus type-1 (HSV-1) and CMV DNA in human excised disc samples. The aim of the present study was to assess the effect of HSV-1 infection on proteoglycan synthesis employing human Nucleus Pulposus (HNPCs) cells as a model of intervertebral disc degeneration. During lytic HSV-1 infection, a significant reduction of Decorin expression was observed 8 h post infection (h.p.i) which furthered deteriorated at 24 h.p.i. Biglycan was also reduced but only 24 h.p.i. Collagen type II, although demonstrated a downward trend, it was not statistically significant, whereas both Versican and Aggrecan showed a substantial decrease at 24 h.p.i. Hyaluronan production was not significantly affected. In a non-productive HSV-1 infection, a substantial reduction of Decorin, Biglycan, Versican and Aggrecan expression was found, similarly to our findings from the lytic infection. Furthermore, collagen type II expression was completely abolished. HAS1 expression was not affected, whereas HAS 2 and 3 were found to be significantly reduced. These results indicate that HSV-1 infection of human NP cells yields a complex effect on host extracellular cell function. The viral-induced changes in proteoglycan and collagen type II concentration may affect cell-matrix interactions and lead to a dysfunctional intervertebral disc which may trigger or promote the degeneration process.

1. Introduction

Intervertebral disc (IVD) degeneration (Fig. 1) is a complex phenomenon characterized by a cascade of biochemical and cellular changes in the disc micro-environment. The extracellular matrix (ECM) of the IVD consists mainly of collagens, proteoglycans and glycosaminoglycans (Sivan et al., 2014). Minority constituents in the IVD are elastin, lipids, matrix remodeling enzymes and glycoproteins. The main types of collagen in the human IVD are I and II, dominating the IVD periphery (AF) and the inner core, respectively (Eyre and Muir, 1977). Collagens constituents form complex compartment-like structures providing tensile strength and enclosing hydrated proteoglycan aggregates which are responsible for resistance to compressive forces and the ability to undergo reversible deformation. The main proteoglycan constituents of the IVD are the large proteoglycans Aggrecan and Versican. Their main characteristic is the ability to form large hydrophilic structures through their ability to bind multiple hyaluronan chains. (Oegema et al., 1979). Aggrecan and Versican are responsible for much of the water-retaining properties of the disc, mediated by its glycosaminoglycan components. Other non-aggregating PGs found in the IVD are the family of small leucine rich proteoglycans (SLRPs) including biglycan, decorin, fibromodulin, keratocan and lumican as well as other matrix PGs, such as perlecan (DiFabio et al., 1987). The SLRPs are very important for the formation of collagen fibrils, growth factor activity regulation and storage (Iozzo, 1997). SLRPs possess protein interacting domains which enable them to affect signaling molecules controlling cell proliferation, differentiation as well as matrix synthesis and remodeling (Iozzo, 1997).

The synthesis and remodeling of the matrix proteoglycans is a dynamic process, which preserves the physiological structure and function of the intervertebral disc. Excessive decline of proteoglycan content, either due to reduced production or increased decay, affects the elasticity and the biomechanical properties of the intervertebral disc. (Roughley et al., 2002). A key element of early disc degeneration is the decrease of nucleus pulposus (NP) proteoglycan content. NP cells are responsible for the initial production and subsequent maintenance of

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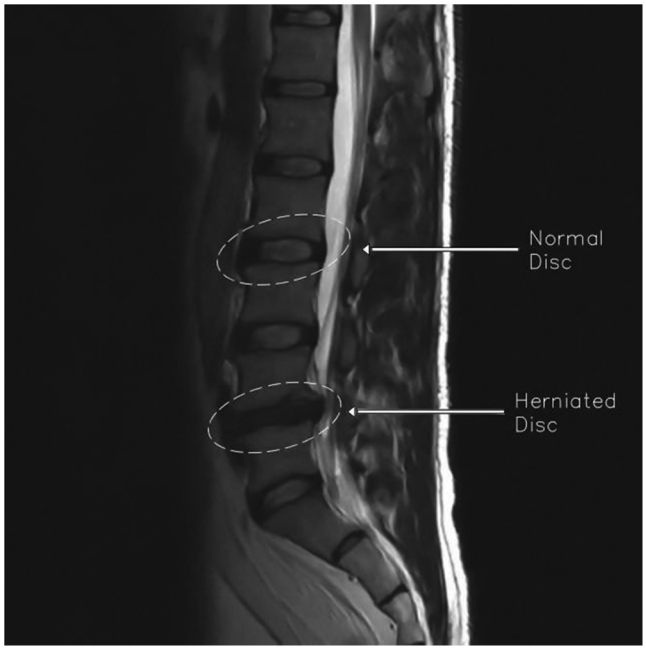

Fig. 1. Sagittal T2 Weighted MR Image of lumbar spine: Decreased L4-L5 disc height with degenerative changes (dehydration and posterior disc bulge). The rest of the intervertebral discs display normal features.

the NP extracellular matrix. (Fraser et al., 1993). Studying the NP cell function and dysfunction is extremely important for the delineation of the disc degeneration mechanism.

During the process of degeneration, NP cells have been shown to produce matrix remodeling enzymes, such as aggrecanases and collagenases, thus limiting aggrecan availability and dismantling collagen type II structures (Cs-Szabo et al., 2002; Le Maitre et al., 2007b). In addition, inflammatory factors have been shown to increase including IL-1b and TNFa. These matrix alterations reduce the ability of NP to retain water and thus alter its mechanistic properties (Antoniou et al., 1996). In conjunction with matrix changes, NP cell physiology has been shown to change, expressing senescence markers that further contribute towards the deterioration of disc degeneration (Le Maitre et al., 2007a). The relationship between matrix remodeling and change of NP cellular phenotype may be part of a feedback loop that ultimately leads to IVD dysfunction (Pattappa et al., 2012).

The concept of herpesvirus infection, as a causative factor of IVD cell dysfunction and thus of intervertebral disc degeneration, was raised several years ago by our group when herpes simplex virus type-1 (HSV-1) and human cytomegalovirus (HCMV) DNA were detected in human intervertebral disc samples harvested during discectomy. In that study, we were not able to prove a linear relationship between the virus presence into the disc and degeneration or the mechanism of how viral DNA might alter disc structure. However, our findings suggested that HSV-1 infection might alter the structural characteristics of the matrix in the disc by modulating apoptosis and local inflammatory response of the matrix producing cells (Alpantaki et al., 2011). A recent case report, documented elegantly that the disc environment is definitely a site where a latent VZV reservoir is present and can be conditionally reactivated (Dhillon and Smith, 2018). Furthermore, it has been shown that HSV-1 has the potential to interfere with proteoglycan metabolism in a study on endothelial cells where significant reduction of heparan sulfate and chondroitin/dermatan sulfate proteoglycan production was achieved by HSV-1 infection in vitro (Kaner et al., 1990). Based on the aforementioned knowledge, the aim of the present study was to investigate the infection capacity of HSV-1, both during lytic and non-productive infection conditions in an experimental cell culture model, using human nucleus pulposus cells (HNPCs) and subsequently to explore whether HSV-1 infection can affect their capacity to produce the characteristic components of the NP extracellular matrix. Our results confirmed the successful establishment of HSV-1 lytic infection in HNPCs while most intriguingly, revealed the differential expression patterns of proteoglycans, dependent on the type of HSV-1 infection, lytic or non-productive.

2. Materials & methods

2.1. Cells and viruses

Primary Human Nucleus Pulposus Cells (HNPCs) were purchased from Innoprot (SKELETAL INNOPROFILE™ Derio – Bizkaia, Spain). They are primary cells isolated from nucleus pulposus of human intervertebral discs, which can undergo up to 15 population doublings. Their purity is assessed and guaranteed by the provider. As regards to marker expression, the provider reported immunofluorecent labelling for fibronectin and vimentin. In addition, the cells test negative for HIV1, HBV, HCV, mycoplasma, bacteria, yeast and fungi. Human Nucleus Pulposus Cells (HNPCs) were grown in Nucleus Pulposus Cell Medium (Cat No P60135, Innoprot, Spain) which is a sterile, liquid medium that contains essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals, 1% penicillin/streptomycin and 10% FBS so as to provide a defined and optimally balanced nutritional environment for proliferation and growth of normal HNPCs in vitro. Moreover, prior to cell seeding, cell culture flasks, plates and coverslips were treated with Poly-L-Lysine (PLL) to facilitate their attachment. Vero and U20S cells were maintained in complete DMEM (10% (vol/vol), Fetal Bovine Serum (FBS), penicillin (1000 unit/ml) and streptomycin (100 µg/ml).

The wild-type HSV-1 *syn 17+* strain was used in this study, propagating and titrating in Vero cells. The mutant virus *dl*0C4 which contains the ICP0 deletion mutant of *dl*1403 and the ECFP-linked ICP4 fusion protein was also used (Everett et al., 2004a). Viral stocks of HSV-1 *dl0C4* were prepared in Vero cells and titrated in U2OS cells in which ICP0 is not required for efficient replication (Yao and Schaffer, 1995). The *dl0C4* virus was used at multiplicities of infection (MOIs) based on its PFU titer in U2OS cells, regardless of the cell type used. The supernatants collected from experimental conditions were titrated according to standard protocols (Everett et al., 2004b).

For viral infection experiments, the cells were infected with HSV-1 at the indicated multiplicity of infection MOI for 2 h and then the inoculum was removed and replaced by fresh medium. When the cells exhibited extensive cytopathic effect, the medium was harvested, clarified by low-speed centrifugation, and stored at −80 °C. Viral growth assays were performed Vero cells according to standard protocols. To characterize the HSV-1 *syn*17+ growth properties onto the human nucleus pulposus cells, single-step growth curves were performed in parallel with Vero infected cells and the viral titres were determined via plaque assay.

2.2. Western blot

Protein extracts were analyzed by Western blot. For whole cell extracts, the cells were washed with cold PBS, collected with 1 mM EDTA in PBS and pelleted at 2500 *g* for 10 min. The cells were then incubated for 10 min at 4oC with M-PER Mammalian Protein Extraction Reagent (Cat.No 78503, Thermo Scientific) along with protease inhibitors (Cat. No 78415, Thermo Scientific) vortexing mildly every 2 min. The cell extracts were then centrifuged for 15 min at 14,000 *g* at 4oC and the supernatant was kept for analysis. All protein extracts were quantified with Cayman Protein Determination kit (Cat.No 704002, Cayman). From each sample, 40 µg of protein were boiled in SDS gel-loading buffer, separated by electrophoresis and transferred on nitrocellulose membranes. The membranes were subsequently blocked in TBST buffer with 5% (w/vol) dried non-fat milk and incubated overnight at 4 °C

with the appropriate primary antibodies. The primary antibodies used for immunoblotting were anti-HSV-1 ICP0 1:8000 (Cat.No H1A027-100, Virusys Corporation), anti-HSV-1 ICP8 (11E2) (Cat sc-53330, Santa Cruz Biotechnology), anti-HSV-1 gG Envelope Protein (7F5) (Cat No sc-56984, Santa Cruz Biotechnology) and mouse anti-actin 1:2000 (Cat.No MAB1501, Millipore). All antibodies were diluted in TBS-0,1% Tween-20 (vol/vol) containing 1% (w/vol) dried non-fat milk. Following incubation with the primary antibodies, the membranes were thoroughly washed and incubated with the secondary antibodies for 1 h at room temperature, washed in TBS-T and developed using Luminata Forte Western HRP Substrate (Cat.No WBLUF0100, Millipore) either on film or by the ChemiDoc™ MP System (Bio-Rad) with the Image Lab v5.0 software (Bio-Rad).

2.3. Immunofluorescence analysis

For immunofluorescence, 1×10^5 human nucleus pulposus cells were plated on glass coverslips placed in 24-well plates. The conditioned medium was aspirated, the cells were washed with PBS, fixed with formaldehyde (4% [vol/vol] in PBS containing 2% sucrose) and permeabilized with Permeabilization Solution (Cat. No 5115, Millipore). The coverslips were incubated for 1 h with primary antibodies diluted in PBS containing 1% (vol/vol) fetal bovine serum at room temperature and they were subsequently washed in the same buffer twice before incubation with the secondary antibodies. Incubation with the secondary antibodies was performed likewise. The primary antibodies used were the same employed for the western blot analysis in 1:100 dilution. The secondary antibody used was Alexa Fluor 488 donkey anti–mouse (Cat. No A21202, Invitrogen) while the nuclei were stained with DAPI (4′,6-diamidino-2-Phenylindole, Cat. No D3571, ThermoFisher Scientific) at a dilution of 1:1000 in PBS. The cells were mounted in Ibidi Mounting Medium (Cat. No 50001, Ibidi) and examined by confocal microscopy (TCS SP2, Leica Microsystems, Germany). The data were collected with sequential scanning to avoid signal overlap, at a resolution of 1024 × 1024 pixels, after a 4–6 fold averaging and the optical slices were between 0.3 and 0.5 µm. The data sets were processed with LCS Lite software (Leica).

2.4. Live-cell microscopy

Each well of a two-well chambered coverglass unit (Lab-Tek, Thermo Scientific) was seeded with 2×10^5 human nucleus pulposus cells and infected at MOI 0.01 PFU/cell with the *dl*0C4 HSV-1 virus. The cells were transferred in a humidified chamber on the microscope stage with 5% CO2 at 37 °C and were observed at the indicated times post infection with an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300 FX digital camera and images were acquired with the IM50 software (Leica) and exported as tiff files.

2.5. RNA isolation and Reverse transcriptase-quantitative PCR (RT-qPCR)

Total ribonucleic acid was isolated from 2×10^5 cells by the TRIzol method (Invitrogen) according to the manufacturer's instructions. The yield and the purity of the RNA preparations were estimated by measuring the A260/A280 ratio. 1 µg of total RNA was used for cDNA synthesis using the Primescript™ RT-PCR System (TAKARA BIO INC) according to the manufacturer's instructions. The primers selected were mRNA specific to avoid misleading results from traces of DNA contamination. For the realtime PCR reaction we utilized the SYBR FAST qPCR (KAPA Biosystems) in a total volume of 20 µl. Standard curves were run in each optimized assay which produced a linear plot of threshold cycle (Ct) against log (dilution). The RT-qPCR reactions were carried out in Agilent Mx3005P™ cycler and all measurements were performed in duplicate. The amount of each target was semi-quantified based on the concentration of the standard curve and was presented as arbitrary units. The value represented as *"arbitrary units"* is directly proportional but not equal to the absolute mRNA copy number of the target. I.e. a target sample with double value *"arbitrary units"* compared to a control sample has exactly double mRNA copy number. The quantity of each target was normalised against the quantity of actin. The oligonucleotides used in the study are as follows: *Aggrecan* R/L ACAGCTGCAGTGATGACCCT/ TTC TTG GAG AAG GGA GTC CA, *Biglycan* R/L GTC CCA GAA GCC TCT CTG CT/ AAC TAG TCA GCC TGC GC CT, *Collagen II* R/L CGG CTT CCA CAC ATC CTT AT/ CTG TCC TTC GGT GTC AGG G, *Versican* R/L CAC TCT TTT GCA GCC TCC TC/ TCT CCC CAG GAA ACT TAC GA, *Decorin* R/L GAG TTG TGT CAG GGG GAA GA/ GGA TAG GCC CAG AAG TTC CT, *Has1* R/L GTC ATG AGG CCC AGG ATG/ ACC CAC TGC GAT GAG ACA G, *Has2* R/L AGC TGT GAT TCC AAG GAG GA/ ACA GAC AGG CTG AGG ACG AC, *Has3* R/L CTA CTT GGG GAT CCT CCT CC/ CGA TTC GGT GGA CTA CAT CC *actin* R/L CACCAACTGGGACGACAT/ ACAGCCTGGATAGCAACG.

3. Results

3.1. Infection of human nucleus pulposus cells by HSV-1

In a previous study, exploring the concept of herpes virus infections *in* the degeneration of intervertebral discs, we were able to identify herpes simplex virus type-1 (HSV-1) and human cytomegalovirus (HCMV) DNA accompanied by overexpression of inflammatory molecules in human intervertebral disc samples (Alpantaki et al., 2011). Given the tight association of HSV-1 with the central nerve system and the spinal cord, we decided to shed light into the potential mechanistic role of this particular herpes virus during disc degeneration. The Human Nucleus Pulposus Cells (HNPCs) isolated from the nucleus pulposus of human intervertebral discs were used in this study. The nucleus pulposus cell culture provides an ideal in vitro model for the study of cellular and molecular events involved in disc degeneration, tissue engineering and cell therapy for spine disc disorders (Agrawal et al., 2007; Kluba et al., 2005; Matsumoto et al., 1999).

As a first step, the permissiveness of human nucleus pulposus cells was tested. HNPCs were infected with wild-type HSV-1 strain *syn*17+ at a multiplicity of infection 1 PFU/cell (MOI = 1). Vero cells, a cell line successfully supporting productive HSV replication, were infected in parallel, serving as a positive control. The expression of all classes of HSV-1 genes, i.e. Immediate-Early (ICP0), Early (ICP8) and Late (gG glycoprotein) genes was examined by western blot analysis, after harvesting protein extracts at the appropriate time points post infection. As shown in Fig. 2, the time courses and the amounts of HSV-1 proteins expressed were comparable in HNPCs and Vero cells. The expression levels of ICP8 and gG proteins appeared slightly lower in HNPCs however, this difference at the protein expression cannot be interpreted as a defect.

The efficient expression and appropriate subcellular localization of HSV-1 proteins was further determined by immunofluorescence microscopy of virally infected cells. Immunostaining of the cells at the indicated times post infection confirmed the typical nuclear dot-like localization of ICP0 at the very early times of infection, the recruitment of ICP8 into viral replication compartments at early times and finally the cytoplasmic distribution of gG at the latest stages of HSV-1 life cycle (Fig. 3). Moreover, the proportion of the infected cells was relatively similar among HNPCs and Vero cells as determined by image analysis (data not shown) (Goulidaki et al., 2015). Taken together, the aforementioned results demonstrate that human nucleus pulposus cells are permissive to HSV-1, enabling the virus to accomplish all stages of lytic infection.

3.2. Growth curve analysis after viral infection of Human Nucleus Pulposus Cells

The replication of HSV-1 was assayed on human nucleus pulposus cells in comparison to Vero cells. Both cell lines were infected in

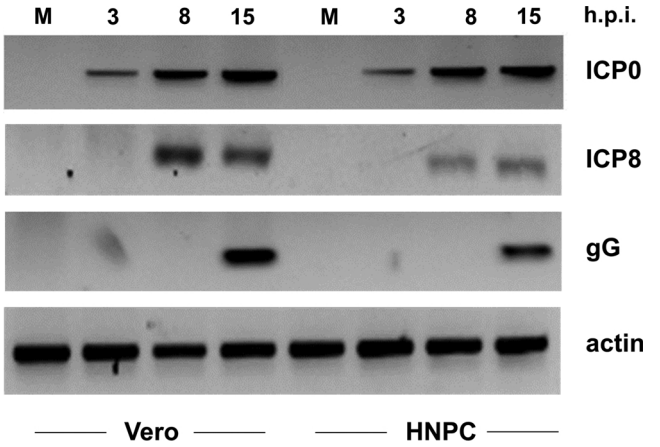

Fig. 2. Comparison of HSV-1 protein expression kinetics in Human Nucleus Pulposus cells and Vero cells. HNPCs and Vero cells were infected with wild-type HSV-1 strain *syn 17+* at an MOI = 1 and total protein extracts were harvested at the indicated time points. ICP0 (Immediate-Early) ICP8 (Early) and gG (Late) protein levels were analyzed by Western blot. Cellular actin levels served as controls for equal protein loading.

parallel with wild-type HSV-1 strain *syn 17+* at MOI = 5 PFU/ml. The culture medium from both cell lines was sampled by removing and replacing a small aliquot at various time points post infection and was subsequently titrated by plaque assay. Growth curve experiments showed that HNPCs successful protein expression is followed by production of progeny (Fig. 4). Compared to Vero cells, the HSV-1 titres obtained from HNPCs were reduced ~2-fold, however, this difference in growth potential is not considered significant. This observation is also consistent with the slightly reduced expression of ICP8 and gG in HNPCs as detected by western blot. Cumulatively, HSV-1 behaves normally in HNPC-infected cells which in turn are capable of shedding progeny virus at the expected time course.

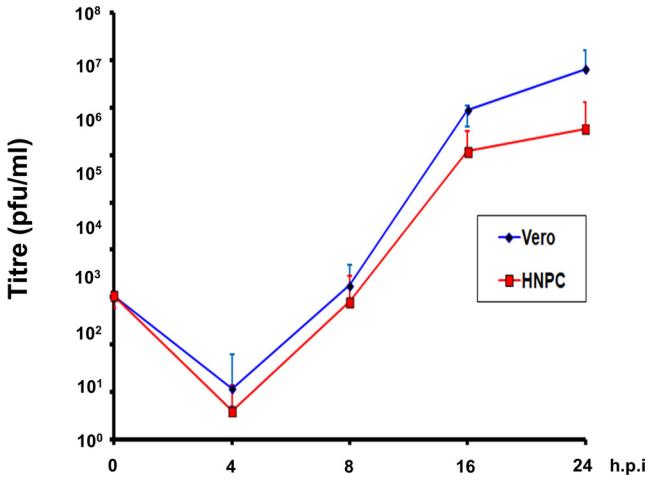

Fig. 4. Growth curve analysis of the HSV-1 progeny virus in Vero and HNPCs. Vero and HNPCs were infected in parallel with viral inocula of wild-type HSV-1 strain *syn 17+* at MOI = 5 PFU/cell. The progeny virus in the supernatants of infected cell cultures were harvested at different time points post infection, as indicated, followed by the quantification by standard plaque assays. Mean values (± SD) of three sets of experiments in triplicate samples are presented.

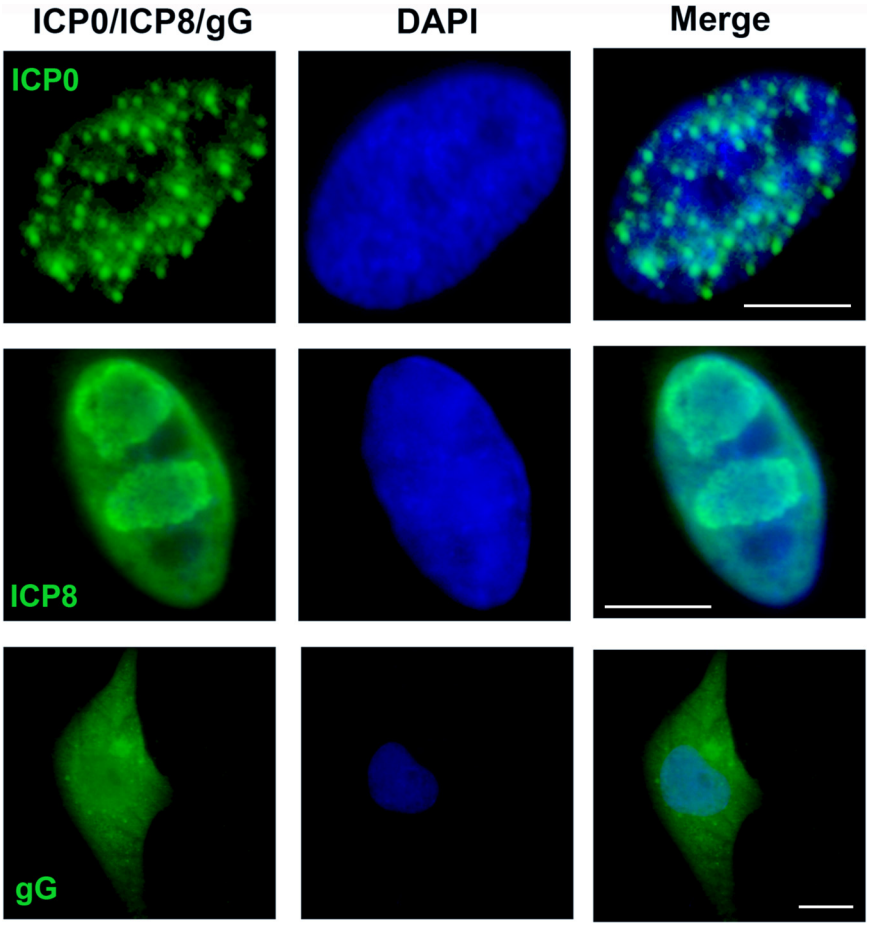

Fig. 3. Expression and subcellular localization of ICP0 (Immediate-Early), ICP8 (Early) and gG (Late) HSV-1 proteins in Human Nucleus Pulposus Cells. Cells were infected with wild-type HSV-1 strain *syn 17+* at an MOI = 1, fixed and stained with the corresponding primary and secondary antibodies. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole). Magnification, ×600 (bars: 5 μm).

3.3. Matrix expression in lytically infected human nucleus pulposus cells

Having documented that HNPCs are efficiently infected by HSV-1, we proceeded to assess the expression profile as regards to specific markers of the NP extracellular matrix (Pattappa et al., 2012). NP tissue is distinguished from other cartilage tissues by an extremely high ratio of proteoglycan to collagen ratio (Mwale et al., 2004). The main collagen variant found in NP is collagen type II (Buckwalter, 1995). Previous studies have established that HNPCs phenotype includes high level expression of Aggrecan and collagen type II as compared to other cells of the IVD (Horner et al., 2002). Other proteoglycans found to be expressed in NP are Biglycan, Versican and Decorin albeit at lower levels (Melrose et al., 2001).

Cells were seeded in 12 well plates coated with poly L lysine. After overnight attachment the cells were infected with wild-type HSV-1 strain *syn*17+ using a range of multiplicity of infection 1–10 PFU/cell (MOI = 1–10) aiming to determine the optimal MOI that can achieve an almost 100% infected cell population at the first circle of infection, concluding at MOI of 5 PFU/cell. The efficiency of infection was assessed by immunofluorescence microscopy 3 h post infection using immunostaining for ICP0. After establishing conditions for 100% efficiency of infection, HNPCs were cultured for 3, 8 and 24 h post infection (h.p.i.) and then harvested for RNA extraction and cDNA synthesis along with uninfected controls. Results of the mRNA semi-quantification by Real-Time PCR are shown in Fig. 5. We observed a significant decrease of Decorin mRNA expression 8 h after infection which was escalated 24 h.p.i. Similarly, Biglycan mRNA expression was downregulated only at the latest stage of the infection (24 h.i.p.). Collagen type II, although demonstrated a downward trend, this was not statistically significant whereas both Versican and Aggrecan demonstrated significant reduction 24 h.p.i. Assessing the expression of hyaluronan, through the analysis of the HAS 1, 2, 3 genes, it did not show any significant effects after HSV-1 infection. In summary, we observed that HNPCs infection with HSV-1 results in significant alterations in mRNA expression of both aggregating and non-aggregation proteoglycans which are essential for the NP matrix structure.

3.4. Matrix expression in non-productively infected human nucleus pulposus cells

In our previous study, the presence of HSV-1 DNA in human intervertebral disc samples was not accompanied by mRNA expression of viral genes, suggesting that the viral genomes probably establish long term latent infections in the disc (Alpantaki et al., 2011). Based on this concept and given the deregulated expression of extracellular matrix genes during lytic HSV-1 infection, we investigated the expression of the same genes in conditions of non-productive infection. Models of quiescent HSV-1 infections are mainly established using mutant viruses for the Immediate-Early regulatory protein ICP0 which induces the expression of all subsequent classes of HSV-1 proteins. The abilities of ICP0 to stimulate the initiation of lytic infection and to induce the reactivation of quiescent viral genomes have led to the suggestion that this molecule is a key determinant for the balance between lytic and latent infection (Everett, 2000; Hagglund and Roizman, 2004). In our study, the HSV-1 *dl*0C4, a derivative of ICP0-null mutant virus *dl*1403 (Stow and Stow, 1986) that expresses ICP4 linked to enhanced cyan fluorescent protein (ECFP) was used. The virus and the properties of this specific ICP0-deficient virus have been described in a thorough and elegant study showing the establishment of non-productive infection in cells infected at low viral input multiplicity (Everett et al., 2004a).

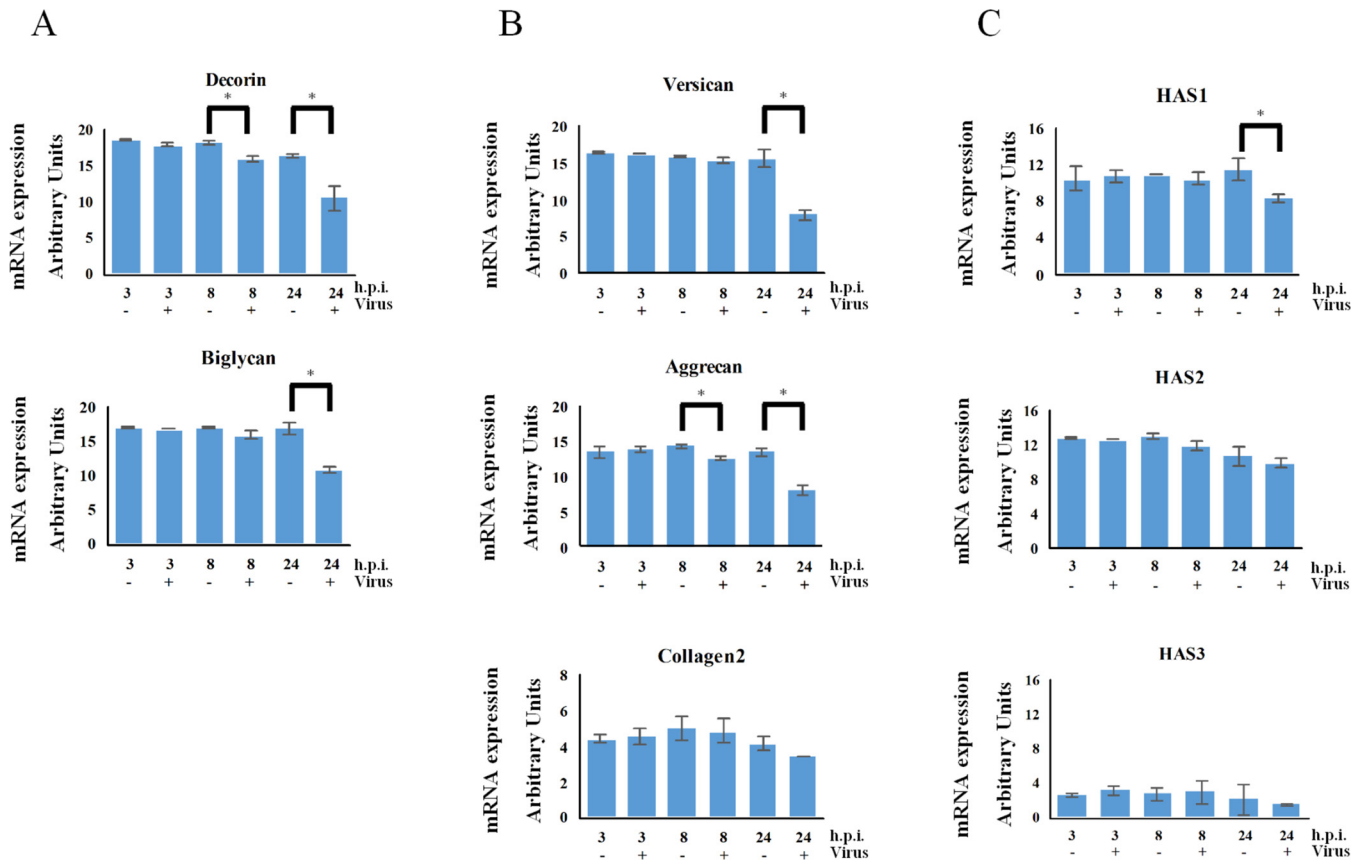

Fig. 5. Real-Time PCR semi-quantification of mRNA expression in HNPCs after wild-type HSV-1 stain *syn 17+* infection at MOI = 5: (A) Decorin, Biglycan, (B) Aggrecan, Versican, Collagen type II and (C) HAS 1, 2, 3 (Means ± SD plotted; n = 2). h.p.i.: hours post infection, * depicts statistical significance $p < 0.05$ and **$p < 0.01$ *t*-test.

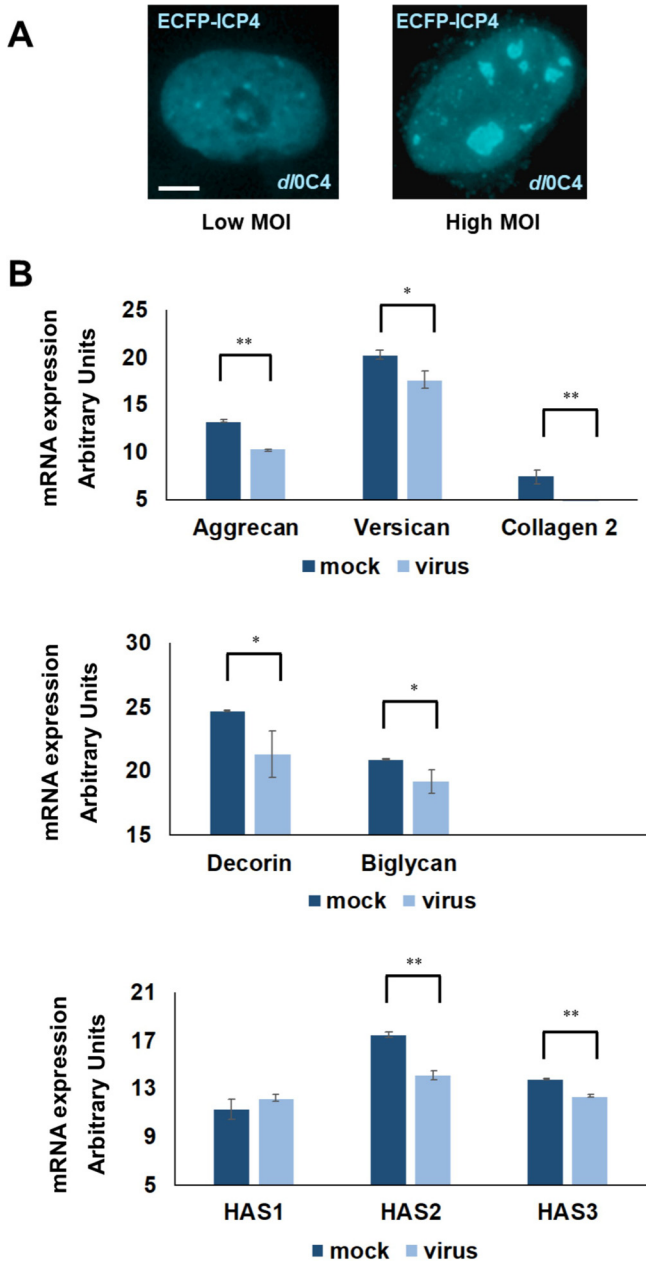

Fig. 6. (A) Establishment of either lytic (a) or non-productive (b) infection in HNPCs using either low (MOI = 2) or high (MOI = 7) multiplicity of infection of the *dl0C4* HSV-1 virus (bars: 5 μm). (B) Real-Time PCR semi-quantification of mRNA expression of matrix molecules in HNPCs non-productively infected cells by HSV-1 for 6 days (Means ± SD plotted; n = 2). * depicts statistical significance $p < 0.05$ and ** $p < 0.01$ *t*-test.

Human nucleus pulposus cells were infected with *dl*0C4 at MOI = 2.5, at an input adjusted to infect 70–80% of the cells. The samples were examined 48 h later, for the appearance of ICP4 expression, the formation of viral replication compartments or viral plaques. Due to ECFP-ICP4 expression, the differentiation between non-productively infected cells and cells entering lytic infection was easy by fluorescence microscopy analysis: in the non-productively infected cells, a very low and even ECFP fluorescence could be detected in the nucleus but no formation of ECFP-ICP4-containing replication compartments (Figure 6Aa), in contrast to lytic cell in which major replication compartments were evident because of the recruitment of ECFP-ICP4 into them (Figure 6Ab). *dl0C4*-infected cells were able to maintain their non-productive infection status for up to 6–7 days, without coexistence of lytic cells in the same sample.

Having established a condition of non-lytic infection, we proceeded to investigate the effect of non-productive HSV-1 genomes on extracellular matrix genes. Infected cells were harvested 6 days after infection for RNA extraction and cDNA synthesis along with uninfected controls. Results of the mRNA semi quantification by Real-Time PCR are shown in Fig. 6. We observed a significant reduction of Decorin, Biglycan, Versican and Aggrecan expression in accordance with our findings from the lytically infected HNPCs. Collagen type II expression was completely abolished. HAS1 expression was not affected, whereas HAS 2 and 3 were found to be significantly reduced. In summary, we observed that HNPCs with non-productively HSV-1 infection results in a pattern of expression compatible with the lytically infected cells, supportive to the model deregulated hyaluronan and collagen type II biosynthesis pathways.

4. Discussion

The potential relationship between chronic infection by low virulence pathogens and intervertebral disc (IVD) degeneration remains contentious with inconsistent literature evidence. In a previous study, we detected HSV-1 and HCMV DNA in human disc samples harvested during discectomy. HSV-1 was the most frequently detected virus. We also found overexpression in inflammatory markers by examining the mRNA levels of TNF-α and IL-6, in all the samples; further supporting the contribution of virus infection in the inflammatory process. We proposed that the presence of the viruses into the disc might create a setting, where the disc become vulnerable to environmental factors which secondarily drive the biological events resulting in degeneration (Alpantaki et al., 2011).

To test our hypothesis in an in vitro model of IVD degeneration, we investigated the effects of HSV-1 infection on proteoglycan and collagen synthesis in human nucleus pulposus cells. First, we demonstrated that HNP cells are permissive to HSV-1, enabling the virus to accomplish all stages of lytic infection. Secondly, during HSV-1 lytic infection we noticed a remarkable reduction of Decorin expression 8 h post infection which further deteriorated at the 24-hour time point of the assay. Biglycan was found to be reduced only in the 24-hour time point. Collagen type II although demonstrated a downward trend it was not statistically significant, whereas both Versican and Aggrecan showed a notable decline 24 h after infection time point. Hyaluronan production was not significantly affected.

In our previous study, none of the patients had evidence of acute infection as indicated by the absence of IgM + antibodies from the peripheral blood and viral mRNA transcripts from the disc samples, suggesting that the virus probably sets a long term quiescent infection into the disc. Based on these findings and considering the overwhelming results of lytic HSV-1 infection on production of extracellular matrix components, we examined the expression of the same genes in non-productive infection conditions which might resemble the model of chronic disc infection. In this part of our investigation, we found out a substantial reduction of Decorin, Biglycan, Versican and Aggrecan expression similarly to our findings from the lytic circle. Furthermore, collagen type II expression was completely abolished (Fig. 6B). HAS1 expression was not affected, whereas HAS 2 and 3 were found to be significantly reduced. In summary, we observed that non-productive HSV-1 infection in HNPCs results in a pattern of expression similar to that of the lytically infected cells, adding to the deregulated hyaluronan and collagen type II biosynthesis pathways model.

Our study is consistent with previous evidence reporting an overall escalating reduction of PGs and collagen II genes expression with advanced degeneration (Vergroesen et al., 2015). Significant alterations in the ECM components occur in degeneration induced animal models. (de Oliveira et al., 2013). Aggrecan reduction in NP leads to alteration in osmotic pressure and hydration levels which impairs disc mechanical properties (Antoniou et al., 1996). Fragmentation of SLRP core proteins

and the subsequent loss of ECM homeostasis leads to fibrocartilaginous metaplasia of the NP and further compromises its biomechanical properties (Erwin et al., 2015). The NP becomes dehydrated, progressively is transformed to fibrotic and rigid and subsequently loses its capacity to distribute compressive forces between the vertebral bodies (O'Connell et al. 2009). The mechanical forces are unevenly distributed to the surrounding AF, causing alteration in AF mechanics and deterioration of its structure, including the formation of fissures, radial tears and eventually disc herniation (Vernon-Roberts et al., 2007). The cellular micro-environment of the NP will gradually become more dysfunctional and the degenerative disc will exhibit a higher level of abnormal components of the matrix and an increase in the matrix degradation mediators such as upregulated levels of proinflammatory cytokines and catabolic enzymes (Hadjipavlou et al., 2008).

Although the inflammatory profile of the IVD degeneration has been extensively studied, the order and the causal relationship of the biological and the biomechanical events that occur in the disease process is rather unknown (Gorth et al., 2015) In a recent publication, the authors proposed a vicious cycle as an etiological disease model which comprises of mechanical overloading, catabolic cell response and degeneration of the extracellular matrix (Vergroesen et al., 2015). They also suggested that low grade infection could possibly provoke NP cells to degrade the extracellular matrix due to a catabolic shift in cellular metabolism and consequently increase the susceptibility of the intervertebral disc to chemical or biomechanical factors, which initiate or promote, the biological cascade resulting in degeneration (Vergroesen et al., 2015).

The hypothesis of low-grade spondylodiscitis as the initial stage of the cascade of IVD degeneration has been raised when Propionibacterium acne was cultured in disc samples obtained during discectomy (Stirling et al., 2001). Recently, other studies have also reported the presence of anaerobic pathogens in NP tissue samples but the possibility of biopsy contamination during tissue harvesting cannot be excluded (Arndt et al., 2012; Ben-Galim et al., 2006; Li et al., 2016). Also, several studies have correlated painful Modic I changes (high intensity bone marrow signal in T2-weighted and low signal intensity in T1-weighted series in MRI scan) with infection (Aebi, 2013; Albert et al., 2013a, 2008; Albert et al., 2013b; Birkenmaier, 2013; Jensen et al., 2008; O'Dowd and Casey, 2013; Sotto and Dupeyron, 2013).

Our findings associate the theory of chronic infection as a contributing cause of degeneration with the degradation of the extracellular matrix which is a predominant finding in disc disease. Low-grade infection, affects the NP cells metabolic status, and might amplify the susceptibility of the intervertebral disc to environmental and genetic factors, which trigger or promote the biological cascade resulting in degeneration (Alpantaki et al., 2011; Vergroesen et al., 2015). A key element in early disc degeneration is the decline in NP proteoglycan content which is associated with a change in the NP cells phenotype (Cappello et al., 2006) NP cells from degenerative tissues displayed significant lower levels of collagen type II compared with NP cells from scoliosis donors (Kluba et al., 2005) which supports our findings. When NP cells receive improper chemical signals they will stop producing, or even start degrading PGs leading to a decline of the intradiscal pressure, NP structural integrity and disc biomechanics (Vergroesen et al., 2015). A previous study reported that HSV-1 infection of human endothelial cells produced a dose-dependent inhibition of total PGs synthesis of up to 85%. A major reduction of heparan sulfate and chondroitin/dermatan sulfate proteoglycan production was noticed. The authors suggested that the viral-induced changes in PGs metabolism may influence cell-matrix interactions (Kaner et al., 1990).

Intervertebral disc degeneration is a complex phenomenon. Multiple interdependent factors, including genetic predisposition, abnormal mechanical forces, aging, metabolic disorders, toxins and possibly infection may act independently, or collectively, as initiators or promoters, of the degeneration process (Hadjipavlou et al., 2008; Smith et al., 2011).

To our knowledge, this is the first study which provides quantitative information about alterations in PGs synthesis by HSV-1 infected HNP cells. We believe that our findings support the theory that virus infection could be involved in the pathogenesis of the degenerative disc disease, since we showed a remarkable reduction of PGs and collagen type II production both in lytic and non-productive infection. Extracellular matrix decay due to PGs degradation is a fundamental event in the pathogenesis of disc degeneration. The results observed in this study indicate that HSV-1 infection of HNP cells yields a complex effect on host cell function but the molecular mechanisms underlying the alterations in PGs production are not clear. A direct causative link between the presences virus-induced changes in PGs production and disc degeneration remains to be established. We assume that changes to host cell genome regulation are involved but further understanding is required. Additional studies in well-designed animal models are necessary to investigate the infection route and to define the underlying mechanisms and support the plausible role of herpes viruses in the pathogenesis of degenerative disc disease. Gaining insight to the pathogenesis of both active and non-productive infection of NP cells may eventually lead to clinical applications. Although it is hard to believe at first glance, low virulence pathogens have been recognized as contributing factors of other conditions that do not primarily impose as infectious diseases and a paradigm shift may be on the horizon (Alpantaki and Hadhipavlou, 2017; Birkenmaier, 2013).

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