Hyaluronan Production in Synoviocytes as a Consequence of Viral Infections

HAS1 ACTIVATION BY EPSTEIN-BARR VIRUS AND SYNTHETIC DOUBLE- AND SINGLE-STRANDED VIRAL RNA ANALOGS*

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One of the hallmarks of arthritis is swollen joints containing unusually high quantities of hyaluronan. Intact hyaluronan molecules facilitate cell migration by acting as ligands for CD44. Hyaluronan degradation products, readily formed at sites of inflammation, also fuel inflammatory processes. Irrespective of whether viruses could be a cause of rheumatoid arthritis, there is clear evidence that links viral infections to this debilitating disease. For this study, live Epstein-Barr virus and a number of double- and single-stranded synthetic viral analogs were tested for their effectiveness as activators of hyaluronan (HA) synthesis. As shown herein, Epstein-Barr virus-treated fibroblast-like synoviocytes significantly increase HA production and release. Real time reverse transcription-PCR data show that HAS1 mRNA levels are significantly elevated in virus-treated cells, whereas mRNA levels for the genes HAS2 and HAS3 remain unchanged. As to the mechanism of virus-induced HAS1 transcription, data are presented that imply that among the double- and single-stranded polynucleotides tested, homopolymeric polycytidylic acid is without effect. Analyses of virus-induced signal cascades, utilizing chemical inhibitors of MAPK and overexpressing mutated IKK and IkB, revealed that the MAPK p38 as well as the transcription factor NF-κB are essential for virus-induced activation of HAS1. The presented data implicate HAS1 as the culprit in unfettered HA release and point out targets in virus-induced signaling pathways that might allow for specific interventions in cases of unwanted and uncontrolled HA synthesis.

Rheumatoid arthritis (RA)2 is a disease of largely unknown etiology. A series of mechanisms have been described that contribute to the progression of this debilitating disorder. A large body of evidence points at contributing factors such as altered apoptotic behavior of cells, the presence of a number of inflammatory cytokines, as well as the massive accumulation of lymphocytes at affected sites (1). Others, pointing at the abnormal production of rheumatoid factors in RA, favor the hypothesis that RA is an autoimmune disease or, referring to data regarding certain major histocompatibility complex class II molecules, link the onset of RA to certain genetic factors (1–7). Alternative hypotheses, however, refer to the fact that archeological signs of RA in the New World can be traced back more than 5000 years, whereas similar evidence is absent in the Old World before the 18th century (8). Such a marked geographic distribution has been interpreted as being consistent with the important role of environmental factors such as viruses in the genesis of RA (8). One of the most compelling hypotheses, however, implicates a special cell type found in the synovium of RA-affected joints, namely activated and proliferating fibroblast-like synoviocytes (FLS), as playing an exceptionally important role in the genesis of RA. In support of this hypothesis, convincing evidence has been presented that FLS isolated from RA patients alone are sufficient to initiate and to propagate RA (9–11).

One of the early signs of RA are swollen joints. A hallmark of these swollen joints is the presence of large amounts of hyaluronan (HA). HA forms a fine layer in healthy joints. In RA-affected joints, however, HA levels can reach enormous levels that in some cases can be more than 100 times higher than what is found in healthy joints. In RA patients, even plasma levels of HA are elevated to such a degree that they could serve as a reliable marker of RA progression (12). Although small amounts of HA are essential for proper joint function, unfettered HA production is associated with a number of detrimental effects. Because HA accumulation is one of the early events in RA, I became interested in analyzing the expression pattern of HAS in synoviocytes isolated from RA patients. My working hypothesis is based on the assumption that in RA, one or more of the HA-coding genes might become unregulated, leading to the unwanted overproduction of HA. As a consequence, either HA itself or its degradation products might propagate inflammation in affected joints. Such a model, in which unfettered HA release sets in motion a series of events, is supported by the well-known fact that HA degradation products clearly possess proinflammatory properties, act as chemoattractants, and have
EBV Induces HAS1

angiogenic properties, all highly undesired features in RA and in many other inflammatory settings (13).

We demonstrated earlier that hyaluronan synthase (HAS)2 and HAS3 are genes that are constitutively activated in FLS but also that mRNA levels of the gene encoding HAS1 are very low or undetectable (14). We showed that among the HAS genes in this cell type HAS1 is the only one that readily responded to a series of pro-inflammatory cytokines such as interleukin (IL) IL-1α, IL-1β, tumor necrosis factor–α, transforming growth factor–β, as well as IL-8, but also to phorbol 12-myristate 13-acetate (14). In addition, we also demonstrated that IL-1β-induced HAS1 activation is a process that depends on the activation of nuclear factor κB (NF-κB), a transcription factor that is know to be essential for the activation of most pro-inflammatory genes (15). These data led me to speculate that it might be the HAS1 product that contributes and/or accounts for inflammatory processes associated with RA.

In recent years, effects of cytokines as well as of particular growth factors on genes encoding HAS have been studied and reported in increasing detail. Although it has been known that viruses can affect HA levels in tissues, I am not aware of studies investigating the effects of virus infection on the particular HAS genes, nor of studies that might have elaborated on the structural requirements and molecular mechanisms of virus-induced changes in HAS expression and HA accumulation. Here, data of in vitro experiments are shown that detail some of the effects and mechanisms of changes of HA in FLS exposed to live virus and synthetic viral analogs.

**EXPERIMENTAL PROCEDURES**

*Reagents—* If not stated otherwise, reagents were from Sigma. Interleukin-1β (IL-1β) was purchased from Strathmann (Strathmann Biotec, Hamburg, Germany). The MAPK inhibitors SB-203580, PD-98059, and the JNK inhibitor II SP600125 were from Calbiochem. Antibodies for p38, ERK, JNK, IκBα, p-IκB used in Western blots were from Cell Signaling (New England Biolabs, Frankfurt am Main, Germany). Antibodies used in EMSA supershift experiments were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-tubulin antibody was from Neo Marker (Fremont CA). Oligonucleotides resembling consensus sequences for NF-κB, AP-1, etc. were from Promega (Promega, Mannheim, Germany) or Santa Cruz Biotechnology. Purified Epstein-Barr virus 4.3 \times 10^{10} virus particles/ml (1.3 mg/ml protein) and a transformation dose (TD_{50}/ml of 5 \times 10^{-25}) was purchased from Advanced Biotechnologies Inc. (Columbia, MD).

**Hyaluronan Measurements**—HA was quantitated via a procedure provided by Corgenix (Corgenix, Westminster, CO), the provider of the HA measurement kits. In short, plates coated with HA-binding protein were incubated with supernatant (10 μl of supernatant diluted with 90 μl of reaction buffer) or standards, respectively, for 1 h (room temperature) in duplicates, washed five times with washing buffer, incubated with a solution containing horseradish peroxidase-conjugated HA-binding protein for 1 h at room temperature, washed again five times, and incubated with 100 μl of the provided substrate solution. After ~20 min, the reaction was stopped by adding an equal amount of sulfuric acid (0.36 N). The resulting absorbance values were measured at 450 nm (630 nm reference). Absorbance values of standards were used to calculate unknown HA levels using a third-order polynomial regression analysis performed with a universal assay calculation program (AssayZap, Biosoft, Cambridge, UK).

**Nucleotide Preparation**—With the exception of 5-, 10-, and 20-mers that were purchased from MWG Biotech AG (Ebersberg, Germany), polynucleotides were purchased from Sigma. All polymers were resuspended in endotoxin-free water. At all times, repetitive pipetting and/or excessive vortexing of solutions containing polynucleotides was avoided. Furthermore, rather than preparing serial dilutions in plastic tubes, polymers were added directly to cell culture dishes. Such a procedure was opted for to minimize loss of nucleotides because of absorption of polymers to pipette tips, plastic tubes, or tissue culture dishes.

**Cell Culture**—A series of human CD90-positive fibroblast-like synoviocytes (FLS) isolated from RA patients was used for the reported experiments. These cells were either purchased from Dominion Pharmakine (Dominion Pharmakine, Derio, Bizkaia, Spain) or were a gift from Prof. G. Steiner (Department of Rheumatology, Medical University of Vienna). FLS were characterized by flow cytometry for the phenotype-specific marker CD90, and such data revealed a purity of ≥98%. Selected experiments were also repeated with cells that had been isolated, characterized, and cultured in our laboratory as described previously (14). Cells were used from passage 3 to 8. Furthermore, FLS grown to high density were utilized for the experimental data presented herein. FLS were cultured in a humidified chamber (37 °C, 5% CO₂) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma) and the antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml). In the early stages of cell cultivation (passage 0–3), fungizone (0.2 μg/ml) was added to cells kept in T75 (75-cm²) flasks. After reaching confluence, FLS were routinely split at a ratio of 1:3. EMSA experiments were done using cells cultured in 10-cm dishes. For all other experiments, FLS were transferred to 6-well cell culture dishes.

**Western Blot Experiments**—SDS-PAGE and Western blotting were carried out essentially as described (16). Proteins were made visible using RenaissancePlus (PerkinElmer Life Sciences) and Kodak BioMax MR films or the chemiluminescence detection device GeneGnome (Syngene, Cambridge, UK). In Western blot experiments, lower concentrations of proteins were loaded on separate gels that served as controls for loading and protein transfer. Such blots were stained with an antibody recognizing tubulin and/or with Ponceau Red.

**Real Time RT-PCR, Data Analysis, and Quality Controls**—Gene expression in FLS was measured by real time RT-PCR on an Mx3000P (Stratagene, Amsterdam, The Netherlands), using SYBR green as reporter fluorophore for quantitating mRNA levels. Results are expressed as relative threshold cycle (ΔCt values) (Ct values of mRNA levels in stimulated cells minus Ct values of a given gene in resting cells). Basal mRNA expression levels in unstimulated FLS were chosen to represent 1× expression of a given gene. Amplification curves and equations for calculations have been reported elsewhere (17). Primers used in real time PCR experiments were selected based on published
data (14, 17, 18) or generated with the help of software provided by the supplier of the primers MWG Biotech AG. After optimization of PCR conditions, RT-PCR was performed using the following standard settings: initial denaturation for 10 min at 95 °C, denaturation for 10 s, annealing for 15 s at 57 °C, and extension for 15 s at 72 °C. Each RT-PCR experiment included a dissociation curve to verify the specificity of the amplification, as well as no template controls. mRNA for hyoxanthine-guanine phosphoribosyltransferase 1 (HPRT) was co-amplified and used as a control for quantification. Primers used in real-time PCR experiments were ordered from MWG Biotech AG and dissolved at a concentration of 100 pmol/μl in Tris-EDTA.

Primer sequences are as follows: HAS1 (sense primer), 5'-GAC TCC TGG GTC AGC TTC CTA AG-3', and HAS1 (antisense primer), 5'-AAA CTG CTG CAA GAG GGT ATT TTC TCT-3'. HAS2 (sense primer), 5'-CTA GTC TTT ACC CAG CAG CAG C-3', and HAS2 (antisense primer), 5'-ACA CTA CGG AGG ATG ACG ATC C-3'; HAS3 (sense), 5'-ACA GGT TTC TTC ACC TTT CCC TTC TCT C-3', and HAS3 (antisense), 5'-GGC AGA TGA TCA TCT CTG C-3'; HPRT1 (sense), 5'-TGA CAC TGG CAA AAC AAT GCA-3', and HPRT1 (antisense), 5'-GTT CTC TTT CAC CAG CAA GCT-3'. The length of amplified fragments is as follows: HPRT, 93 bp; HAS1, 117 bp; HAS2, 107 bp; and HAS3, 166 bp. The correct length of the PCR product was confirmed by agarose gel electrophoreses. Standards and samples were assayed in a 25-μl reaction mixture containing 2× Brilliant SYBR green QPCR Master Mix, 30 nM reference dye (carboxy-X-rhodamine), 1.5 μl of forward and reverse primer, cDNA (2.5 μl), and double distilled H₂O.

**EMSA**—Preparation of nuclear extract, execution of EMSA, and EMSA specificity control experiments have been described in detail on a number of occasions (16, 19, 20). In short, oligonucleotides resembling consensus binding sites for NF-kB (5'-AGT TGA GGG GAC TTT CCC AGG C-3', AP-1 (c-Jun) (5'-CGG TTT ATG AGT CAG CCG GAA-3'), and CRE (5'-AGA GAT TGC CTG ACC TAG GTC-3')) were purchased from Promega (Promega, Mannheim, Germany). Mutated NF-kB oligonucleotides (5'-AGT TGA GGC GAC TTT CCC AGG C-3') were purchased from Santa Cruz Biotechnology. In addition, double-stranded oligonucleotides synthesized by MWG Biotech (5'-CAC GGG GGA ATT TCT CTC TGC-3') have been used as well. This element is identical to the sequence found -1331 to -1320 bp upstream of the HAS1 start codon that resemble a NF-kB binding domain (≥80% minimum matrix conservation). The sequence has been located with the help of Alibaba2.1. The underlined sequence in this region of the HAS1 promoter (5'-GGG AAT TCT TCC-3') contains, among others, prospective binding sites for NF-kB, RelA and NF-kB2.

All double-stranded oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-32P]ATP according to the protocol provided by Promega. After labeling and purification by chromatography, 5 μg of nuclear extracts were incubated with ~100,000 cpm of labeled probe in the presence of 1.5 μg of poly(dl,dc). The resulting mixture was separated on a native 6% polyacrylamide gel. For specific competition, 7 pmol of unlabeled oligonucleotides were included. For nonspecific competition, 7 pmol of double-stranded nucleotides were used. For supershift assays, 1 μl of specific supershift antibodies (Santa Cruz Biotechnology) were added to the nuclear extract solution ~15 min prior to the addition of the labeled probe.

**Results**

**EBV and Synthetic Double-stranded Viral RNA (dsRNA) Analogs Induce HAS1 Synthesis and HA Release in FLS**—EBV has been linked to the onset of RA (22). Whether exposure of FLS to EBV changes HAS expression patterns and to what degree HA synthesis is affected have been the focus of experiments shown in Fig. 1. FLS, grown to high density, were left untreated (Fig. 1, lane w/o), treated with IL-1B (5 ng/ml), or were exposed to purified, active EBV virus at concentrations ranging from 0.3 to 30 μg/ml medium. FLS were exposed to the virus for 1 h, and for the remaining hours of these experiments the virus concentration was 1/6 of the indicated initial concentration. The reason is that the total amount of medium initially was 0.5 ml per tissue culture dish, after 1 h; however, medium was added to the final amount of 3 ml used in all other culture dishes. Experiments were terminated after 8 h. HAS1 mRNA levels were significantly elevated in response to EBV treatment. HAS2 and HAS3 mRNA levels, however, were largely unchanged (data not shown). As shown in Fig. 1A, FLS exposed to 30 μg/ml (1×10⁹ virus particles/ml) of viable EBV respond similarly to cells exposed to IL-1B, one of the strongest inducers of HAS1 in FLS (14). IL-1B data were generated from three independent experiments; mRNA levels of the EBV dose curve are the means ± S.D. of two independent experiments.

Next, I tested whether elevated HAS1 mRNA levels also translate into higher HA synthesis by FLS exposed to EBV. Shown in Fig. 1B are data that demonstrate that similar to IL-1B (5 ng/ml), EBV (30 μg/ml) also induces the release of significantly increased amounts of HA into the culture supernatant (n = 3; p ≤ 0.05). In addition, cells that were exposed to the synthetic viral RNA analog poly(I:C) also responded with significantly increased HA synthesis (n = 3; p ≤ 0.05). HA was measured in supernatant of cells exposed to the indicated stimuli for 10 h. EBV concentrations (μg/ml) are indicated on the x axes.

Shown in Fig. 1C are additional data that resulted from initial experiments exploring time requirements for the activation of HAS genes by EBV. Over the course of such experiments (3 days) in FLS, HAS1 is the only gene that quickly responded to EBV exposure, whereas steady state mRNA levels of HAS2 and
HAS3 remained largely unaffected. Steady state mRNA levels for HAS1, HAS2, and HAS3 were monitored at 12, 24, 48, and 72 h following exposure of FLS to an initial concentration of 30 μg/ml of active EBV.

Double- and Single-stranded Viral RNA Activates HAS and HA Release—The synthetic dsRNA analog poly(I,C) has been used in the past to analyze virus-induced activation and signaling pathways (23, 24). As shown above, similar to EBV, poly(I,C) induces the release of HA in FLS. Shown in Fig. 2A are data that demonstrate that HAS1 mRNA levels in poly(I,C)-treated FLS are dose-dependently elevated and that low nanogram concentrations of this viral analog are sufficient to significantly increase HAS1 mRNA levels in FLS. Asterisk indicates the lowest concentration of poly(I,C) that resulted in significant activation of HAS1 mRNA. Data in B indicate that the single-stranded poly(C) molecule accounts for the majority of effects of double-stranded poly(I,C). Shown in C are HA levels found in the supernatant of FLS exposed to the indicated single-stranded viral analogs for 12 h.

After confirming that poly(I,C) and EBV act in similar ways regarding HAS activation and HA release, structural requirements of the synthetic viral mRNA analogs were tested. Single-stranded poly(A), poly(I), poly(U), and poly(C) were used to analyze their effectiveness as inducers of HAS1 transcription. Shown in Fig. 2B are data of experiments in which FLS were treated with 10 and 0.6 μg of ssRNA analogs for 8 h. FLS were left untreated (Fig. 2B, lane w/o) or were exposed to poly(C), poly(A), and poly(I). These data indicate that poly(C) is the most potent inducer of HAS1 translation followed by poly(A). Poly(I), however, only activates HAS1 significantly at the very high concentration of 10 μg/ml.

The fact that changes in mRNA levels induced by ssRNA translate into changes in HA production/release is shown in Fig. 2C. HA levels in cells treated with poly(C) are significantly higher than in control cells (Fig. 2C, lane w/o) (n = 6; p ≤ 0.05). Levels of HA in the supernatant of poly(A)-treated cells are lower but still significantly higher than HA in untreated cells.

FIGURE 2. dsRNA and ssRNA analogs are potent inducers of HAS1 and HA release. Shown in A are data demonstrating that low nanogram concentrations of the double-stranded viral analog poly(I,C) are sufficient to increase HAS1 mRNA levels in FLS. Asterisk indicates the lowest concentration of poly(I,C) that resulted in significant activation of HAS1 mRNA. Data in B indicate that the single-stranded poly(C) molecule accounts for the majority of effects of double-stranded poly(I,C). Shown in C are HA levels found in the supernatant of FLS exposed to the indicated single-stranded viral analogs for 12 h.

FIGURE 1. EBV induces HAS transcription and translation. Shown are data that demonstrate that in response to exposure to EBV, FLS respond with the transcription of HAS1 and the release of HA. mRNA levels were analyzed in FLS exposed to increasing concentrations of EBV (0.3, 3, and 30 μg/ml) for 8 h (A). Where indicated, IL-1β (5 ng/ml) has been added to FLS that served as positive controls. Data from untreated cells are shown in lane w/o. Shown in B are data that demonstrate that significantly higher levels of HA can be detected in the supernatant of FLS treated with EBV than in the supernatant of cells that were left untreated (lane w/o). In addition to EBV, the synthetic viral RNA analog poly(I,C) (5 μg/ml) also acts as a potent inducer of HA release. Shown in C is a time course experiment monitoring steady state HAS mRNA levels in FLS exposed to active EBV for up to 3 days.
Viral RNA Activates HAS1 through a MAPK p38 and Stress-activated Protein Kinase/JNK-dependent Pathway—Analyzing signaling pathways provides basic information with regard to regulatory mechanisms of a given gene as well as possible targets for future drug intervention. Shown in Fig. 3 are data that demonstrate that blocking the MAPK p38 pathway by chemical means nearly completely prevented poly(I,C)-induced HAS1 mRNA accumulation. Blocking the JNK pathway reduced poly(I,C)-induced HAS1 mRNA by roughly 50%. However, blocking the ERK pathway was without significant effect. In this type of experiment, FLS were preincubated with the indicated MAPK inhibitors for 60 min, afterward poly(I,C) (0.6 μg/ml) was added where indicated. RNA was isolated 8 h afterward. The MAPK p38 and JNK inhibitors were used at 5 μM and the ERK inhibitor at 10 μM, respectively.

Double- and Single-stranded RNA Analogs Activate NF-κB in FLS—Activation of NF-κB is a hallmark of many inflammatory processes (15). Whether and to what degree viral analogs are able to activate nuclear translocation of NF-κB in FLS was tested next. FLS were treated with equal concentrations (2.5 μg/ml) of either double-stranded (poly(I,C)) or single-stranded (poly(I), poly(A), poly(U), poly(C)) viral analogs. Shown in Fig. 4A is one of two EMSA experiments demonstrating well defined differences in the ability of single-stranded polynucleotide to activate NF-κB. Among the polynucleotides used, poly(C) is the most potent inducer of NF-κB activation followed by poly(A). However, effects on NF-κB-DNA complex formation were negligible in poly(U), and especially in poly(I)-treated FLS. Also of interest, treating FLS with the double-stranded poly(I,C) molecule resulted in NF-κB-DNA complexes that were approximately half of protein-DNA complexes formed by cells that were exposed to the single-stranded poly(C) molecule. Such data support the conclusion that only poly(C) accounted for the effects on NF-κB activation in poly(I,C)-treated FLS. Neither exposure to double-stranded nor to single-stranded polynucleotides resulted in changes in protein-DNA binding patterns in EMSA experiments utilizing the consensus CRE or AP-1-binding elements (Fig. 4A). FLS were left untreated (Fig. 4A, lane MED) or treated for 45 min with the indicated substances. IL-1β (5 ng/ml) was included as a positive control.

Consensus NF-κB elements were used for EMSA experiments shown in Fig. 4A. That the nuclear extract of poly(I,C)-treated FLS also interacts with the DNA element (CAC GGG GGA ATT TCT CTC TGC), an NF-κB-binding site that can be found in the promoter region of HAS1, is shown in Fig. 4B. As shown in Fig. 4B, left panel, consensus and HAS1-NF-κB elements compete for protein binding in EMSA experiments. Aliquots of nuclear extracts of poly(I,C)-treated FLS were preincubated (15 min) with unlabeled consensus NF-κB or AP-1 oligonucleotides. Although adding AP-1 elements had no effect on the shifted complex, consensus NF-κB elements subsequently prevented the binding of proteins to the prospective NF-κB sequence found in the HAS1 promoter. That proteins of the NF-κB family are indeed present in the shifted DNA-protein complex is shown in Fig. 4B, right panel. Shown there are supershift experiments that indicate the presence of p65 in the protein complex that interacts with HAS1-NF-κB elements.

A number of additional controls (competition and supershift assays) for EMSAs utilizing the consensus NF-κB elements have been shown before (17, 19). Parts of such control experiments are also shown in Fig. 4B. In Fig. 4, lane free Pr, indicates the positions of unbound γ-32P-labeled oligonucleotides and/or lanes in which nuclear extracts were omitted from the reaction mixture.

Poly(C)-induced HAS1 Activation Relies on the Activation of the Transcription Factor NF-κB—HAS1 is among those genes that can be activated in NF-κB-dependent as well as independent ways (17). Because, as shown in Fig. 3, NF-κB is activated by poly(C), I tested whether this transcription factor is essential for viral RNA-induced HAS1 transcription. To this effect two adenovirus constructs were used expressing either IκBα or a mutated versions of IKK-2. As demonstrated before, overexpression of a mutated IKK-2 or IκBα protein is well suited to test the involvement of NF-κB (25, 26). Fig. 5 shows the mean ± S.D. of experiments that indicate that NF-κB is essential for viral RNA-mediated HAS1 activation. In these experiments, 5 μg/ml poly(C) was used as an inducer of HAS1 transcription. As indicated in Fig. 5, the x axis, FLS were left untreated or transfected with the two adenovirus constructs (mIKK, IκB). Three days later, cells were stimulated with poly(C) for 8 h. Although FLS exposed to poly(C) responded with the activation of HAS1 (Fig. 5, lane w/o), cells in which the activation and translocation of NF-κB was blocked were unable to do so. As shown in Fig. 5, both mIKK and IκB dramatically diminished

FIGURE 3. MAPK play an essential role in poly(I,C)-induced HAS1 activation. Shown here is a comparison of HAS1 mRNA levels in FLS left entirely untreated (lane w/o), treated with poly(I,C) only (lane poly I,C), or exposed to specific MAPK inhibitor (Inh) prior to stimulation with the double-stranded viral analog.

(n = 6; p = 0.019). The final concentration of each of the compounds used was 5 μg/ml. Culture medium was collected 12 h later; IL-1β (5 ng/ml) was used as a positive control and to provide some measure of comparison (Fig. 2C, lane IL-1).
poly(C)-induced HAS1 mRNA levels. That transfection with these adenovirus constructs did not change basal HAS1 mRNA levels is shown in Fig. 5, lanes mIKK and IkB.

Polymers Shorter Than 20 bp Are Ineffective—In an initial attempt to investigate minimum length requirements of poly(C) structures that would activate HAS1, 5-, 10-, and 20-mer poly(C) oligonucleotides were used. As shown in Fig. 6A, compared with untreated cells (lane w/o), steady state HAS1 mRNA levels are not significantly altered in FLS that are treated for 8 h with 10 μg/ml of the indicated poly(C) polymers. With regard to HAS1 activation, these cells are fully functional, as is shown in control experiments demonstrating significantly increased levels of HAS1 mRNA in IL-1β (5 ng/ml)-treated cells.

In FLS, HAS1 Is Not Activated in Response to Endotoxin—A number of experiments were performed to rule out the possibility that endotoxin contamination could play a role in the reported findings (at high polynucleotide concentrations results of endotoxin measurements were inconclusive). Endotoxin as well as Staphylococcus aureus suspensions have been shown to be potent inducers of proinflammatory genes. Shown in Fig. 6B are data in which FLS were treated for 8 h with 10 and 1000 ng/ml of endotoxin serotype 055:B5 or with 1000 ng/ml of endotoxin serotype 127:B8. In addition to using purified endotoxin, FLS were also exposed to solutions containing 50 μg/ml of fixed S. aureus (Fig. 6B, lane SA (50 μg/ml)) and to IL-1β (5 ng/ml). As shown in Fig. 6B, even the enormous concentrations of endotoxin used in these experiments did not result in significantly elevated steady state HAS1 mRNA levels in this cell type (n = 3; p = 0.05). HAS1, however, was highly up-regulated in these experiments in cells that were exposed to IL-1β.

DISCUSSION

Viruses are an amazingly diverse group of organisms. They have been categorized into seven groups based on their genetic material, e.g. dsDNA, ssDNA, dsRNA, ssRNA with DNA intermediate, ssDNA with RNA intermediate, etc. Viral infections directly account for, or are partially implicated in, an array of diseases as varied as viruses themselves. RA is a debilitating disease whose origin has also been linked to viral infections (22). At present, the best evidence linking viral infection and RA is provided for EBV. This dsDNA virus has repeatedly been
implicated in arthritis. Patients suffering from RA have a 10-fold higher EBV DNA load in mononuclear cells than do mononuclear cells from healthy individuals; this elevation is stable and not influenced by the presence or absence of rheumatoid factor, age, duration of RA, disease activity, or RA treatment (22, 27). RA patients also have much higher numbers of EBV-infected B cells in their circulation and a significantly elevated load of EBV DNA in saliva (27, 28). Furthermore, a number of studies have shown that levels of EBV DNA and RNA are considerably higher in the synovium of RA patients than in healthy controls (29–32). Interestingly, EBV DNA loads are also highest in RA patients with at least one copy of the HLA-DRB1 epitope, the strongest known genetic risk factor for RA (30).

Irrespective of whether viruses are the cause or a consequence of RA, the above reports regarding the high viral load in synovium seemed important and led me to probe for a possible link between viral infection and HA production. To my knowledge, there is only one published report investigating the link between viral infections and HA synthesis. de La Motte et al. reported (24) that infection of smooth muscle cells with the respiratory syncytial virus resulted in increased adhesion of mononuclear cells, an event that has been found to be linked to increased HA synthesis.

HA degradation products are obviously detrimental in that they fuel inflammation (33–35). However, the concept that one or more of the intact HA molecules also contribute to inflammatory processes is rather new. It has, however, been known for a long time that HA is the main ligand for CD44. Nevertheless, efforts have nearly exclusively been directed at finding ways to prevent CD44 binding/activation, rather than on analyzing HA regulation. The reason for this seems to be that it is widely assumed that HA is ubiquitously present on all cells. At present, several years after the discovery of the three genes encoding HAS, rather little is known about the functional differences among HA molecules synthesized by these genes. Based on the unlikelihood that three genes encode for molecules with identical biological func-

EBV Induces HAS1

From the demonstration that poly(C) oligonucleotides activate NF-κB/HAS1, the following question arises: what length of poly(C) string suffices to produce this activation? As shown here, single-stranded polymers of 20 or less bases are ineffective as HAS1 inducers. It is, however, interesting that a number of viruses reportedly do contain longer homopolymeric poly(C) tracts (36, 37). Among these are members of the family of Picornaviridae that contain single-stranded poly(C) stretches ranging from 80 to more than 400 residues (37). Similarly, encephalomyocarditis and mengo-virus have also been reported to have the unusual feature of containing poly(C) stretches in their 5’-untranslated region (38). Reportedly, the above viruses, together with the related cardioviruses of the Picornavirus family, are the only known eukaryotic and prokaryotic genomes to contain such poly(C) tracts (39). However, similar features, namely long stretches of nucleotides, have also been shown to be present in other viruses, e.g., a leader sequence of at least 30 poly(A) nucleotides in vaccinia virus (40). Whether viruses with homopolymeric(C) tracts are indeed especially potent inducers of HAS1 or whether other nucleotide combinations would be equally sufficient has yet to be established. However, so far
no homopolymeric poly(C) tract has been reported to be present in EBV.

During the initial time course experiments, analyzing mRNA levels of HAS genes in response to viruses, it became evident that maximal steady state levels of HAS1 mRNA can be seen very early. Indeed, the time course of EBV-induced HAS1 activation in FLS is very similar to the one induced by pro-inflammatory cytokines. This seems to indicate that, at least for HAS1 induction in FLS, virus replication is not essential. That viruses or viral analogs can exert effects that are independent of virus replication has been shown before. For example, poly(I,C)-induced VCAM transcription in smooth muscle cells behaves in many ways very similar to HAS1 transcription in FLS. Vastly increased VCAM mRNA levels can be seen as early as 4 h and, after reaching maximal levels at around 8 h, taper off to nearly basal values (24). Although it is unclear how EBV, or for that matter polynucleotides, interacts with FLS, gp350 is the major envelope glycoprotein on EBV that specifically binds to CD21. Binding to this structure initiates viral replication. However, binding to CD21 also initiates a number of cellular and humoral immune responses that have been shown to be independent of viral replication. In monocyte-derived macrophages, for example, NF-κB activation is among such replication-independent events (41). In an other study, investigating EBV effects on endothelial cells, it has been shown that EBV but also the EBV latent membrane protein 1 rapidly activate NF-κB and a number of NF-κB-dependent genes (42).

Further support for the hypothesis that viral polynucleotides, fulfilling certain sequence requirements, could play a role in the activation of HAS and arthritis comes from the demonstration that nonmethylated bacterial DNA and synthetic DNA containing CpG motifs are able to induce arthritis (43). The absence of a poly(C) stretch with the required qualities as well as methylation of large parts of human DNA might explain why human DNA (data not shown) and autologous RNA were largely ineffective as inducers of HAS1.

Taken together the presented data strengthen the suggested link between HAS1 and inflammation in that exposure to virus as well as synthetic viral analogs activate only HAS1, leaving mRNA levels of the two other HAS genes unaltered. This is relevant because a causal link between activation of HAS genes and inflammation has already been established through the demonstration that virus-induced leukocyte adhesion in inflammation depends on newly synthesized HA (24). Also, p38 MAPK is an important signaling molecule leading to the activation of many proinflammatory genes (44, 45). As shown here, p38 is essential in that HAS1 induction in FLS by viral analogs depends to a large degree on the activation of this kinase. Finally, a further indirect indicator of the (patho)physiological functions of HAS1 is provided by the demonstration that virus-induced HAS1 depends on the activation of NF-κB, a transcription factor that is essential for the activation of most proinflammatory genes.

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REFERENCES

1. Bird, H. A., and Snaith, M. L. (1999) in Challenges in Rheumatoid Arthritis (Bird, H. A., and Snaith, M. L., eds) 1st Ed., pp. 1–399, Blackwell Scientific, Oxford
2. Muller-Ladner, U., Kriegsmann, J., Gay, R. E., and Gay, S. (1995) *Rheum. Dis. Clin. North Am.* 21, 675–690
3. Chaiamnuay, S., and Bridges, S. L., Jr. (2005) *Pathobiology* 72, 203–216
4. Turesson, C., and Matteson, E. L. (2006) *Mayo Clin. Proc.* 81, 94–101
5. Alamanos, Y., and Drosos, A. A. (2005) *Autoimmun. Rev.* 4, 130–136
6. Ma, Y., and Pope, R. M. (2005) *Curr. Pharm. Des.* 11, 569–580
7. Klareskog, L., Alfredsson, L., Rantapaa-Dahlqvist, S., Berglin, E., Stolt, P., and Padyukov, L. (2004) *Ann. Rheum. Dis.* 63, Suppl. 2, 28–31
8. Perl, A. (1999) *Ann. Rheum. Dis.* 58, 454–461
9. Geiler, T., Kriegsmann, J., Keyszer, G. M., Gay, R. E., and Gay, S. (1994) *Arthritis Rheum.* 37, 1664–1671
10. Pap, T., Muller-Ladner, U., Gay, R. E., and Gay, S. (2000) *Arthritis Rheum.* 42, 361–367
11. Seemayer, A. C., Kuchen, S., Kuenzler, P., Rihoskova, V., Rethage, J., Aicher, W. K., Michl, B. A., Gay, R. E., Kyburz, D., Neidhart, M., and Gay, S. (2003) *Ann. J. Pathol.* 162, 1549–1557
12. Laurent, T. C., Laurent, U., and Fraser, J. R. (1996) *Ann. Med.* 28, 241–253
13. Agren, U. M., Tammi, R. H., and Tammi, M. I. (1997) *Free Radic. Biol. Med.* 23, 996–1001
14. Stuhlmeier, K. M., and Pollaschek, C. (2004) *J. Biol. Chem.* 279, 8753–8760
15. Siebenlist, U. S., Brown, K., and Franzoso, G. (1995) in *Inducible Gene Expression, Environmental Stresses and Nutrients* (Bauerle, P. A., ed) pp. 93–141, Birkhauser Boston, Inc., Cambridge, MA
16. Markovic, M., and Stuhlmeier, K. M. (2006) *J. Mol. Med.* 84, 821–832
17. Stuhlmeier, K. M., and Pollaschek, C. (2005) *J. Biol. Chem.* 280, 42766–42773
18. Stuhlmeier, K. M., and Pollaschek, C. (2004) *Rheumatology* 43, 164–169
19. Stuhlmeier, K. M. (2007) *J. Immunol.* 179, 655–664
20. Stuhlmeier, K. M., Csizmadia, V., Cheng, Q., Winkler, H., and Bach, F. H. (1994) *Eur. J. Immunol.* 24, 2186–2190
21. Stuhlmeier, K. M. (2005) *J. Immunol.* 174, 7376–7382
22. Costenbader, K. H., and Karlson, E. W. (2006) *Arthritis Res. Ther.* 8, 204
23. Jiang, Z., Zamanian-Daryoush, M., Nie, H., Silva, A. M., Williams, B. R., and Li, X. (2003) *J. Biol. Chem.* 278, 16713–16719
24. de La Motte, C. A., Hascall, V. C., Calabro, A., Yam, Y.-L., Bierman, J., and Strong, S. A. (1999) *J. Biol. Chem.* 274, 30747–30755
25. Wrighton, C. J., Hofer-Warbinek, R., Moll, T., Eyten, R., Bach, F. H., and de Martin, R. (1996) *J. Exp. Med.* 183, 1013–1022
26. Oitzinger, W., Hofer-Warbinek, R., Schmidt, J. A., Koshelnick, Y., Binder, R. B., and de Martin, R. (2001) *Blood* 97, 1611–1617
27. Tosato, G., Steinberg, A. D., Yarchao, R., Heilman, C. A., Pike, S. E., De Seau, V., and Blaese, R. M. (1984) *J. Clin. Invest.* 73, 1789–1795
28. Newkirk, M. M., Watanabe Duffy, K. N., Leclerc, J., Lambert, N., and Shiroya, J. B. (1994) *Br. J. Rheumatol.* 33, 317–322
29. Blaschke, S., Schwarz, G., Moncke, D., Binder, L., Muller, G., and Reuss-Borst, M. (2000) *J. Rheumatol.* 27, 866–873
30. Sael, I. G., Krimmel, M., Steidle, M., Gerneth, F., Wagner, S., Fritz, P., Koch, S., Zacher, J., Sell, S., Einsele, H., and Muller, C. A. (1999) *Arthritis Rheum.* 42, 1485–1496
31. Takei, M., Mitamura, K., Fujiwara, S., Horie, T., Ryu, J., Osaka, S., Yoshino, S., and Sawada, S. (1997) *Int. Immunol.* 9, 739–743
32. Takeda, T., Mizugaki, Y., Matsubara, L., Imai, S., Koike, T., and Takada, K. (2000) *Arthritis Rheum.* 43, 1218–1225
33. Termeer, C., Benedix, F., Sleeman, J., Fieber, C., Voith, U., Ahrens, T., Miyake, K., Freundberg, M., Galanos, C., and Simon, J. C. (2002) *J. Exp. Med.* 195, 99–111
34. Noble, P. W., McKee, C. M., Cowman, M., and Shin, H. S. (1996) *J. Exp. Med.* 183, 2373–2378
35. Scheibner, K. A., Lutz, M. A., Boodo, S., Fenton, M. J., Powell, J. D., and Horton, M. R. (2006) *J. Immunol.* 177, 1272–1281
36. Black, D. N., Stephenson, P., Rowlands, D. J., and Brown, F. (1979) *Nucleic Acid Res.* 7, 3275–3280
EBV Induces HAS1

37. Mellor, E. J., Brown, F., and Harris, T. J. (1985) J. Gen. Virol. 66, 1919–1929
38. Duke, G. M., Osorio, J. E., and Palmenberg, A. C. (1990) Nature 343, 474–476
39. Martin, L. R., Neal, Z. C., McBride, M. S., and Palmenberg, A. C. (2000) J. Virol. 74, 3074–3081
40. Ahn, B. Y., and Moss, B. (1989) J. Virol. 63, 226–232
41. D’Addario, M., Ahmad, A., Xu, J. W., and Menezes, J. (1999) FASEB J. 13, 2203–2213
42. Xiong, A., Clarke-Katzenberg, R. H., Valenzuela, G., Izumi, K. M., and Millan, M. T. (2004) Transplantation 78, 41–49
43. Deng, G. M., and Tarkowski, A. (2000) Arthritis Rheum. 43, 356–364
44. Saklatvala, J. (2004) Curr. Opin. Pharmacol. 4, 372–377
45. Kaminska, B. (2005) Biochim. Biophys. Acta 1754, 253–262