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Short communication

Identification of gene expression profiles in HeLa cells and HepG2 cells infected with Coxsackievirus B3

Alexander Rassmann[a,d], Ulrike Martin[b], Hans-Peter Saluz[a], Stefan Peter[c], Thomas Munder[a,e], Andreas Henke[b,*]

[a] Leibniz Institute for Natural Product Research and Infection Biology-Hans-Knöll-Institute, Department of Cell and Molecular Biology, Jena, Germany
[b] Department of Virology and Antiviral Therapy, Jena University Hospital, Friedrich Schiller University Jena, Jena, Germany
[c] HELIOS Clinical Centre Erfurt, Department of Children and Youth Medicine, Erfurt, Germany
[d] Bioscientia Institut für Medizinische Diagnostik GmbH, Gotha, Germany
[e] University of Applied Science Jena, Department of Medical Engineering and Biotechnology, Jena, Germany

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Viral infections of host cells cause multiple changes of cellular metabolism including immediate defense mechanisms as well as processes to support viral replication. Coxsackievirus B3 (CVB3) is a member of the Picornavirus family and is responsible for a wide variety of mild or severe infections including acute and chronic inflammations. Thereby, intracellular signaling can be changed very comprehensively. In order to compare the influence of CVB3 replication on gene expression pattern of two different cell lines, DNA microarray systems were used to study a set of 780 genes related to inflammation. Expression analysis of HeLa cells and HepG2 cells infected with CVB3 identified 34 genes whose mRNA levels were altered significantly upon infection. The expression of additional 16 genes in HepG2 cells and 31 genes in HeLa cells were found to be influenced during CVB3 replication as well. All genes expressed differentially were sorted with regard to their functions and interpreted in view of known contributors to the infection process. The activation of the tumor necrosis factor pathways by CVB3 represents one peculiar observation, including apoptosis, stress, and inflammation responses.

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Coxsackievirus B3 (CVB3), as member of the *Picornaviridae*, is a small, non-enveloped enterovirus containing a single positive-stranded RNA genome, which encodes 11 viral proteins. Most infections proceed without any symptoms but CVB3 is also associated with different forms of acute and chronic inflammatory diseases, including pancreatitis, aseptic meningitis, and myocarditis. Thereby, a wide variety of virus/host cell interactions may occur, which are under investigation today. So far several data have been published to characterize gene profiles in cells (Kim et al., 2005) and tissue (Lang et al., 2008) infected with CVB3. For example, DNA microarrays were used to detect changes in gene expression in myocardial tissue of mice infected with CVB3 (Taylor et al., 2000).

The present study provides further information on host gene expression changes, which might be involved in cellular responses to CVB3 infections. Gene expression data of two different cell lines infected with CVB3 were obtained and compared with each other. Genes, which are involved preferentially in inflammation, apoptosis, and control of related processes in infected versus non-infected HeLa cells as well as HepG2 cells, were tested using a cDNA microarray system. Genes expressed differentially were sorted and discussed in view of the infection process.

At first, viral replication efficacy was compared between HeLa cells (derived from human cervix carcinoma) and HepG2 cells (derived from human hepatocellular carcinoma). For this purpose, monolayers of both cell types were infected with CVB3 using a multiplicity of infection (m.o.i.) of 5. After 45 min, cells were washed twice to remove all unbound viruses. Then, samples were taken at different time post infection (p.i.) and the total amount of infectious virus in each sample was measured in triplicate by standard $TCID_{50}$ assays. As shown in Fig. 1, one-step growth-curve analyses revealed that CVB3 replication in HepG2 cells is delayed clearly in contrast to HeLa cells.

In order to generate comparable experimental conditions for cDNA analyses, HeLa cells were obtained at 3 h p.i. and HepG2 cells at 6 h p.i., respectively. In both cell lines, this time indicates the state 2 h prior the start of massive progeny release. Control cells were mock-infected. Then, cells were washed with PBS and total RNA was isolated (Qiagen, Hilden, Germany), followed by DNase I treatment (Ambion, Life Technologies, Frankfurt, Germany). For every experiment 10 μg of total RNA, isolated either from infected or

* Corresponding author at: Department of Virology and Antiviral Therapy, Hans-Knöll-Straße 2, 07745 Jena, Germany. Tel.: +49 3641 9395713; fax: +49 3641 9395702.
E-mail address: Andreas.Henke@med.uni-jena.de (A. Henke).

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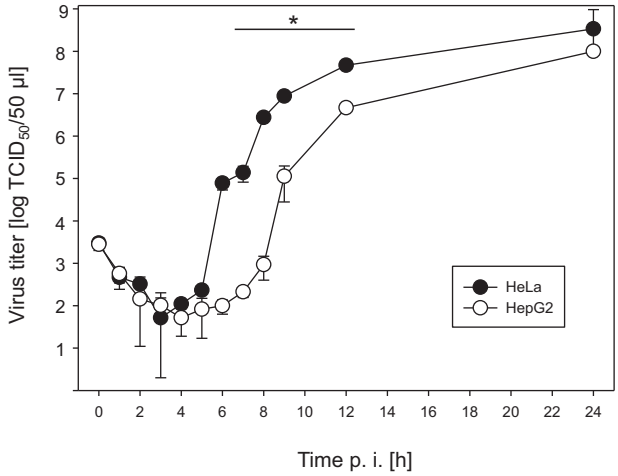

Fig. 1. One-step growth curve experiments.

control cells, were reverse transcribed into cDNA and labeled subsequently with AlexaFluor 647 or AlexaFluor 555 monofunctional NHS-esters, respectively. Thereafter, labeled cDNA molecules were co-hybridized to custom made arrays (Lab-Arraytor® 60 inflammation, SIRS-Lab, Jena, Germany) and gene expression profiles of cells either infected with CVB3 or without infection were analyzed. One of those arrays comprises 3 identical separate arrays; each consisting of probe spots of 780 genes (see supplementary data, Table 1) distributed over 4 sub-arrays. Spots are composed of single-stranded oligo nucleotides, 55–70 bases long, integrating transcription of human genes involved in inflammation, apoptosis, cell cycle control and related processes. Data pre-processing included spot detection and background subtraction spot flagging according to defined signal-to-noise threshold values. Normalization and transformation of all signals obtained from both laser channels were performed as well as averaging of data from array replicates and determination of thresholds.

To minimize the biological variance and to reach conclusions on the host responses to viral infection, experiments were repeated independently 3 times with HepG2 cells and 2 times with HeLa cells. All data were compared between both cell lines and sorted according to their cellular functions. As shown in Table 1a, the transcriptional activation of the tumor necrosis factor (TNF) pathway is detectable in HeLa cells and HepG2 cells infected with CVB3 (represented by the genes TNFRSF10D, TRADD, TRAF4 and TRAF6). This confirms and extends the data of Seko et al. (1999). In addition, an activation of signaling based on NFκB was found in both cell lines infected with CVB3 as well, which is demonstrated by the up-regulation of NFKBIA, LTB4R, and down-regulation of G3BP2 transcription. This result is in accordance to data published previously (Esfandiarei et al., 2007). The role of NFκB was investigated as a potential downstream mediator of signals based on the phosphotidylinositol 3-kinase (PI3K/Akt) cascade, which might be involved in regulating cellular injuries induced by CVB3. It was reported that CVB3 infection induced the translocation of NFκB into the nucleus of infected cells. Inhibition of the PI3K/Akt pathway decreases NFκB activation induced by CVB3. Further, NFκB inhibition suppresses significantly host viability, suggesting a pro-survival role for NFκB. The NFκB pathway is also involved in TNF activation (Adcock, 1997; Liu et al., 2005). Further it was found that short-term treatment of cells with TNF-α as a potent activator of NFκB promotes host cell viability without affecting virus replication (Esfandiarei et al., 2007). An activation of NFκB signaling induced by the pathogen has already been reported for different viruses (e.g. reviewed in Hiscott et al. (2006) and Unterholzner and Bowie (2008), respectively). The up-regulation of the FOS gene transcript and of other transcription and translation factors or modulators (e.g. EGR1) supports also the hypothesis on TNF pathway activation as well (Adcock, 1997; McManus et al., 2002). Previously, a modification of the protein 14-3-3 ε in cells infected with CVB3 was shown using a proteome wide approach (Rassmann et al., 2006). This protein is also regulated in a pathway dependent on TNF-α signalling (Benzing et al., 2002). In addition, it has been demonstrated that CVB3 induces the activation of the mitogen-activated protein kinase (MAPK) p38 (Rassmann et al., 2006; Si et al., 2005). Even if the transcription is down-regulated due to an infection (see Table 1a), the activation of the MAPK p38 by phosphorylation could be shown (Rassmann et al., 2006; Si et al., 2005). The activation of the MAPK p38 via TRAF6 as an up-stream factor leads to an increase of interleukin (IL)-8. In HepG2 cells, an up-regulation of IL-8 mRNA (1.75-fold) could be detected (see Table 1b). IL-8 is a pro-inflammatory chemoattractive protein, which stimulates immune cells (Elssner and Vogelmeier, 2001; Gidron et al., 2002).

The present results suggest strongly the activation of TNF pathways in cells infected with CVB3, which could induce apoptotic events. One important aspect of apoptosis is the activation of caspases (Cohen, 1997). The present study indicates that caspase 10 gene transcription was up-regulated in HeLa cells and HepG2 cells after CVB3 infection (Table 1a). However, the transcription of caspase 3 was up-regulated only in HeLa cells (Table 1c), in accordance with results obtained by Yuan and coworkers (Yuan et al., 2003), but was down-regulated in HepG2 cells (Table 1b). This observation may indicate that viral replication could be more advanced in HeLa cells at 3 h p.i. in comparison to HepG2 cells at 6 h p.i. But the expression of caspases itself is not enough to induce apoptotic pathways. Additional cleavage processes are necessary to generate fully active enzymes. Nevertheless, it could be possible that CVB3 inhibits activation of apoptosis until a specific state of infection occurs. Afterwards, the transcription of genes related to apoptosis like caspase 3 is increased followed by their conversion into the active form of the protein as it was shown previously (Carthy et al., 1998). The up-regulation of the MDM4 gene transcription only in HepG2 cells supports the hypothesis that CVB3 decreases the initiation of apoptosis during early stages of infection. MDM4 is an inhibitor of the tumor suppressor activity of p53 and is targeted by the adenoviral E1A to stabilize p53 (Li et al., 2004). Apoptosis has been linked frequently to diseases induced by CVB3. For example, apoptotic processes are present in myocardial tissue of patients with dilated cardiomyopathy (Olivetti et al., 1997). It was also demonstrated that apoptotic cell death occurs inside and outside of inflamed areas in the myocardial tissue of mice infected with CVB3 (Gebhard et al., 1998; Henke et al., 1995; Huber et al., 1996). From the viral point of view, both the induction and the inhibition of apoptosis is reasonable for viral replication: on the one hand, the delay of apoptosis enables the virus to generate as much progenies as possible without inducing premature cell death (O'Brien, 1998). On the other hand, the induction of apoptosis contributes to viral release from host cells and reduces inflammatory immune responses at a given time during replication (Jarasch et al., 2007; Martin et al., 2007). Two mechanisms of induction of apoptosis can be triggered by CVB3: the intrinsic pathway requiring virus replication and the extrinsic pathway signaling through death receptors, like TNF receptors. For example, the over-expression of the pro-apoptotic protein Siva was already reported during infections with CVB3 under in vitro and in vivo conditions (Henke et al., 2001, 2000; Martin et al., 2004). This host cell protein interacts with the viral protein VP2 and binds to the CD27 receptor, which is a member of the TNF family (Camerini et al., 1991; Gravestein et al., 1993; Prasad et al., 1997; Smith et al., 1994).

Different stress responses and pro-inflammatory pathways are activated during CVB3 infections. For example, in HeLa cells infected with CVB3 the expression of the cystein-rich protein gene

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