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Downregulation of Endothelin Receptor mRNA Synthesis in C6 Rat Astrocytoma Cells by Persistent Measles Virus and Canine Distemper Virus Infections

NICOLE N. MEISSNER AND KLAUS KOSCHEL*

Institut für Virologie und Immunbiologie, der Universität Würzburg, D-97078 Würzburg, Germany

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Persistent infections of C6 rat astrocytoma cells with measles (subacute sclerosing panencephalitis [SSPE]) virus (C6/SSPE cells) or canine distemper virus (C6/CDV cells) cause a loss of endothelin-1 (ET-1) binding to its specific receptors (ETR_A type) and subsequent ET-1-induced Ca^{2+} signaling. It was the aim of this study to investigate the underlying mechanism of this phenomenon in more detail. By using an RNase protection assay, it was found that ETR_A mRNA disappears, whereas other cellular mRNA species, e.g., β-actin mRNA, were not influenced. The data show that the loss of the ET-1 signaling pathway in C6/SSPE and C6/CDV cells is due to a receptor downregulation at the transcriptional level.

Endothelins (ET) are a group of very potent vasoconstrictive peptide factors discovered in 1988 (25, 26). Specific receptors were found not only on smooth muscle cells but also on other target cells, including endothelial cells, fibroblasts, mesangial cells, and cells of the central nervous system (15). ET are distributed heterogeneously throughout the nervous system. Both endothelin-1 (ET-1) and ET-3 are found in neuronal tissue, with high concentrations of ET-1 in the brain stem and spinal cord (17). In response to ET binding both short- and long-term effects are observed (for a review, see reference 20). In the neural tissue, ET obviously play an important role as neuromodulators, since they have mitogenic effects on primary astrocyte cultures and induce a release of gonadotropin-releasing hormone, substance P, antidiuretic hormone, and other hormones from the hypothalamus and the pituitary gland (21). A loss of ET type A receptors (ETR_A) from the surfaces of neural cells could therefore have serious consequences for the affected individual.

To study the influences of persistent virus infections on the functions of cells in the central nervous system, rat C6 astrocytoma cells (ATCC CCL 107) (3), uninfected or persistently infected with either measles (subacute sclerosing panencephalitis [SSPE] strain Lec) virus or canine distemper virus (CDV), designated C6/SSPE and C6/CDV cells, respectively (7), all free of mycoplasmas (4), were used. In a previous report we discussed the specific loss of the ET-1-signaling pathway in C6/SSPE cells due to a loss of specific receptor binding compared with uninfected C6 cells (22). As we also described before (22), C6 cells cannot be directly infected with measles virus because the specific receptors necessary for virus adsorption are missing on these cells. However, they easily become infected through cell-cell contact by cocultivating them with C6/SSPE cells in a 100:1 or 40:1 ratio, without the occurrence of a cytopathic effect in the culture. The C6/SSPE cells only marginally express F protein on their surfaces, and only traces of F mRNA are detectable (26a). These results support the observation that the absence of F protein by restriction of F mRNA is a prerequisite for the development of the persistence of measles virus (8). The cocultivation allows us to study the behavior of the ETR_A and its mRNA during the establishment of viral persistence to find out why the ETR_A are downregulated during infection. There are several possible explanations for this downregulation: (i) changed receptor species, (ii) inhibition of the insertion of receptor protein into the plasma membrane, (iii) inhibition of the translation of ETR_A mRNA, and (iv) inhibition of ETR_A mRNA transcription. The work presented here addressed these possibilities.

Binding of ET-1 to C6, C6/SSPE, and C6/CDV cells. Earlier investigations in this laboratory (22) showed that C6/SSPE cells have a very small amount (<5%) of specific binding of human ^{125}I-ET-1 (2,000 Ci/mmol; Amersham) compared with uninfected C6 cells. A similar result was obtained with C6 cells persistently infected with another paramyxovirus, CDV. The specific ET-1 binding of C6/CDV cells is compared with that of C6 and C6/SSPE cells in Table 1. It was reduced to about 18% of the level of binding in uninfected cells.

Determination of the amount of ETR_A mRNA. To study the loss of binding in more detail, we determined the amount of ETR_A mRNA in uninfected C6 (control) and C6/SSPE cells as well as in C6/CDV cells by an RNase protection assay (18). As a further control we used A10 cells (ATCC CRL 1476), a rat aortic smooth muscle cell line from which the ETR_A gene had been isolated by Lin et al. (13). The amount of β-actin mRNA in the same samples was determined simultaneously as an internal standard, assuming that this constitutively expressed mRNA was not significantly affected by the persistent infection.

Negative-strand RNA probes for the detection of ETR_A and β-actin mRNA were generated by in vitro transcription as follows. Plasmid pcDNAETRA containing the full-length cDNA of a rat ETR_A clone from A10 cells (13) (a gift from H. Y. Lin, Massachusetts Institute of Technology, Cambridge) was linearized by *Msp*I, and pSP64bact72, a pSP64 vector with a 280-bp inclusive GC tail rat β-actin cDNA insert (6, 19) (a gift from T. Hünig of our institute), was linearized by *Rsa*I. Negative-strand RNA probes were synthesized by SP6 polymerase by using [α-^{32}P]rCTP for labeling. Subsequently, the DNA templates were digested by RQ DNase, and the RNA reaction products were separated on a preparative denaturing 3% polyacrylamide gel. Transcripts of appropriate lengths

* Corresponding author. Mailing address: Institut für Virologie und Immunbiologie, der Universität Würzburg, Versbacher Str. 7, D-97078 Würzburg, Germany. Fax: 931-210-2243.

TABLE 1. Specific binding of ^{125}I-ET-1 to C6, C6/CDV, and C6/SSPE cells

Cells	Level of specific binding of ^{125}I-ET-1 (cpm/mg of protein)[a]			% Specific binding (mean ± SD)
	Expt 1	Expt 2	Mean ± SD	
C6	7,806	8,391	8,098 ± 413	100 ± 5
C6/CDV	1,545	1,416	1,480 ± 91	18.3 ± 6
C6/SSPE	587	557	572 ± 21	7.1 ± 4

[a] Each value was corrected by subtraction of the value of unspecific binding determined in the presence of a 1,000-fold surplus of unlabeled human ET-1 (Sigma). The amount of protein was determined by the method of Lowry et al. (14).

were identified by autoradiography and isolated from the gel for use as probes for the RNase protection assay.

For ETR_A mRNA and β-actin mRNA detection, 30 μg of total RNA (isolated as described by Kriegler (12) and 2×10^5 cpm of [α-^{32}P]rCTP-labeled RNA probes were hybridized and digested with RNase T_1 and RNase A as described by Melton et al. (18). The reaction products were analyzed by electrophoresis (40 W) on a denaturing (urea) 6% polyacrylamide gel followed by autoradiography.

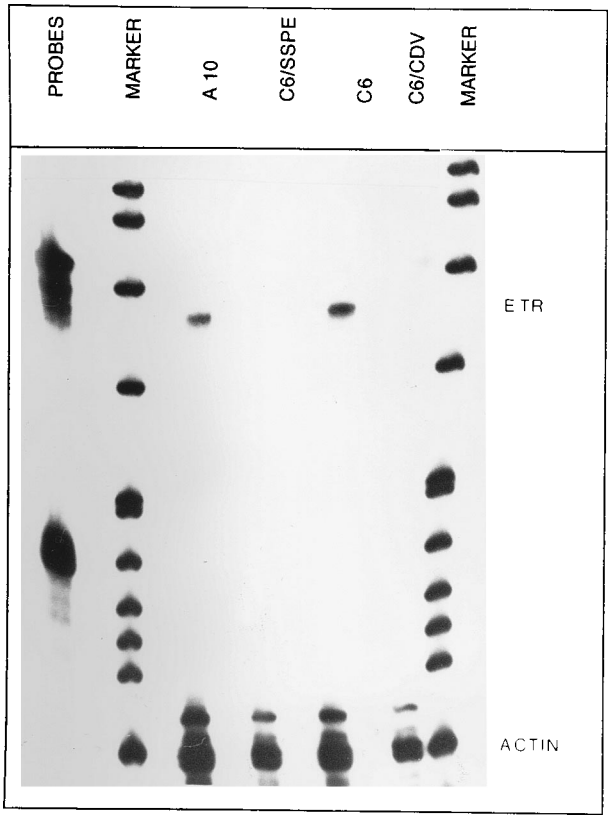

FIG. 1. Autoradiographic analysis of the amounts of ETR_A mRNA and β-actin mRNA from A10, C6, C6/SSPE, and C6/CDV cells (lanes 3 through 6, respectively). Equal amounts of total cellular RNA from the different cell types were hybridized with a 372-bp *Msp*I fragment probe for ETR_A mRNA and a 200-bp *Rsa*I fragment probe for β-actin mRNA. After hybridization, the mixtures were treated with RNase A and RNase T_1 in the RNase protection assay and subsequently analyzed on a 6% polyacrylamide gel. Lane 1, radiolabeled, undigested, transcribed, unhybridized probes for ETR_A mRNA and β-actin mRNA; lanes 2 and 7, end-labeled pBR322 plasmids after *Hpa*II digestion, used as a size marker.

The results of the autoradiography are shown in Fig. 1. No ETR_A mRNA signal was detected in the RNA extracts of C6/SSPE and C6/CDV cells, whereas β-actin mRNA was present in comparable amounts in RNA extracts of C6, C6/SSPE, and C6/CDV cells. These data are principally in agreement with the binding data shown in Table 1. From these results, we conclude that either persistent virus infections inhibit the specific transcription of ETR_A mRNA while the transcription of β-actin mRNA is not inhibited, or such infections lead to a very accelerated specific degradation of ETR_A mRNA.

To rule out the possibility of the selection of ETR-negative C6 cells during the course of viral persistence, we established a new persistent virus infection in C6 cells and monitored the specific ET-1 binding and the level of ETR_A mRNA expression as a function of time after infection.

Establishing viral persistence in C6 cells. Viral persistence was established in C6 cells by coculturing them with C6/SSPE cells at ratios of 100:1 to 40:1. The progression of infection was followed by fluorescent antibody staining for membrane and cytoplasmic viral antigens (2). After 3 to 5 cell culture passages, all cells were infected without the occurrence of a cytopathic effect. Uninfected and infected cells showed the same growth kinetics (data not shown). Therefore, the overgrowth of uninfected cells by infected cells is unlikely. That the uninfected cells indeed become persistently infected was confirmed by a cocultivation experiment using hygromycin B-resistant C6 cells (C6-pCMVhygro$^+$) (11) which were transfected with the hygromycin phosphotransferase *hph* gene (16) and drug-sensitive C6/SSPE cells. Starting the experiment by culturing the cells without hygromycin B for two passages gave the C6/SSPE cells a chance to infect the C6-pCMVhygro$^+$ cells. For selection, hygromycin B was then added to the cell culture medium. Drug-sensitive C6/SSPE cells died in the following passages. The remaining drug-resistant cells were all infected as demonstrated by immunofluorescence for measles virus proteins. Only one infectious virus particle was produced by about 500 to 1,000 C6/SSPE cells per 24 h. Furthermore, supernatants of C6/SSPE cells, collected 24 h after plating, could not infect C6 cells directly. Therefore, the mechanism of infection by cocultivation has to be a transmission of virus or viral ribonucleoprotein complexes containing polymerase by direct cell-cell contact. This could be very similar to the in vivo situation in which, in spite of the progression of the infection, no infectious viruses but only defective particles were found (1).

ET-1 binding to C6 cells during establishment of persistent infection. As we reported previously (22), ET-1 binding to the cells strongly decreased during the first 12 passages (Fig. 2). However, at later passages temporary strong fluctuations in the ET-1 binding were observed before a steady state with a very low level of binding was reached. This phenomenon seems to reflect the similar behavior of viral mRNA and viral proteins, which are known to fluctuate during the establishment of viral persistence in newly infected cells. In our experiments, this was studied by [^{35}S]methionine labeling of newly synthesized viral proteins and immunoprecipitation using polyclonal measles virus-hyperimmune sera from patients and monoclonal antibodies (a gift of E. Norrby, Stockholm, Sweden) followed by autoradiography of gels after separation by polyacrylamide gel electrophoresis (PAGE) (12a, 27) (data not shown).

ETR_A mRNA during establishment of viral persistence. Expression of ETR_A mRNA in the cells was studied in parallel to ET-1 binding. Results of the RNase protection assay are shown in Fig. 3. Controls consisted of RNA from uninfected C6 and long-term-infected C6/SSPE cells (Fig. 3, lanes 1 and 2). β-Actin mRNA was used as an internal standard in each sample.

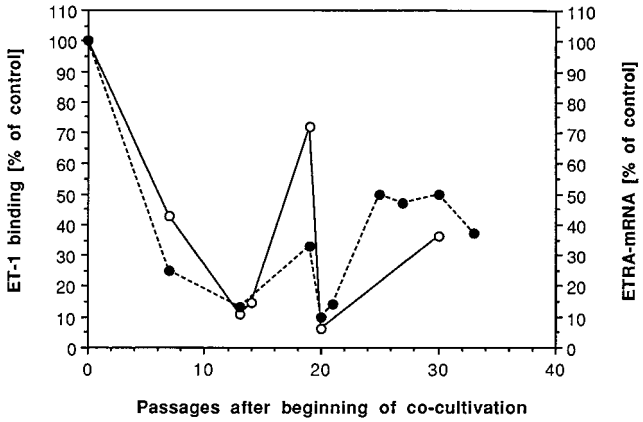

FIG. 2. Specific binding of ^{125}I-ET-1 (open circles) and the amount of ETR_A mRNA (solid circles) as a function of time after establishment of a persistent measles (SSPE) virus infection in C6 cells. ETR_A mRNA was measured by the RNase protection assay and PAGE. Values of uninfected controls were taken as 100%.

During the course of the persistent virus infection, ETR_A mRNA levels decreased (passages 8, 14, and 15). In passage 15, ETR_A mRNA levels strongly decreased. However, passage 20 showed a strong increase in ETR_A mRNA which was followed by a decrease in passage 21 and an additional increase in passage 31. The increase of ETR_A mRNA levels in some passages was directly associated with a higher level of binding of ET-1, as can be seen in Fig. 2, which also contains the densitometrical data from Fig. 3. The amount of β-actin mRNA, however, remained constant during the course of the experiment.

The fluctuation observed for ETR_A mRNA and ET-1 binding is an argument against the suggestion that the loss of ETR was a random phenomenon of single cells which were then preferentially selected.

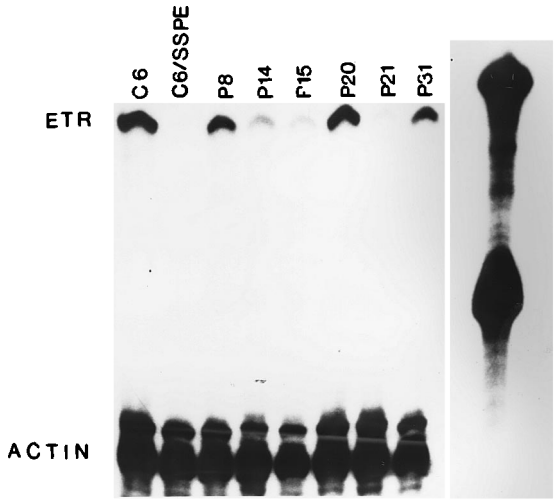

FIG. 3. Autoradiographic analysis of the amount of ETR_A mRNA and β-actin mRNA in uninfected C6 and C6/SSPE cells and in cells during passage (P) 8 (when all cells are infected), 14, 15, 20, 21, and 31 after starting the cocultivation of C6 and C6/SSPE cells (100:1). Total cellular RNA was isolated and the amounts of ETR_A mRNA and β-actin mRNA were estimated by the RNase protection assay using a 372-bp-long ETR probe (*Msp*I fragment) and a 200-bp-long β-actin probe (*Rsa*I fragment). Analysis of the protected fragments was carried out on a denaturing (urea) 6% polyacrylamide gel.

This report clearly shows that the reason for the previously described loss of specific ET-1 binding in the persistently infected C6/CDV and C6/SSPE cells (22) is based on a receptor downregulation either by inhibition of transcription itself or by specific degradation of ETR mRNA. There is no degradation or inhibition of transcription of cellular mRNA in general, as shown by the unchanged amount of β-actin mRNA in such cells. This is of interest, since during viral persistence the infected cell is not lysed by the virus, and the pathomechanism leading to disease might be based in early phases on specific virus-host interactions, which can result in the loss of some differentiated (luxury) functions of the cell without leading to cell death (10).

Our cocultivation method, used to establish a persistent measles virus infection without free virus, might be useful as a model for the spread of the measles (SSPE) virus in the central nervous systems of children and adolescents, causing an inflammatory degenerative central nervous system disease (11) in which no free virus is observed. However, one must keep in mind that C6 cells are transformed cells, and it is difficult to know whether the properties of our model are comparable to those of the in vivo situation.

The inhibition of transcription of certain cellular genes by paramyxoviruses has not been described before, whereas similar inhibition by other RNA viruses is well known. For example, mice which are persistently infected with lymphocytic choriomeningitis virus show growth retardation and hypoglycemia. This phenomenon is due to a drastically reduced production of growth hormone caused by a fivefold-decreased amount of growth hormone mRNA in the pituitary glands of infected animals (24). The advantage of the downregulation of ETR for the virus remains speculative. Interesting in this respect is the fact that in unstimulated lymphocytes, measles virus replication is suppressed, which might favor the establishment of viral persistence (9). Vainionpää et al. (23) concluded that second-messenger signaling by cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) stimulates measles virus replication in the infected cell. Thus, ETR downregulation might be advantageous for the establishment of viral persistence in C6 rat glioma cells because the cells are then less responsive to exogenous ET-1 stimuli, resulting in a drastically reduced PIP_2 cleavage and therefore also in the loss of Ca^{2+} signaling in the cells by this factor.

In an attempt to find a viral factor responsible for the ETR downregulation in the course of the establishment of a persistent viral infection in C6 cells, immunoprecipitations of measles virus proteins and ETR binding studies were performed in parallel for 30 passages of the cells. For Sendai virus, a paramyxovirus related to measles virus, the P protein served as a modulator by either inhibiting or initiating viral transcription (5). Curran et al. (5) assumed that the viral transcription factor was similar to other eucaryotic transcription factors and might therefore interact with some host transcription. However, our results could not clearly demonstrate such a function of measles virus or CDV P protein in respect to the observed downregulation of ETR_A mRNA. For elucidating the possible influences of single viral proteins on specific cellular functions in persistently infected C6 cells, stable expression of single viral proteins by transfection experiments may be helpful.

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