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Varicella-Zoster virus gene expression at variable periods following death in a rat model of ganglionic infection

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Abstract We used a rat model of Varicella-Zoster virus (VZV) ganglionic infection, which mirrors some of the features of VZV latency in humans, to determine the temporal pattern of expression of a VZV immediate-early gene (63) and a VZV late gene (40) at 0, 24 and 48 h after death of the animal. The immediate-early VZV gene 63 is known to be abundantly expressed during human ganglionic latency, while the late VZV gene 40 is not expressed during human latency. Using both RNA in situ hybridisation (ISH) and nested RT-PCR, it was found that at all time points in both thoracic and lumbar ganglia, the number of ganglia positive for VZV gene 63 was higher than for gene 40. The expression of gene 40 did not increase with time postmortem (pm) These results provide indirect support for the hypothesis that patterns of expression of VZV genes detected in human tissue at even 48 h pm reflect the pattern of expression during human ganglionic latency.

Keywords Varicella-Zoster virus · Ganglion · Latency · Reactivation · Viral gene expression

Varicella-Zoster virus (VZV) is a human herpesvirus that causes varicella (chickenpox) as a primary infection, following which the virus remains in latent form in the trigeminal ganglia (TG) and dorsal root ganglia (DRG) for the life of the host [1]. After a variable interval, the virus can reactivate to produce herpes zoster (shingles), which may lead to post-herpetic neuralgia, which is a major cause of human morbidity [1]. It is known that latent VZV is located predominantly in neuronal cells, with only occasional satellite cells infected [2]. Evidence to date also indicates that there is a highly restricted pattern of viral gene expression during human latency, with VZV genes 21, 29, 62, 63 and 66 being expressed, and no evidence of late viral gene expression [3, 4]. Results obtained on human tissues, which are usually obtained between 12 and 48 h postmortem (pm), always raise the possibility that the patterns of viral gene expression observed in such tissues reflect a particular stage of the reactivation process that may have been triggered by death of the individual. This is one of the major problems of investigating VZV latency in man.

The results of our previous explantation study [5] where human ganglia obtained pm were explanted onto permissive cells at 37°C for periods up to 11 days, a system whereby explantation should induce reactivation, suggest that viral reactivation immediately after death probably does not occur, since the profile of gene expression changed over time from an initially highly restricted to a more generalised pattern. A direct approach to this problem, however, requires the use of animal models. The rat model of VZV ganglionic infection [6] has proved to be a useful tool in investigating factors determining the initiation of VZV latency, and mirrors some, but by no means all, of the features of VZV latency in humans [7 and see below]. In this study, we used the rat

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model to investigate the dynamics of VZV gene expression over time pm. We hypothesised that if viral reactivation had not occurred in the immediate pm period, an immediate-early, but not a late, VZV gene, would be expressed in the ganglia between 0 h and 48 h after death of the animal. If this were shown to be the case, then it would support the notion that delays of up to 48 h before human ganglia are analysed for VZV gene expression are unlikely to be problematic in terms of triggering viral reactivation. We therefore analysed the pattern of expression of two VZV genes, ORF63, an immediate-early gene which is known to be abundantly expressed during latency [8], and ORF40 a late gene not expressed in latency [5, 9] to determine whether the time spent pm at 4°C has any influence on the viral reactivation. If there is predominant expression of VZV ORF 63 over time pm this would give credibility to the idea that one is studying truly latent virus. But if there were a progressive evolution from IE to L gene expression, then this would suggest the possibility that results observed in tissue harvested pm actually represent reactivated virus.

Inoculation with VZV-infected (a clinical isolate) Vero cells in both footpads of 17 rats was performed as previously described [6, 7]. Rats were euthanased with CO_2 gas and pentobarbital (200 mg/kg, I.P), and all tissues were removed 18 months postinfection. Thoracic and cervical ganglia were removed and either snap frozen (0 h) for RNA extraction and nested RT-PCR, or fixed and embedded for RNA in situ hybridisation (ISH), or else removed and left at 4°C for 24 or 48 h in DMEM prior to freezing or fixation. In 3/17 animals the rats were left in situ at 4°C for 48 h before removing ganglia, fixing and embedding for ISH and their organs harvested for RT-PCR. In addition, blood was taken from all animals at the time of death (0 h only) for DNA extraction and PCR. Spleen, liver, kidney and lung were removed and either snap frozen (0 h), or else left at 4°C for 48 h prior to removal for RNA extraction and subsequent nested RT-PCR. Identical procedures were performed on eight control rats inoculated with mock-infected Vero cells.

ISH was performed as previously described [2, 5]. For nested RT-PCR, RNA was extracted from frozen tissue using Trizol and extensively DNAsed, and the absence of DNA was then confirmed using PCR. cDNA was synthesized using Superscript II according to the manufacturer's instructions, and nested PCR performed using primers for either VZV ORF 63 or VZV ORF 40. The primers used are listed in Table 1.

The results of these experiments are summarised in Table 2. It can be seen that for ISH studies, at all time points after death, 0 h, 24 h and 48 h, a higher number of both thoracic and lumbar ganglia expressed VZV gene 63 compared to VZV gene 40. The results with RT-PCR showed more marked differences between expression of the two genes. At all time points, in both thoracic and lumbar ganglia, a higher number of ganglia expressed VZV gene 63 compared to gene 40. It should be noted that the six VZV 63 positive ganglia at 24 h and the positive thoracic ganglion at 48 h were also positive at 0 h. Therefore, there was no increase in the number of animals expressing VZV gene 63 at 24 h. At 48 h after death, 2/6 of the ganglia expressed VZV gene 63 compared to a total absence of expression of VZV gene 40. There was no significant difference in the expression of gene 63 at 0 h and 48 h by ISH ($P = 0.628$, chi-squared test) or in the expression of gene 40 at 0 h and 48 h by ISH ($P = 0.765$, chi-squared test). There was no significant difference in the expression of gene 63 at 0 h and 48 h by RT-PCR ($P = 0.333$, chi-squared test), or in the expression of gene 40 at 0 h and 48 h by RT-PCR ($P = 0.643$), Fisher exact probability test). For the three rats which had been left at 4°C for 48 h, 3/9 of ganglia had expression of gene 63 and 1/11 of gene 40 using ISH. These rats have been included in Table 2.

In addition, at the 0 h time point, 3/15 blood samples were positive for VZV DNA, and 7/56 organs from 6/14 VZV-infected animals were positive for VZV RNA. In comparison, at 48 h 1/9 organs from 1/3 VZV-infected animals were positive for VZV RNA. All tissues studied for control, uninfected, rats were negative for VZV DNA and RNA by ISH and RT-PCR.

It was essential to know that the sensitivity of the two VZV primer sets was comparable. Accordingly, RT-PCR was performed on VZV of known copy number and it was determined that the two primer sets were of comparable sensitivity both being able to detect at least 50 copies of VZV DNA.

We had reasoned that over a 48 h period following death of the animal, the expression of the late gene 40 would not increase in the absence of viral reactivation. Both our ISH and RT-PCR results appear to support this. Using nested RT-PCR, 0/5 ganglia expressed gene 40 at 48 h compared with 1/9 studied at 0 h, and using RNA ISH 2/17 ganglia expressed gene 40 at 48 h compared with 1/12 at 0 h. It has to be recognised that expression of gene 40 at the 0 h time-point may indicate persistent infection, a limitation of the rat model. Nevertheless, we detected no convincing evidence of increased expression of gene 40 over the time studied.

Table 1 VZV oligonucleotide primers used in this study

Primer	5′–3′ sequence	Location
63 O[a]S[b]	GTTTTGTACTCCGGGTTG	110721–110738
63 OAS[c]	TTACATCCGATGGCGTAG	111089–111106
63 I[d]S	GCTCGTTGAGGACATCAACCGTGTT	110757–110781
63 IAS	CATCGTCGCTATCGTCTTCACCAC	111059–111082
40 OS	ATGACAACGGTTTCATGTCCCGT	71540–71561
40OAS	TGGGCCATCACGTGCTATCAT	71882–71902
40I AS	GCGACAGACAGTCCAAGTTCTAGA	71785–71808
40I AS	GCGACAGACAGTCCAAGTTCTAGA	71785–71808

[a] O Outer primer

[b] S sense primer

[c] AS anti-sense primer

[d] I Inner or Nested primer

It could be argued that an experimental design which would more closely mimic the situation in humans would have been to leave the ganglia in situ in the rat for 24 or 48 h at 4°C, as was done for three of the rats. However, in the great majority of rats, the ganglia were dissected and kept in medium for the stated time periods for the following reasons: (a) we believed this would increase the chances of viral reactivation as all host influences inhibiting reactivation would have been removed (b) to ensure that all ganglia were kept under identical conditions from the start which would have been much more difficult if each ganglion was dissected out sequentially and (c) since all rats were exsanguinated at 0 h, leaving ganglia and organs in situ would not have adequately reflected the normal environment. In fact, a comparison of the results of detection of gene 40 after 48 h using the two different methods shows that 1/6 ganglia were positive in those dissected out at 0 h and kept at 4°C and 1/11 ganglia were positive in those left in situ at 4°C then dissected. Future work with this model could include a study of viral reactivation using the latter method.

Table 2 Summary table of numbers of ganglia positive for VZV RNA by ISH or RT-PCR at 0, 24 and 48 h

RNA ISH 63			
Timepoint	Lumbar	Thoracic	Total ganglia
0 h	3/6	1/5	4/11
24 h	1/3	1/3	2/6
48 h	3/6	3/7	**6/13
RNA ISH 40			
Timepoint	Lumbar	Thoracic	Total ganglia
0 h	0/6	1/6	1/12
24 h	1/3	0/4	1/7
48 h	1/9	1/8	**2/17
RT-PCR 63			
Timepoint	Lumbar	Thoracic	Total ganglia
0 h	3/6	4/6	7/12
24 h	*3/3	*3/3	6/6
48 h	1/3	*1/3	2/6
RT-PCR 40			
Timepoint	Lumbar	Thoracic	Total ganglia
0 h	0/5	1/4	1/9
24 h	0/2	0/1	0/3
48 h	0/2	0/3	0/5

* These positive ganglia are from the same rats that were positive at the 0 h timepoint

** These results included ganglia from three rats which were kept at 4°C for 48 h before thoracic and lumbar ganglia were dissected, fixed and paraffin-embedded

Gene 63 expression is the hallmark of VZV latency, and its frequent detection in the rat ganglia was predicted. It is possible that over the 48 h period some degradation of the RNA could lead to a reduction in the detection of VZV in these samples. However, beta-actin RT-PCR on these RNAs were positive with very little or no indication of degradation of the RNA. Our results provide indirect credibility to the notion that in the human situation we are probably studying true viral latency rather than a viral reactivation pm. In the absence of a good animal model of reactivation with isolation of infectious VZV following a reactivation stimulus, we have to accept the presence of VZV RNA in the peripheral tissue together with transcription of late genes, such as VZV ORF 40, as possible evidence for reactivation in the animal. The presence of viral nucleic acids in a minority of peripheral tissues, particularly VZV RNA, at 0 h pm, indicates some persistence of virus in the rat even 18 months postinfection in these animals. This could either be the result of reactivation, or else a low level of persistent, as opposed to latent, virus. The figure of 7/56 (12.5%) organs positive for VZV nucleic acid in this study is in contrast to the 1/13 (7.7%) samples found positive in our previous study [10]. This may be explained by the fact that, in the present study we used nested RT-PCR, which is a far more sensitive method than the single round DNA PCR used previously. In addition the virus

strain used was different in both studies, being the Oka strain in the previous study and a clinical isolate in the current study. It would be worthwhile in a future study to analyse VZV expression quantitatively in this system since if the virus load in peripheral tissue is significantly lower than in peripheral ganglia, then this would add credibility to the notion that the rat model reflects key features of VZV latency in humans.

While there are some clear similarities between VZV latency in humans and the rat model, such as the largely neuronal localisation of the virus, the fact that the virus may remain in the ganglia for at least 2 years [10], and the fact that a similar subset of genes is usually expressed in both systems during ganglionic latency [5, 7, 11], there are also several differences. Firstly, more satellite cells are found to be positive in infected ganglia in the rat model than in human latency, [7] as well as in axons [12], something not observed in human ganglia. In addition, unlike the human situation, the late VZV gene 40 has been shown to be expressed in the rat model, in 1/5 rats at 1 month postinfection [7]. The presence of viral RNA in a proportion of rat peripheral tissues observed in this study, is also problematic, as mentioned above. VZV has also not been shown to reactivate in rats, which is an important criterion of viral latency. Despite these caveats, the rat model has been very useful in evaluating the contribution of virus genes to the establishment of putative ganglionic latency [10, 13, 14]. For the purposes of this study the rat model used here has been referred to as one of ganglionic infection.

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References

1. D.H. Gilden, B.K. Kleinschmidt-DeMasters, J.J. LaGuardia, R. Mahalingam, R.J. Cohrs, N. Engl. J. Med. **342**, 635–645 (2000)
2. P.G.E. Kennedy, E. Grinfeld, J.W. Gow, Proc. Natl. Acad. Sci. USA **95**, 4658–4662 (1998)
3. R.J. Cohrs, M. Barbour, D.H. Gilden, J. Virol. **70**, 2789–2796 (1996)
4. R.J. Cohrs, D.H. Gilden, P.R. Kinchington, E. Grinfeld, P.G. Kennedy, J. Virol. **77**, 6660–6665 (2003)
5. P.G.E. Kennedy, E. Grinfeld, J.E. Bell, J. Virol. **74**, 11893–11898 (2000)
6. C. Sadzot-Delvaux, M.P. Merville-Louis, P. Delrée, P. Marc, J. Piette, G. Moonen, B. Rentier, J. NeuroSci. Res. **26**, 83–89 (1990)
7. P.G.E. Kennedy, E. Grinfeld, S. Bontems, C. Sadzot-Delvaux, Virology **289**, 218–223 (2001)
8. A. Vafai, R.S. Murray, M. Wellish, M. Devlin, D.H. Gilden, Proc. Natl. Acad. Sci. USA **85**, 2362–2366 (1988)
9. R.J. Cohrs, M.B. Barbour, R. Mahalingam, M. Wellish, D.H. Gilden, J. Virol. **69**, 2674–2678 (1995)
10. E. Grinfeld, C. Sadzot-Delvaux, P.G.E. Kennedy, Virology **323**, 85–90 (2004)
11. C. Sadzot-Delvaux, S. Debrus, A. Nikkels, J. Piette, B. Rentier, Neurology **45**, S18–20 (1995)
12. S. Debrus, C. Sadzot-Delvaux, A.F. Nikkels, J. Piette, B. Rentier, J.Virol. **69**, 3240–5 (1995)
13. J.I. Cohen, T. Krogmann, J.P. Ross, L. Pesnicak, E.A. Prikhod'ko, J. Virol. **79**, 6969–6975 (2005)
14. J.I. Cohen, T. Krogmann, S. Bontems, C. Sadzot-Delvaux, L. Pesnicak, J. Virol. **79**, 5069–5077 (2005)