Attempted modulation of herpes simplex virus (HSV) infection of neurons in culture by fibroblast growth factor

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It has been suggested that fibroblast growth factor (FGF) receptors may mediate entry of herpes simplex virus (HSV) to susceptible cells. We investigated the possible modulation of acute lytic HSV infection of cultured rat neurons by basic FGF, using cell-specific markers and indirect immunostaining. Dissociated neural cell cultures were prepared from the medial septal region of the basal forebrain, the hippocampus and dorsal root ganglion (DRG). The proportion of neurons in the cultures was enhanced by the addition of cytosine arabinoside (2μM) in the hippocampal and DRG cultures and by inversion of the coverslips in septal cultures. The percentage of MAP2⁺ and neurofilament⁺ neurons in these cultures varied between 9 and 95%. Cultures were treated with basic FGF (4–5ng ml⁻¹) continuously and infected with wild-type HSV-1, untreated uninfected cultures acting as controls. Both FGF binding and FGF receptors were demonstrated in hippocampal, and to a much lesser extent, DRG neurons. In repeated experiments it was found that FGF treatment did not have a significant effect on lytic infection in any of the neuronal populations studied as assessed by the development of viral antigen expression and comparison of identified neurons in FGF, treated versus untreated, infected cultures. Our data show that FGF does not have a ‘neuroprotective’ effect on HSV infection of either central or peripheral neurons.

Keywords: herpes simplex virus; neuron; fibroblast growth factor

The most important neurological disease caused by herpes simplex virus (HSV) is herpes simplex encephalitis (HSE), usually produced by HSV-1, which if untreated, has a mortality rate in the region of 50% with severe sequelae in those surviving the infection (Kennedy et al., 1988; Kennedy, 1988). The information gained from studies of in vitro HSV-host neural cell interactions can provide data which may be relevant to the neuropathogenesis of HSE. The modulation of HSV infection of neural cells in culture may also have implications for neuroprotection from viral infection in vivo. Further, there is some very indirect evidence implicating a viral role in the pathogenesis of Alzheimer’s disease and there is some pathological similarity between this disease and HSE (Itzhaki, 1988).

We have shown previously that nerve growth factor (NGF) treatment of cultured rat phaeochromocytoma (PC12) cells altered the susceptibility of these cells to acute lytic HSV infection (Clements and Kennedy, 1989). This modulation appeared to be mediated by an NGF-induced slowing of the lytic cycle in these cells, possibly due to a delay in the transition from early to late HSV polypeptide synthesis. In the current study we have examined the possible modulating effect of another growth factor, namely fibroblast growth factor (FGF), on HSV infection of cultured neurons obtained from three different sources. The possible effects of FGF in these systems are of interest since there is some evidence to suggest that FGF receptors may mediate entry of HSV-1 into susceptible cells (Kaner et al., 1990). We therefore postulated that FGF pretreatment of cultured neurons might lead to a less permissive HSV infection in these cells compared with untreated cultures due to either non-specific factors or the saturation of FGF receptors on neurons.
Hippocampal neurons have previously been shown to possess FGF receptors (Walczik et al., 1989), and these cells undergo pathological changes in both HSV encephalitis and Alzheimer’s disease (Booss and Esiri, 1986; Tomlinson, 1992). The cholinergic neurons of the basal forebrain are of considerable interest since they undergo degeneration in Alzheimer-type dementias (Coyne et al., 1983; Pearson et al., 1983). It has also been shown that adult medial septal cells of the basal forebrain group do not die after excitotoxic ablation of their target neurons in the hippocampus (Sofroniew et al., 1990) and it is possible that trophic support for these cells may be obtainable in the form of growth factors from sources other than target neurons. Although there is little evidence for FGF receptors on medial septal cells (Ferguson and Johnson Jr., 1991), FGF can prevent the degeneration of these cells after injury (Anderson et al., 1988). In addition, DRG neurons were studied as a source of peripheral rather than central neurons to provide comparative data.

Dissociated cell cultures obtained from the hippocampus, medial septal region and DRG were prepared from E18 Sprague-Dawley rats as previously described in detail (Deloume et al., 1991; Kennedy et al., 1983). Embryonic rat hippocampal neurons of this age are known to possess FGF receptors (Wanaka et al., 1991). Embryonic rat DRG neurons of this age do possess FGF receptors, but, unlike hippocampal neurons, rapidly lose them postnatally by P1 (Wanaka et al., 1991). The cultures growing on glass coverslips were transferred to serum-free medium and medial septal and DRG cells were cultured with NGF (Sigma) 100 ng ml^{-1} continuously. Experimental cultures were treated with basic FGF (bFGF) at a concentration of 4–5 ng ml^{-1} 4 h after the cultures were initiated. Two different sources of bFGF were used namely Boehringer (measured concentration on ELISA in medium used, 4 ng ml^{-1}) and R&D (measured concentration in medium used 5 ng ml^{-1}). The experimental results obtained with both bFGFs were very similar.

The FGF used had demonstrable bioactivity as when added to cultures of BHK cells, at the experimental concentration, it stimulated an increase in cells of 45% compared to unstimulated cells in a period of 4 days. In addition before adding bFGF there was no detectable (<5 pg ml^{-1}) bFGF in either the FCS or SATO medium used. Hippocampal and DRG cultures were treated with 2 μM cytosine arabinoside for 48 h at day 2 and day 5. Septal cultures treated with cytosine arabinoside do not survive this procedure (possibly due to cytosine arabinoside blocking essential NGF receptors). Septal neurons cultured on inverted coverslips do not require antimitotic treatment to inhibit non-neuronal cells.

Cells were infected on the seventh day of culture. Wild-type HSV-1 (Glasgow strain 17) of non-syn-}
Double-labelling experiments were also carried out by sequential labelling with biotinylated bFGF and fluorescein labelled anti-biotin antibody followed by anti-bFGF receptor antibody and rhodamine conjugated secondary antibody.

The percentage of cells labelled with antibodies was assessed using a Zeiss Axioplan fluorescent microscope (Welwyn Garden City, Herts, UK) equipped with epi-illumination and phase contrast optics as previously described (Clements and Kennedy, 1989; Kennedy et al., 1983). At least 1,000 cells/coverslip were counted. Statistical analysis was carried out using Student’s t-test.

Following incubation of uninfected (initially hippocampal and DRG) cultures with biotinylated bFGF it was found that approximately 85% of hippocampal neurons bound bFGF on their surface compared with approximately 2% of neurons in DRG cultures. This labelling was inhibited by preincubation of cultures with excess bFGF (100 ng ml⁻¹) and could be proportionately reduced by incubating the biotinylated bFGF with anti-bFGF prior to application. No binding was seen when non-biotinylated bFGF was used. Following incubation of cultures with anti-bFGF receptor antibody, it was found that 80–90% of neurons in hippocampal cultures were labelled, whereas in DRG cultures only 5–10% of neurons were labelled and with a much lower intensity of staining. In double labelling experiments with anti-bFGF receptor antibody and biotinylated FGF it was found that approximately 85% of hippocampal neurons were co-labelled with both fluorescent antibodies, although FGF staining intensity was greater than anti-receptor staining.

The results of FGF treatment on HSV infection of medial septal neurons are summarised in Table 1. A consistent feature in these experiments was the wide variation in counts of both infected and uninfected cells with large standard deviations. The untreated cultures comprised approximately 80% MAP2⁺ neurons, >10% GFAP⁺ astrocytes and occasional FN⁺ or GC⁺ cells. The average number of cells/coverslip prior to infection was similar with and without FGF (data not shown). Following infection it can be seen that there was no significant difference in the percentage of total cells infected. Infection was defined as a cell showing viral antigen expression. When double-labelling experiments were carried out using a combination of neurofilament/MAP2 and anti-herpes antibodies, it was found that although the percentage of identified infected neurons with and without FGF treatment, appeared different, this difference was not significant (P = 0.05), indicating that FGF did not alter the susceptibility of medial septal neurons to infection and/or slow the viral lytic cycle. It was also calculated that the infected neurons comprised 22–31% of the total cell population in these cultures.

The results of FGF treatment on HSV infection of hippocampal neurons are summarised in Table 2. The untreated cultures comprised approximately 66% MAP2⁺ neurons, 25% GFAP⁺ astrocytes and a small number of GC⁺ and FN⁺ cells. Following infection the percentage of the total cells infected

### Table 1: Effect of FGF treatment on HSV infection of septal neurons

<table>
<thead>
<tr>
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<th>No FGF</th>
<th>With FGF</th>
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<tr>
<td>i</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>% Neurons in uninfected culture</td>
<td>79.2</td>
<td>15.6</td>
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<td>ii</td>
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<td></td>
<td>68</td>
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<tr>
<td>% Neurons in infected culture</td>
<td>46.1</td>
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<tr>
<td>iii</td>
<td></td>
<td></td>
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</tbody>
</table>

Figures are based on a total of 10 observations in 5 independent experiments

*P values compare percentages with and without FGF treatment

### Table 2: Effect of FGF treatment on HSV infection of hippocampal neurons

<table>
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<tbody>
<tr>
<td>i</td>
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<td>SD</td>
<td>Mean</td>
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<td>% Neurons in uninfected culture</td>
<td>70.8</td>
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<td>63.6</td>
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<tr>
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<td>37.3</td>
<td>30.6</td>
<td>36</td>
</tr>
<tr>
<td>iii</td>
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<td></td>
<td>29.6</td>
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</table>

Figures are based on a total of 9 observations in 5 independent experiments

*P values compare percentages with and without FGF treatment
Figure 1  Rat hippocampal culture. Cells in culture were treated with basic FGF and infected with HSV. They were then double-labelled with a combination of anti-MAP2 and anti-HSV antibodies followed by the appropriate fluorescein and rhodamine labelled secondary antibodies. In (A) infected neurons are shown labelled with anti-MAP2 but only three of them (B) express HSV antigens. In the uninfected control cultures (C) neurons are labelled with anti-MAP2 and in (D) show no expression of HSV antigens. (x 570).

Table 3 Effect of FGF treatment on HSV infection of DRG neurons

<table>
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<th></th>
<th>*P value</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>% Neurons in uninfected culture</td>
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<td>9.5</td>
<td>82.1</td>
<td>8.3</td>
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<td>ii</td>
<td>% Neurons in infected culture</td>
<td>67.8</td>
<td>25.9</td>
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<td>iii</td>
<td>% Total cells infected</td>
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<td>15.7</td>
<td>34.8</td>
<td>2.5</td>
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<td>iv</td>
<td>% Neurons infected</td>
<td>35.1</td>
<td>16.9</td>
<td>45.5</td>
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Figures are based on a total of 9 observations in 5 independent experiments

was similar with and without FGF treatment with no significant difference. The percentage of identified infected neurons was similar with and without FGF treatment (Figure 1). We interpret this result as indicating no positive evidence of an effect of FGF on neuronal infection. The percentage of infected neurons was 29–32% of the total of the cell population in these cultures which is similar to the case with the medial septal neurons.

The results of FGF treatment on HSV infection of
DRG neurons are summarised in Table 3. These experiments were carried out to provide comparative data with the two central neuronal cultures and also since we had previously obtained data using NGF in this culture system (Clements and Kennedy, 1989). The untreated cultures comprised approximately 85% neurons, >10% Schwann cells and occasional FN fibroblasts. As was the case in the other culture systems the total number of cells prior to infection with and without FGF was comparable (data not shown). The percentage of infected identified neurons was 35–45% of the total cell population and was similar with and without FGF treatment.

We have previously shown that NFG can modulate the HSV lytic cycle in PC12 cells but not DRG neurons where its effects were minimal (Clements and Kennedy, 1989). In the current experiments the effects of FGF could be assessed as an independent factor. Hippocampal neurons are well recognised to possess FGF receptors (Wanaka et al., 1991; Walicke et al., 1989); this was confirmed in the current study by use of appropriate antibody staining. Further, FGF, but not NGF, promotes the survival of dissociated hippocampal neurons in culture and also enhances neurite extension (Cheng and Mattson, 1991). Although FGF has also been reported to have trophic effects on septal neurons in culture (Walicke, 1988), FGF receptor mRNA has not been demonstrated in these neurons (Wanaka et al., 1991). For these reasons we considered that comparison of FGF's neuroprotective effects during HSV infection in the two culture systems would be of interest. Neurons obtained from rat DRG were also studied as a source of peripheral neurons to compare with those obtained from central sources and these cultures would not be expected to possess FGF receptors. The very low FGF receptor expression in cultured DRG neurons was also confirmed in this study. FGF is also of interest for two other reasons. First, there is evidence to suggest that it plays an important role in the interaction between neuronal and glial cells in the CNS through its additional ability to stimulate the proliferation and maturation of these cells in vitro (Pettmann et al., 1986). Second, and relevant to the current study, the FGF receptor has been implicated (Kaner et al., 1990) as a means of cellular entry of HSV-1 although more recent evidence has added contrary data. Thus, HSV infection of cultured rat L6 cells as well as smooth muscle cells, fibroblasts, endothelial cells and neuroblastoma cells can all occur in the absence of FGF receptor (Mirda et al., 1992; Muggeridge et al., 1992). It was therefore of particular interest to investigate whether treatment of different cultured neurons with FGF might affect their susceptibility to HSV infection. It was important to actually demonstrate FGF binding to neuronal cells, in particular to FGF receptors in neurons in these cultures, and such expression was confirmed in the positive control experiments. In replicate independently performed experiments it was found that FGF pretreatment of all three cultures did not have a significant effect on either the survival of identified neurons or the number of these cells which were infected with HSV.

These findings are consistent with the view that viral entry into susceptible cells is not mediated by the FGF receptor (Shieih and Spear, 1991). It should be emphasised that we have only addressed this question in a limited and indirect study, but using a number of culture systems. We cannot exclude a potential role for other growth factors, but our results are consistent with recent data in non-neuronal cells. Further, in this study we did not address the specific question of FGF cell binding, to high affinity specific receptors or to heparin sulphate, a receptor for HSV (Shieih and Spear, 1991). We have assumed in this study that the FGF used was present in saturating amounts during the course of HSV incubation. But even if this were the case, we recognise that under the conditions used we cannot exclude the possibility that HSV displaced from the FGF receptor could still attach to a secondary receptor and cause the observed infection. Two of the key questions which remain are, firstly, how HSV is able to penetrate the neuronal surface to gain entry into the neuron and, secondly, how this virus–receptor interaction can be overcome, possibly by the use of drugs or neurotrophic factors.

Acknowledgements

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