Effective transduction of osteogenic sarcoma cells by a baculovirus vector

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Efficient gene delivery of a baculovirus-derived vector (BV-p53-lacZ) to a human osteogenic sarcoma cell line, Saos-2, was serendipitously found while evaluating the vector for gene delivery to human p53-null tumour cells in a previous study. Therefore, we investigated other human, rat and mouse osteogenic sarcoma and other types of tumour cell lines for transduction efficiency via baculovirus vectors containing a lacZ reporter gene under the control of either a cytomegalovirus or Rous sarcoma virus promoter. The expression of β-galactosidase protein, assessed by X-Gal staining and β-galactosidase ELISA, demonstrated an extremely high level of transduction efficiency in some osteogenic sarcoma cell lines, such as U-2OS, Saos-2 and Saos-LM2. These human osteogenic sarcoma cell lines showed levels of β-galactosidase expression 5–40 times greater than HepG2 cells, which were previously thought to be the mammalian cells most susceptible to baculovirus-mediated gene delivery. The level of acetylated histone proteins in these tumour lines did not correlate well with the high level of reporter gene expression. These results strongly suggest that some osteogenic sarcoma cells are highly susceptible to baculovirus-mediated gene delivery and that a baculovirus-derived vector is an efficient gene delivery vehicle into human osteogenic sarcoma cells.

INTRODUCTION

The insect baculovirus Autographa californica nucleopolyhedrovirus (AcNPV) is a rod-shaped, enveloped virus with a double-stranded genomic DNA (~134 kb). It has been widely utilized for the large-scale production of recombinant proteins in insect cells or as biopesticides (Smith et al., 1983; Pennock et al., 1984; Carbonell et al., 1985; Matsuura et al., 1987; Bishop et al., 1988; Luckow & Summers, 1988; Cory & Bishop, 1997). Although the possibility of using AcNPV to transduce mammalian cells has been explored extensively in the past, baculovirus-mediated gene expression was not reported in mammalian cells until reporter gene (lacZ) expression was achieved in human hepatocytes (Hofmann et al., 1995) and in HepG2, a human liver tumour cell line (Boyce & Bucher, 1996), under the control of the cytomegalovirus (CMV) immediate early (IE) or Rous sarcoma virus (RSV) promoter, respectively. Studies by Shoji et al. (1997) showed that non-hepatic cell lines, such as HeLa and COS7, could be transduced efficiently by AcNPV if a strong CAG promoter composed of the CMV IE enhancer and chicken β-actin promoter was used. Recently, Condrea et al. (1999) and Sarkis et al. (2000) showed that a baculovirus vector could also efficiently transduce a large variety of cell lines, including neuroblastoma cell lines and human primary neural cell cultures. Although transgene expression has been demonstrated in non-hepatic cells, hepatic cells were believed to be the most susceptible to baculovirus infection.

In a previous study evaluating a baculovirus-derived vector (BV-p53-lacZ) for gene delivery to human p53-null tumour cells (Song & Boyce, 2001), the Saos-2 osteogenic sarcoma cell line was accidentally found to be extremely susceptible to a budded virus form of baculovirus. The level of lacZ reporter gene expression in Saos-2 cells was 18 times greater than that in HepG2 cells. The real mechanism of cell entry of baculoviruses in insects and mammals remains substantially unknown. A receptor-mediated endocytosis pathway has been suggested as the mechanism of cell uptake of baculoviruses and it has also been suggested that entry is mediated by electrostatic interactions involving heparan sulfate in mammalian cells (Duisit et al., 1999). Tani et al. (2001) also suggested that certain phospholipids on the cell surface may play an important role in transduction of mammalian cells via a baculovirus vector. The requirement for endolysosomal maturation for the transport of baculoviruses after infection and transfer of baculovirus capsids to the nuclear pore with the help of actin filaments have been demonstrated in both insect and mammalian cells (Volkman & Goldsmith, 1985; Charlton & Volkman, 1993; van Loo et al., 2001). The level of reporter gene expression...
probably varies depending on how efficiently the baculovirus genome enters the nucleus and on how active the promoter driving the reporter gene is in the host cell.

Here we have examined several osteogenic sarcoma and other types of tumour cell lines for the transduction efficiency of a baculovirus vector containing a lacZ reporter gene under the control of either the CMV or RSV promoter. Our results demonstrated that transduction efficiency mediated by a baculovirus vector is extremely efficient in some osteogenic sarcoma cell lines. Among these cell lines, U-2OS cells showed the highest level of transduction efficiency. These results suggest that a baculovirus-derived vector may be an excellent tool for gene delivery to osteogenic sarcoma cells.

METHODS

Preparation of BV-pCMV-lacZ and BV-pRSV-lacZ. The baculovirus transfer vector plasmids were constructed containing a β-galactosidase open reading frame under the control of the CMV or RSV promoter together with SV40 polyadenylation signals in the background of the pBacPAK9 baculovirus transfer vector (Clontech). The β-galactosidase coding region was amplified by PCR using primers containing Ascl and Pael sites and cloned into the BVP33-lacZ plasmid (Song & Boyce, 2001) cut with Ascl and Pael restriction enzymes to form BV-pCMV-lacZ. The RSV promoter fragment was amplified by PCR using primers containing BglII and Ascl sites and cloned into the BV-pCMV-lacZ plasmid cut with BglII and Ascl to form BV-pRSV-lacZ. Purification and amplification of the recombinant baculoviruses were carried out as previously described (Boyce & Bucher, 1996).

Western blot analysis and antibodies. Mock- or virus-infected cell monolayers were lysed, separated by 12% SDS-PAGE and transferred to nitrocellulose paper. They were then probed with the appropriate primary antibodies before reactive protein detection using a horseradish peroxidase-conjugated secondary antibody and ECL detection (Amersham). Monoclonal anti-β-galactosidase antibody (Chemicon) was used at a dilution of 1:5000 for Western blot analysis. Polyclonal anti-β-actin (Sigma) and anti-acetylated histone 4 antibodies (Chemicon) were used at dilutions of 1:5000 and 1:800, respectively, for Western blot analysis.

X-Gal staining. Mock- or virus-infected cells were fixed in 4% paraformaldehyde in PBS at 24 h post-infection. After rinsing three times with PBS, the cells were stained by addition of X-Gal (1 mg ml⁻¹) in staining solution (35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM MgCl₂ in PBS) for at least 4 h at 37°C.

β-Galactosidase assay. The expression of β-galactosidase protein was measured using a biochemical assay. Cells (3 × 10⁵ per well) were grown in a six-well plate 1 day before infection with BV-pCMV-lacZ or BV-pRSV-lacZ at an m.o.i. of 10 or 100. Cells were mock- or virus-infected in 1 ml of medium for 2 h, and then the virus-containing medium was removed and replaced with fresh medium. After 24 h, the cells were washed three times with cold PBS and lysed. Levels of β-galactosidase activity were measured using the high-sensitivity β-galactosidase assay kit (Stratagene) with chlorophenol red-β-D-galactopyranoside. The extracted protein was quantified by a protein assay (Bio-Rad) based on the Bradford method. β-Galactosidase activity was expressed as units (mg protein)⁻¹.

RESULTS

Construction of recombinant baculoviruses expressing the lacZ reporter gene under the control of the CMV or RSV promoter

To express β-galactosidase reporter proteins in osteogenic sarcoma cells, recombinant baculoviruses containing a lacZ reporter gene under the control of the CMV or RSV promoter were constructed by cotransfection of the baculovirus transfer vector (Fig. 1A) and a linear intact baculovirus genome into Sf9 insect cells. Production of β-galactosidase protein was confirmed by Western blot analysis of Saos-2

See Fig. 1 for the construction of baculovirus transfer vectors for generation of the recombinant baculoviruses BV-pCMV-lacZ and BV-pRSV-lacZ.
cells infected with the recombinant baculoviruses (Fig. 1B). Protein extracts from Saos-2 cells infected with BV-pCMV-lacZ or BV-pRVS-lacZ at an m.o.i. of 100 showed high levels of β-galactosidase production (Fig. 1B, lanes 2 and 4). In contrast, mock-infected cell extracts showed no detectable β-galactosidase expression (Fig. 1B, lanes 1 and 3).

**Susceptibility of osteogenic sarcoma cell lines to the recombinant baculoviruses**

In our previous study, a human osteogenic sarcoma cell line, Saos-2, was found to be highly susceptible to a recombinant baculovirus infection. In order to investigate whether other osteogenic sarcoma cell lines are also susceptible to baculovirus infection, six human, two rat and two mouse osteogenic sarcoma or sarcoma cell lines were tested for transduction efficiency, as mediated by recombinant baculoviruses containing a lacZ reporter gene under the control of either the CMV or RSV promoter, by X-Gal staining 24 h after exposing cells to the viruses at an m.o.i. of 75. As shown in Figs 2 and 3, the intensity of X-Gal staining in U-2OS, Saos-2 and Saos-LM2 cells was stronger than in HepG2 human hepatocellular carcinoma cells, which were previously thought to be the mammalian cells most susceptible to baculovirus infection. These X-Gal staining results indicated that four of six human osteosarcoma cell lines (U-2OS, Saos-2, Saos-LM2 and G292) showed high levels of transduction efficiency following infection with the recombinant baculoviruses. NIH3T3 mouse fibroblast cells were used as a negative control and

![Fig. 2. X-Gal staining of osteogenic sarcoma cells infected with baculovirus BV-pCMV-lacZ. Cells were infected with a recombinant baculovirus containing the lacZ reporter gene under the control of the CMV promoter and stained with X-Gal 24 h post-infection. Left panels, mock-infected cells; right panels, cells infected with BV-pCMV-lacZ at an m.o.i. of 75. The cell lines used are indicated.](http://vir.sgmjournals.org)
CHP212 human neuroblastoma cells as a positive control. The CHP212 cell line was recently described as an efficient target of baculovirus infection (Sarkis et al., 2000).

Quantification of the transduction efficiency of osteogenic sarcoma cell lines mediated by BV-pCMV-lacZ and BV-pRSV-lacZ

To confirm the X-Gal staining results and to measure transduction efficiency quantitatively, expression of the β-galactosidase reporter protein was measured by ELISA using extracts prepared from cells infected at an m.o.i. of 10 or 100 (Table 1). Mock-infected cells were used as a control to determine background β-galactosidase protein activity (data not shown). The most dramatic finding was that one of the human osteosarcoma cell lines, U-2OS, expressed levels of β-galactosidase under the control of the RSV or the CMV promoter that were >20- or 40-fold higher, respectively, than HepG2 human cells. The level of β-galactosidase production in U-2OS cells was about 100-fold greater than in CHP212 cells. Other osteogenic sarcoma cell lines, such as Saos-2 and Saos-LM2, also showed much higher β-galactosidase expression than HepG2 cells. Another human osteogenic sarcoma cell line, G292, expressed β-galactosidase protein at a slightly lower level than HepG2 cells, but this was higher than in CHP212 cells. Among the rat and mouse osteosarcoma and sarcoma cell lines, RR1022 rat sarcoma cells showed the highest level of β-galactosidase expression, which was slightly less than that of G292 cells (Table 1). These results strongly suggest that baculovirus vectors are capable of transducing some human osteogenic sarcoma cells with high efficiency.
Table 1. Baculovirus-mediated expression of pCMV-lacZ and pRSV-lacZ reporter genes in osteogenic sarcoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BV-pCMV-lacZ</th>
<th>BV-pRSV-lacZ</th>
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<tbody>
<tr>
<td></td>
<td>M.o.i. 10</td>
<td>M.o.i. 100</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>0.005 ± 0.001</td>
<td>1.309 ± 0.065</td>
</tr>
<tr>
<td>CHP212</td>
<td>0.064 ± 0.031</td>
<td>1.315 ± 0.318</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.127 ± 0.078</td>
<td>4.104 ± 1.066</td>
</tr>
<tr>
<td>U-2OS</td>
<td>27.182 ± 5.193</td>
<td>159.172 ± 20.152</td>
</tr>
<tr>
<td>Saos-LM2</td>
<td>2.985 ± 0.946</td>
<td>10.21 ± 3.577</td>
</tr>
<tr>
<td>G-292</td>
<td>0.107 ± 0.054</td>
<td>2.468 ± 0.826</td>
</tr>
<tr>
<td>MG-63</td>
<td>0.026 ± 0.001</td>
<td>0.452 ± 0.151</td>
</tr>
<tr>
<td>ROS</td>
<td>0.045 ± 0.007</td>
<td>0.194 ± 0.084</td>
</tr>
<tr>
<td>RR1022</td>
<td>0.059 ± 0.013</td>
<td>1.978 ± 0.174</td>
</tr>
<tr>
<td>CCRF-S-180</td>
<td>0.015 ± 0.001</td>
<td>0.741 ± 0.025</td>
</tr>
<tr>
<td>Sarcoma-180</td>
<td>0.008 ± 0.002</td>
<td>0.051 ± 0.022</td>
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Measurement of the transduction efficiencies of other types of tumour cells mediated by BV-pCMV-lacZ or BV-pRSV-lacZ

To determine the transduction efficiencies of other types of tumour cells using recombinant baculoviruses, four prostate, two colon and two breast tumour cell lines were tested. As shown in Table 2, three of the four prostate tumour cell lines showed a level of β-galactosidase expression as high as that shown in G292 osteosarcoma cells. On the other hand, the other colon and breast tumour cells showed low levels of reporter protein expression. The expression of β-galactosidase reporter proteins under the control of the CMV promoter were in most cases higher than those controlled by the RSV promoter in the tumour cell lines used in this study. However, SK-BR-3 human breast adenocarcinoma cells showed higher β-galactosidase production with the RSV promoter than with the CMV promoter. These results suggest that some human osteosarcoma cells are more susceptible to baculovirus infection than other types of human tumours.

Measurement of acetylated histone proteins in osteogenic sarcoma cell lines

The results above indicate heterogeneity of reporter gene expression within the same type of tumour cell lines. For example, of the six human osteosarcoma cell lines, one showed an extremely high level of β-galactosidase reporter protein, three showed high to intermediate levels and two showed very low levels. Evidence that an epigenetic regulation influences reporter gene expression in mammalian cells transduced with a baculovirus vector has recently been presented (Sarkis et al., 2000). Therefore, to explore the

Table 2. Baculovirus-mediated expression of pCMV-lacZ and pRSV-lacZ reporter genes in prostate, colon and breast tumour cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BV-pCMV-lacZ</th>
<th>BV-pRSV-lacZ</th>
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<tbody>
<tr>
<td></td>
<td>M.o.i. 10</td>
<td>M.o.i. 100</td>
</tr>
<tr>
<td>DU145</td>
<td>0.029 ± 0.005</td>
<td>0.185 ± 0.031</td>
</tr>
<tr>
<td>PC-3M</td>
<td>0.399 ± 0.098</td>
<td>2.215 ± 0.504</td>
</tr>
<tr>
<td>PC-3</td>
<td>0.258 ± 0.064</td>
<td>2.019 ± 0.248</td>
</tr>
<tr>
<td>PC-3M-MM2</td>
<td>0.576 ± 0.157</td>
<td>2.199 ± 0.482</td>
</tr>
<tr>
<td>HT-29</td>
<td>0.012 ± 0.003</td>
<td>0.391 ± 0.246</td>
</tr>
<tr>
<td>LoVo</td>
<td>0.031 ± 0.018</td>
<td>1.015 ± 0.247</td>
</tr>
<tr>
<td>Hs578T</td>
<td>0.036 ± 0.014</td>
<td>0.196 ± 0.042</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>0.042 ± 0.028</td>
<td>0.262 ± 0.061</td>
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state of acetylation in cells, the level of acetylated histone 4 proteins was measured using antibodies against acetylated histone 4 by Western blot analysis of cell extracts (Fig. 4). Our results showed that some of the osteogenic sarcoma cell lines, U-2OS, MG63 and HOS, contained various levels of acetylated histone 4 proteins, whereas other osteogenic sarcoma cell lines did not. The level of acetylation of histone 4 in U-2OS cells was about 5-fold greater than in MG63 and HOS cells. CHP212 cells showed about the same level of acetylated histone 4 as U-2OS cells. These results suggest that the level of acetylation of histone proteins does not correlate well with the level of reporter protein expression in mammalian cells transduced with a baculovirus vector, indicating that the chromatin structure of the baculovirus genome in the infected cells may not be critical for expression of the transgene.

**DISCUSSION**

This study has explored the baculovirus-mediated transduction efficiency of osteogenic sarcoma cell lines and demonstrated that some osteogenic sarcoma cell lines exhibit extremely high levels of transduction efficiency using a baculovirus vector. The expression of the reporter gene in these cell lines was at least 5–40 times greater than in the HepG2 cell line, which was previously thought to be the mammalian cell line most susceptible to baculovirus infection. However, β-galactosidase reporter protein expression was found to be heterogeneous in osteogenic sarcoma cell lines and this did not correlate well with the acetylated state of histone proteins in these cell lines.

It would be interesting to find out why U-2OS osteogenic sarcoma cells show an extremely high level of transduction efficiency for baculovirus-mediated gene transfer. It is likely that entry of the baculovirus, endolysosomal maturation and transport of baculovirus capsids to the nuclear pore in U-2OS cells are very efficient. Moreover, the promoters of CMV and RSV used for driving the transgene expression must be highly active in U-2OS cells. Thus far, no baculovirus cell-surface receptor has been found on mammalian cells. U-2OS cells would therefore be a good candidate for any search for receptor-mediated endocytosis at entry were a dominant effect for the high level of reporter gene expression. We tested the state of acetylation of histone 4 proteins in U-2OS cells to find out whether any epigenetic regulation was involved in reporter gene expression, since the level of acetylation is thought to be an important regulator of the expression of a reporter gene transferred using a baculovirus vector (Condreay et al., 1999; Sarkis et al., 2000). This study demonstrated that the level of acetylation of histone 4 proteins is very high in U-2OS and CHP212 cells compared with the level in NIH3T3 fibroblasts (Fig. 4). However, the level of acetylated histone proteins in other highly susceptible osteogenic sarcoma cell lines, such as Saos-2, Saos-LM2 and G292, was undetectable. These results suggest that the amount of acetylation of histone proteins may not be a crucial factor in the regulation of reporter gene expression via a baculovirus vector.

In our previous study evaluating a p53-containing baculovirus vector for its anti-tumour effect in p53-null tumour cell lines (Song & Boyce, 2001), one of the osteogenic sarcoma cell lines, Saos-2, was found serendipitously to be highly susceptible to baculovirus infection. The Saos-2 cell line used was obtained from ATCC and showed levels of β-galactosidase protein expression 18 times greater than in HepG2 cells. In the present study, the Saos-2 cell line was obtained from the Korean Cell Line Bank and showed only about 5- to 7-fold difference with the CMV or RSV promoter, respectively. This difference could have been due to either different recombinant baculovirus preparations or the different sources. Transduction efficiency in rat and mouse osteogenic sarcoma or sarcoma cells showed lower transduction levels than human cells. This species preference of the baculovirus has also been observed by other groups (Hofmann & Strauss, 1998; Sarkis et al., 2000).

One of the limiting factors of gene therapy is the lack of an efficient gene delivery system. The problem has been an inability to deliver therapeutic genes efficiently to target cells and to obtain sustained expression (Mulligan, 1993; Verma & Somia, 1997). Although no ideal vector is currently available, gene therapy is still recognized as a promising alternative for the treatment of incurable diseases. Therefore, development of new vectors that are specifically designed to meet all the requirements for the disease and conditions to be treated is necessary. Another important requirement for an ideal vector is the ability to obtain a high level of recombinant virus particles for in vivo application.
One of the advantages of the baculovirus vector system includes the ability to grow the virus to high titres (10^{10} p.f.u. ml^{-1}), thus facilitating production of large quantities of baculovirus particles, which would be an asset for in vivo application of the vector. The results of the present study suggest that a baculovirus-derived gene delivery vector offers a highly efficient tool for delivering a therapeutic gene to osteogenic sarcoma cells in vivo.

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