

## Effects of ectopic decorin in modulating intracranial glioma progression *in vivo*, in a rat syngeneic model

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Given the failure of conventional treatments for glioblastoma, gene therapy has gained interest considerable in recent years. Gliomas are associated with a state of immunosuppression, which appears to be partially mediated by an increase in secretion of transforming growth factor- $\beta$  (TGF- $\beta$ ) from glioma cells. Decorin, a small proteoglycan which can bind to and inactivate TGF- $\beta$ , has been successfully used as an antitumor strategy on stably transfected tumor cells and has been shown to cause growth suppression in neoplastic cells of various histological origins. In this paper, we investigated the use of gene therapy to deliver the decorin transgene in a site-specific manner in an experimental model of intracranial gliomas. Our aim was to inhibit the glioma-associated immunosuppressive state, and prolong the survival of tumor-bearing rats.

We studied the effects of decorin gene transfer in the rat CNS-1 glioma model. To assess the effect of ectopic expression of decorin on glioma progression *in vivo*, stably transfected CNS-1 cells expressing decorin were implanted into the brain parenchyma of syngeneic Lewis rats. The rats implanted with CNS-1 cells expressing decorin survived significantly longer than those in the control groups which received CNS-1 cells that did not express decorin ( $P < .0001$ ). We then investigated whether the survival observed with decorin expressing cells could be mimicked *in vivo*, using recombinant adenoviruses (RAds) expressing the decorin gene under the control of two different promoters: the human immediate-early cytomegalovirus (h-IE-CMV) and the glial fibrillary acidic protein (GFAP). *In vivo* results showed that administration of RAd expressing the human decorin under the control of h-IE-CMV promoter has a small, but significant effect in prolonging the survival of experimental tumor bearing rats ( $P < .0001$ ). Our data indicate that ectopic decorin expression has the potential to slow glioma progression *in vivo*. Our results also indicate that expression of decorin has to be present in all cells which constitute the intracranial tumor mass for the inhibition of tumor growth and prolongation of the life expectancy of tumor-bearing rats to be effective.

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Decorin plays different roles in regulating matrix organization, growth factor activity, angiogenesis, and cell proliferation and differentiation.<sup>1–3</sup> Overexpression of decorin inhibits TGF- $\beta$ -induced proliferation and slows growth of a Chinese hamster ovary cell line.<sup>4</sup> In gene transfer studies of human colon cancer, cells stably transfected with decorin exhibited reduced growth rates *in vitro* and failed to generate tumors in severe combined

immunodeficiency (SCID) mice.<sup>5</sup> Decorin has been shown to arrest cells in the G1 phase of the cell cycle, and growth suppression could be restored by treatment with decorin antisense nucleotides.<sup>6–8</sup> The mechanism of action of decorin-induced growth suppression appears to be mediated by interaction with the epidermal growth factor receptor (EGFR),<sup>8–10</sup> which leads to an upregulation of p21, a potent inhibitor of cyclin-dependent kinases.<sup>6,7</sup> In agreement with this, it has been shown that adenovirus-mediated gene transfer of decorin attenuates EGFR activity and suppresses *in vivo* tumorigenesis.<sup>11</sup> The function of decorin to inactivate the oncogenic ErbB2 protein in breast carcinoma cells has also been reported.<sup>12</sup> Additionally, it has been indicated that decorin could adversely affect *in vivo* tumor growth by suppressing the endogenous tumor cell production of angiogenic

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stimulus.<sup>3</sup> Finally, it has been shown that ectopic expression of decorin in C6 rat glioma cells resulted in the inhibition of tumor formation *in vivo*.<sup>13</sup>

These unique features make decorin a very good candidate gene for many experimental cancer gene therapy applications. Malignant gliomas frequently secrete TGF- $\beta$  which creates a state of immunosuppression in the glioma micro-environment, and due to the invasive nature of glioma, throughout the surrounding brain parenchyma.<sup>14–16</sup> Decorin, by inhibiting TGF- $\beta$  or EGFR activity could display antiglioma activity. We therefore proposed to test the hypothesis of using decorin as a therapeutic gene to inhibit intracranial glioma growth in a syngeneic rat model.

The development of an appropriate gene therapy approach requires not only a suitable therapeutic gene, but an effective gene delivery system. Malignant brain tumors are attractive targets for local gene therapy because of their exclusive location in the CNS. Adenoviral vectors are highly efficient at *in vivo* transduction and can be concentrated to high titers, thus it is possible to stereotactically inject high titers of viruses directly into the area of brain infiltrated by tumor cells.<sup>17–19</sup> In addition to the high efficiency of transduction, adenoviruses mediate high-level transgene expression and pose little risk of insertional mutagenesis.

Unlike transient transgene expression observed following systemic administration of adenoviral vectors, in the absence of prior systemic immune priming to adenovirus, first-generation adenoviral vectors injected into the brain parenchyma can sustain prolonged transgene expression for at least 6–18 months.<sup>17,20–23</sup> We have assessed this for up to 3 months after CNS-1 tumor implantation in the survivors after gene therapy using RAD/HSV1-TK plus ganciclovir. The reasons for this are two-fold. Firstly, although the tumors are fast growing, due to the fact that they are encased within the cranium, they have a limited space to grow. Second, due to the fact that the treated animals survive long term, either the tumor is completely eliminated or the growth of the tumor is severely impaired. If the tumor is completely eliminated, transgene expression is observed in the surrounding normal brain.<sup>17,18</sup> Therefore, RADs are very good gene transfer vectors to test experimental therapies in animal models of brain pathologies, such as brain tumors and neurodegenerative diseases.

Finally, a relevant animal model is critical for most gene therapy approaches, particularly in cancer gene therapy. We chose the rat CNS-1 glioma<sup>17,24</sup> model to conduct our studies since it is accepted as a highly reproducible syngeneic model with immunological and invasive characteristics similar to those of human gliomas.<sup>25</sup> This constitutes one of the most stringent animal models to study the effectiveness of novel therapies for glioblastoma multiforme.

## Results

### *Generation of a CNS-1 glioma clonal cell subline engineered to stably express human decorin*

Human decorin cDNA was cloned into the BCMGS neo-expression plasmid<sup>26</sup> and transfected into CNS-1 glioma

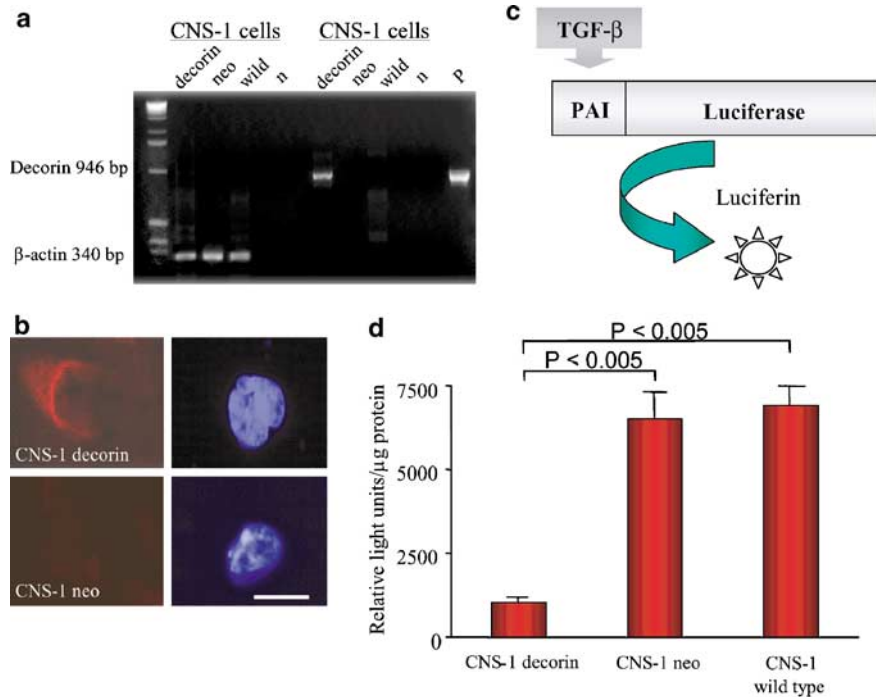
cells by electroporation. The introduction of transgene into CNS-1 cell line was confirmed by PCR amplification of the human decorin gene. A 946 bp human decorin-specific PCR product was detected in decorin-transfected cells, but not in untransfected or neo-transfected cells (Fig 1a,b). The capability of decorin-transfected CNS-1 cells to express decorin was assessed by immunocytochemical analysis (Fig 1b). The ability of secreted decorin to suppress TGF- $\beta$  bioactivity was evaluated using a mink lung epithelial cell line stably transfected with a plasminogen activator inhibitor-1 promoter-Luciferase construct (MLE/PAI/L cells). Exposure of MLE/PAI/L cells to TGF- $\beta$  induces a dose-dependent increase in Luciferase activity<sup>27</sup> (Fig 1c). The conditioned medium containing secreted decorin was collected from decorin CNS-1 cell cultures and incubated with MLE/PAI/L cells. Wild-type and neo-transfected CNS-1 cells were used as controls. Lysates from MLE/PAI/L cells incubated with CNS-1 cell supernatant were tested for Luciferase activity. Following incubation with supernatant from decorin-transfected cells a statistically significant ( $P < .005$ ) reduction in Luciferase activity was seen (Fig 1d).

### *Assessment of the growth rate of decorin-transfected CNS-1 cells in vitro*

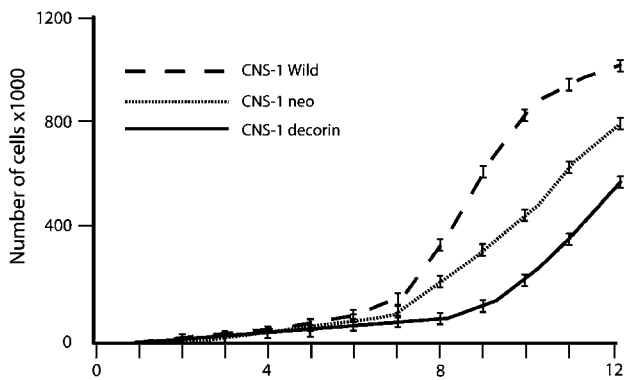
The growth rate of decorin-transfected CNS-1 cells was assessed and compared with the growth rate of control (neo-) transfected CNS-1 cells and wild-type CNS-1 cells. Cells were seeded into 12-well plates at a density of 2500 cells/well and incubated at 37°C in 5% CO<sub>2</sub> with constant humidity. Cells were counted every 24 hours during 12 consecutive days. Decorin-transfected cells were the slowest growing cell population (Fig 2). In comparison with the neo-transfected cells or wild-type CNS-1 cells, the growth rate was inhibited by 25 and 40%, respectively ( $P < .0001$ ) as analyzed using the Student's *t*-test. The experiment was repeated two times, giving identical results.

### *Effects of ectopic decorin expression on CNS-1 glioma growth in Lewis rats*

To assess the effect of ectopic expression of decorin on glioma tumor regression in a syngeneic glioma model, stably transfected CNS-1 cells expressing decorin were implanted into the striatum of Lewis rats as described previously.<sup>17</sup> Wild-type and neo-transfected CNS-1 cells were used as experimental controls. Animals were monitored daily, and rats that demonstrated signs of distress were perfused-fixed and their brains retained for histological analysis. All control animals implanted with wild-type CNS-1 cells or neo-transfected CNS-1 cells had to be euthanized for progressive tumor growth within 30 days. Lewis rats implanted with CNS-1 cells expressing decorin survived significantly longer than those in the control groups (Fig 3a). The surviving rats (60% of decorin CNS-1 group) were perfused-fixed 90 days post-tumor implantation; upon histopathological analysis one of these animals was shown to still carry remaining tumor tissue indicating that decorin overexpression slows the growth of CNS-1 glioma cells *in vivo*. In order to assess



**Figure 1** Evaluation of bioactivity of CNS-1 cells expressing decorin. (a) Decorin expression in rat CNS-1 glioma cells. A volume of 20  $\mu$ l of the PCR reaction were loaded on a 2% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide, and the DNA fragments fractionated by electrophoresis. The gel indicates that the decorin gene is amplified in transfected CNS-1 cells (946 bp band) compared to the negative band in control lanes;  $\beta$ -actin serves as an internal control; “n” is a negative control lacking genomic DNA in the PCR reaction, and “p” shows amplification from the plasmid (pAL119/decorin), used here as positive control. (b) Immunocytochemical detection of decorin in CNS-1 cells. Cells were paraformaldehyde fixed, permeabilized with Triton X-100, labeled with a sheep anti-human decorin polyclonal antibody followed by an anti-sheep FITC-conjugated secondary antibody and counterstained with 4'-6-diamidino-2 phenylindole (DAPI). The positive signal (in CNS-1 decorin cells) was not detected in neo-transfected cell samples (CNS-1 neo cells). Corresponding DAPI staining of the same cells show cell nuclei. Scale bar represents 10  $\mu$ m. (c) Schematic representation of the Luciferase assay experiment. (d) Exposure of MLE/PAI/L cells to supernatant from decorin-transfected cells results in a statistically significant reduction in Luciferase activity. Results are shown as means  $\pm$  SEM ( $n=3$ ).

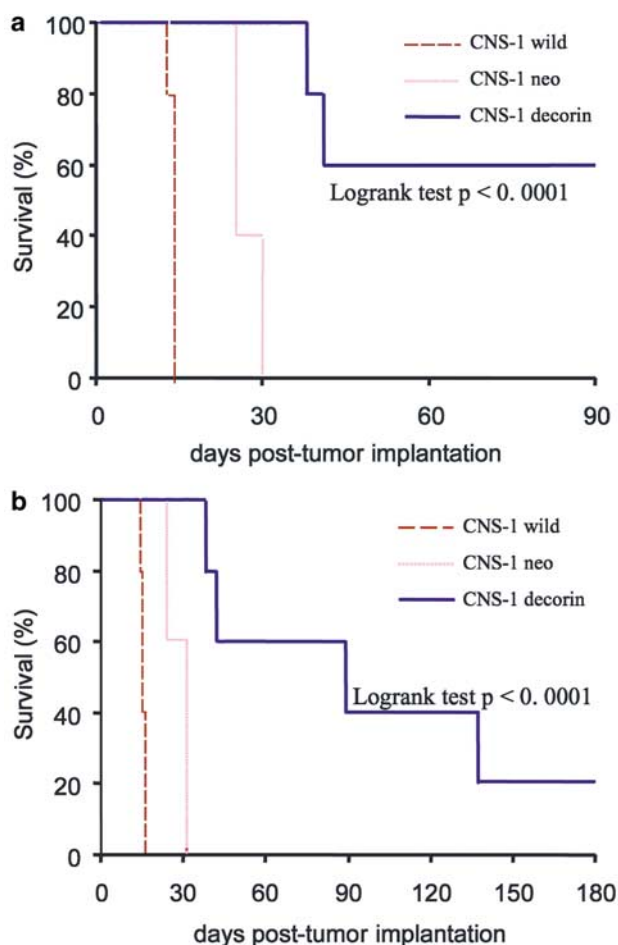


**Figure 2** *In vitro* proliferation of transfected CNS-1 cells. Cultured cells were subjected to daily count in quadruplicate. Growth curves were generated for each of the stably transfected and wild type CNS-1 cells over a 12-day period. Data are represented as the mean of four values (SEM is less than 10%). There was significant difference in cell-doubling times among these cell lines *in vitro* ( $P<.0001$ ).

longer survival of Lewis rats implanted with decorin-transfected cells, the experiment was repeated and surviving animals were allowed to survive for up to 180 days (Figs 3b and 4h). In the second long-term survival

experiment (Fig 3b), most animals died at <180 days. Only one rat survived for 180 days and was shown to be tumor free (Fig 4h). Kaplan–Meier survival curves were devised for decorin-expressing CNS-1 tumor-bearing animals and control groups, and significance was determined using the logrank test ( $P<.0001$ ).

CNS-1 wild-type and CNS-1 neo-implanted animals evaluated at 14 days post-tumor implantation showed tumor masses infiltrated with ED1, CD8 $\alpha$ , CD8 $\beta$  and CD161 cells (Fig 4d,e). These animals succumbed to the tumors between 2 and 4 weeks post-tumor implantation (Fig 3a,b), had large solid masses on the left cerebral hemisphere that filled the entire striatum and displaced the cerebral ventricles (Fig 4a). One asymptomatic rat from the CNS-1/decorin group that was killed on day 90, had a small tumor in the striatum infiltrating the cerebral cortex (Fig 4b). Other asymptomatic animals from the same group that were killed on either day 90 or 180 (Fig 4c,h) were tumor free; only a scar remained in the injection site of CNS-1 cells. The number of infiltrating immune cells was inversely correlated with the size of the remaining CNS tumor (Fig 4d–h). Activated macrophages and microglial cells that either infiltrate or surround the tumor were identified using a mouse monoclonal anti-rat ED1. These cells appeared with increased density in the larger



**Figure 3** Kaplan–Meier survival curves for Lewis rats implanted with decorin-transfected CNS-1 cells, observed for up to 90 (a) or 180 (b) days post cell implantation of transfected or wild-type CNS-1 cells into the striatum of Lewis rats. Lewis rats implanted with CNS-1 cells expressing decorin survive significantly longer than those in the control groups ( $n=5$ , logrank test  $P<.0001$ ; in both a and b).

tumors (Fig 4d,e). Using a mouse monoclonal anti-rat CD8 $\beta$  antibody, regions of densely staining CD8 $^+$  cells were detected within tumors. The density of CD8 $^+$  cells in CNS-1 neo-implanted rats that die between 26 and 31 days post-implantation was considerably higher than in wild-type CNS-1-implanted rats which die before day 15. Brain sections were also stained with mouse monoclonal anti-rat CD8 $\beta$  and CD161 antibodies to detect T-lymphocytes and NK cells. Interestingly, the number of CD8 $\beta$  and CD161-positive cells in CNS-1/neo-implanted rats was much higher than in wild-type CNS-1-implanted rats (Fig 4d,e); this could indicate that neo is being recognized as a tumor neo-antigen. If this was the case, however, neo-antigenicity was not enough to lead to tumor rejection.

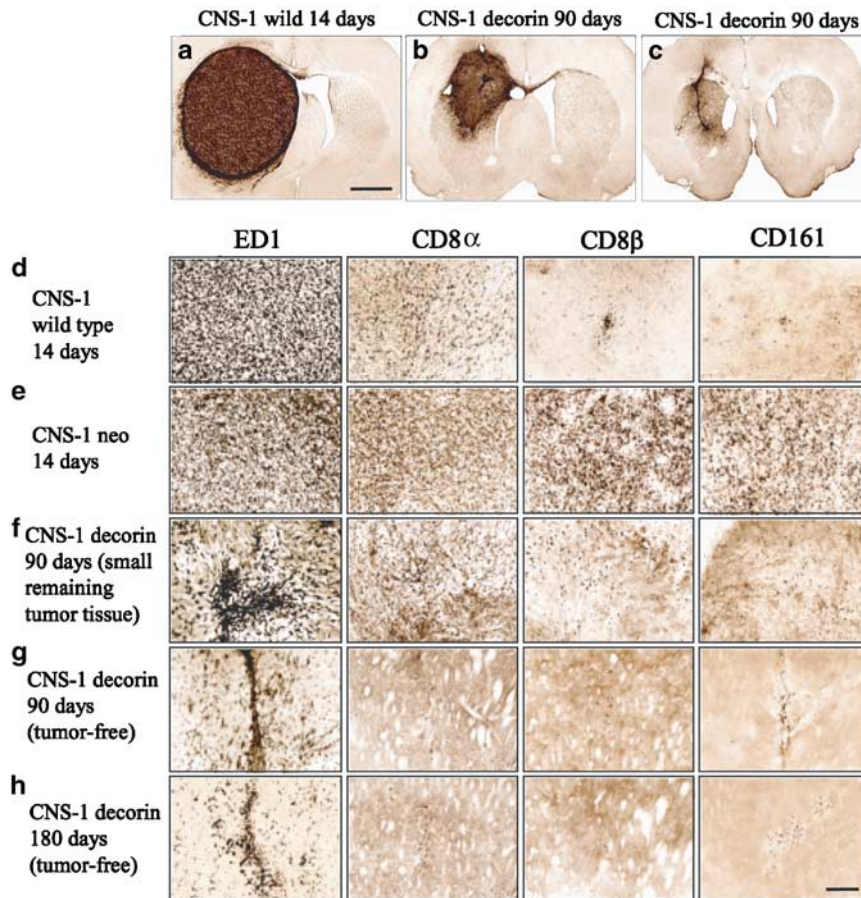
#### Assessment of bioactivity of RAd expressing decorin in vitro

In view of the *in vivo* effects of decorin expressed within stably transfected and implanted CNS-1 cells, we decided

to implement a gene therapy approach to either eliminate or slow glioma growth *in vivo* using decorin gene transfer. To this end, two first-generation RAd vectors encoding the human decorin, RAd/GFAP/decorin and RAd/hCMV/decorin, were constructed. The presence of decorin in the viral genome was confirmed by Southern blotting *Hind*III-digested viral DNA with a decorin-specific probe (Fig 5, panels b and d, lane I). The ability of RAd encoding decorin to express biologically active protein and suppress TGF- $\beta$  bioactivity was evaluated using MLE/PAI/L cells. Following incubation of MLE/PAI/L cells with conditioned medium (containing 0.1 ng/ml recombinant TGF- $\beta_3$ ) from HeLa cells infected with RAd encoding the human decorin at MOI of 100 and 300, luciferase activity was measured (Fig 6). Supernatant from HeLa cells infected with a RAd encoding  $\beta$ -galactosidase, RAd/hCMV/ $\beta$ -gal, were used as controls. At MOI of 100, RAd/GFAP/decorin did not significantly suppress the biological activity of TGF- $\beta$ . However, when a higher MOI of RAd/GFAP/decorin was used, a statistically significant inhibition of TGF- $\beta$  occurred. Incubation of RAd/hCMV/decorin with MLE/PAI/L cells resulted in a statistically significant inhibition of TGF- $\beta$  at MOI of 100 and 300 (Fig 6). HeLa cells were used to prepare conditioned media and as a source of decorin after infection with RAd/hCMV/Decorin or RAd/GFAP/Decorin, since they are easily infected by RAd and further, the RAd do not replicate within these cells. Final confirmation that the decorin-expressing RAd were biologically active was performed in the brain glioma model in Lewis rats *in vivo*.

#### Efficacy of RAd-mediated decorin expression to inhibit rat CNS-1 glioma growth in vivo

Having shown that RAd/GFAP/decorin and RAd/hCMV/decorin express biologically active decorin and suppress TGF- $\beta$  activity, we then investigated whether the survival observed with decorin expressing CNS-1 cells could be replicated *in vivo* using RAd expressing the human decorin gene product to transduce established CNS-1 gliomas. We decided to test the efficiency of two RAd expressing decorin, one driven by the pan-cellular hCMV promoter and the other by the glial cell-type specific promoter, GFAP, with the aim of restricting expression of the therapeutic transgene within cells of glial origin. CNS-1 cells are GFAP positive (results not shown). Lewis rats were injected intratumorally with  $8 \times 10^7$  infectious units (iu) of either of the vectors 3 days post-CNS-1 cell implantation. The survival rate of rats treated with RAd/hCMV/decorin was significantly better compared to the survival rate of rats treated with RAd/hCMV/ $\beta$ -gal ( $P<.005$ ; Figure 7a). There was no statistically significant difference in survival between the rats treated with RAd/GFAP/decorin or RAd/hCMV/ $\beta$ -gal ( $P<.12$ ). The long-term efficiency of RAd/hCMV/decorin to inhibit glioma growth was also compared with the efficiency of a RAd/hCMV/HSV1-TK<sup>17</sup> and ganciclovir (GCV). The survival of animals treated with either RAd/hCMV/decorin ( $P<.0001$ ) or RAd/hCMV/HSV1-TK



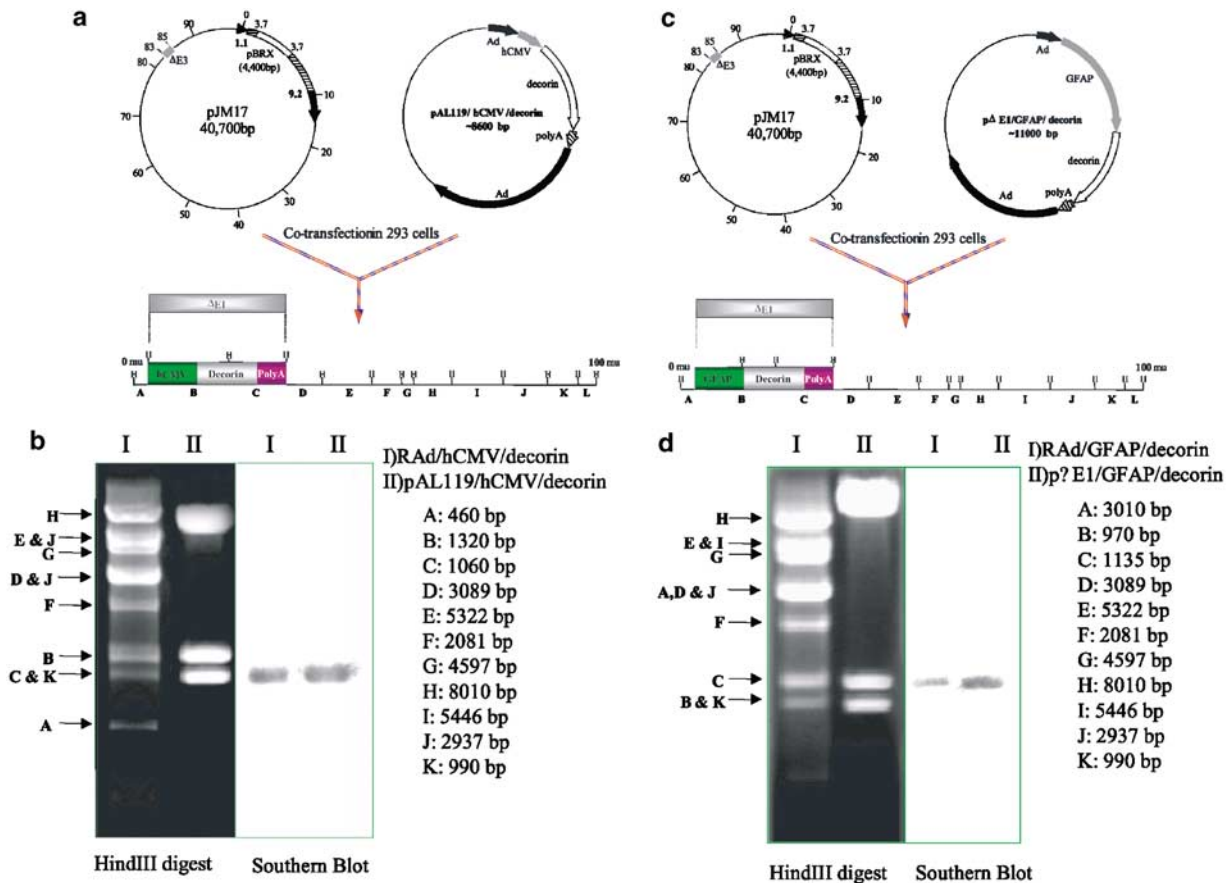
**Figure 4** Representative photomicrographs demonstrating brain histology and immunohistochemical staining for ED1, CD8, CD8 $\beta$  and CD161-positive cells in rats implanted with CNS-1 gliomas. (a) At death, all animals had large solid tumours at the site of cell implantation in the striatum. Surviving rats at 90 days post-tumor implantation had a small remaining tumor (b) or were tumor free (c). Scale bars represent 2 mm. (d–h) The distribution of activated ED1 immunoreactive cells was much higher than the distribution of CD8 immunopositive cells in all rats. In the presence of a growing tumor (d–f), the infiltration of tumor and peritumoral tissue with inflammatory immune cells is higher in tumor-bearing animals (d–f) than in animals that eliminated the tumor (g,h). In animals that eliminated the tumor (g,h), only ED1<sup>+</sup> cells remained in the visible tissue scar. Infiltration of tumors was higher in animals implanted with CNS-1 neo cells when compared to CNS-1 cells; these animals died at the same time as those implanted with control untransfected CNS-1 cells.

( $P < .0001$ ) was better than survival of animals treated with RAD/hCMV/ $\beta$ -gal (Fig 7b). Analysis of rat brains showed that the animals which survived for up to 12 months, were tumor free. Massive enlargement of associated lateral ventricle was observed in the RAD/hCMV/decorn-treated animals that survived for 12 months, but not in RAD/hCMV/HSV1-TK-treated animals (Fig 8a). This enlargement could be due to an ongoing inflammation process in the brains of these animals. All rats treated with RAD/hCMV/HSV1-TK and 20% of the rats treated with RAD/hCMV/decorn were completely tumor free at 12 months. These animals had infiltration of ED1-positive macrophages/microglia in the scar left at the injection site where both the tumor cells and the viruses were delivered, and throughout the striatum (Fig 8b). Rats which were completely tumor free at 12 months also had a small number of CD8 $\beta$ <sup>+</sup> cells present along the injection site (Fig 8b). These results indicate that following successful tumor treatment only

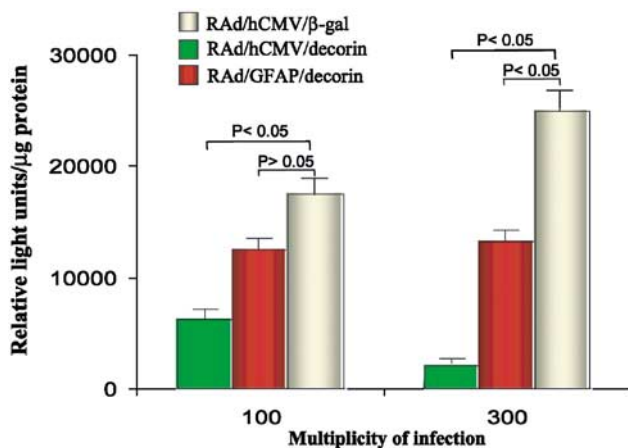
small amounts of activated macrophages (ED1) or CD8 T-cells (CD8 $\beta$ ) remain in the tissue of animals injected with RAD/hCMV/decorn or RAD/hCMV/HSV1-TK when compared to saline or RAD/hCMV/ $\beta$ -gal-treated controls.

## Discussion

The purpose of this study was to examine whether intratumoral expression of decorn can result in tumor regression in an intracranial tumor model and thereby prolong the survival of CNS-1 tumor-bearing Lewis rats. To assess the effect of intratumoral expression of decorn on glioma tumor progression *in vivo*, we used stably transfected glioma cells expressing decorn. *In vivo* experiments with CNS-1 cells expressing decorn clearly indicated that sustained expression of decorn from stably



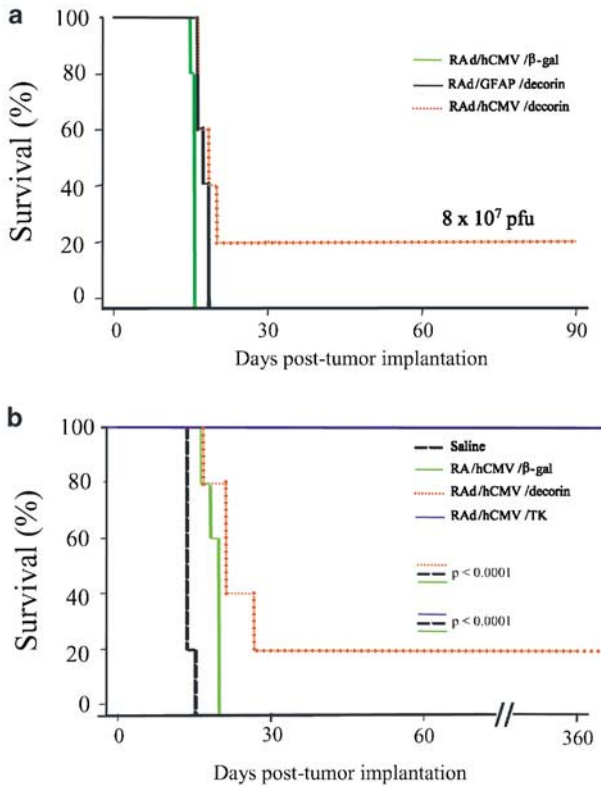
**Figure 5** (a) Schematic diagram of cotransfection of pJM17 and pAL119/hCMV/decorin to generate the RAD/hCMV/decorin vector genome. (b) Southern blot analysis to verify the correct insertion of the decorin expression cassette within the RAD vector. (c) Schematic diagram of cotransfection of pJM17 and pΔE1/GFAP/decorin to generate the RAD/GFAP/decorin vector genome. (d) Southern blot analysis to confirm the correct insertion of the decorin expression cassette within the RAD vector.



**Figure 6** Assessment of the biological activity of RADs encoding decorin. Luciferase activity in MLE/PAI/L cells following incubation with conditioned medium from HeLa cells infected with RADs encoding decorin or β-gal. Both RAD/hCMV/decorin and RAD/GFAP/decorin inhibited TGF-β signaling; RAD/hCMV/decorin was more effective than RAD/GFAP/decorin.

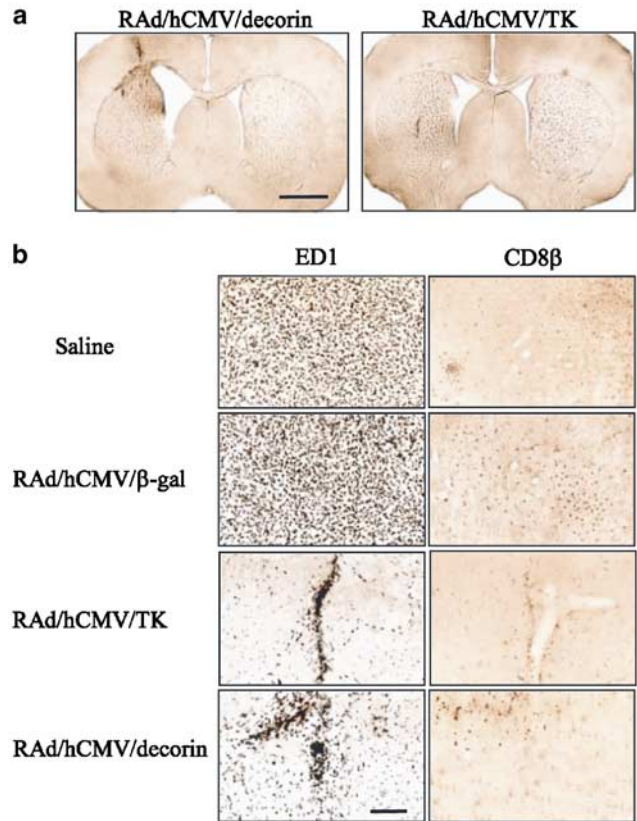
transfected tumor cells is able to increase the survival of tumor-bearing Lewis rats, but it is not sufficient to prevent tumor growth in all animals. The improved survival in Lewis rats that received decorin-transfected CNS-1 cells, is similar to results obtained in tumor xenografts and in the C6 glioma model using C6 cells expressing decorin.<sup>11,13</sup> However, in the C6 glioma model decorin expression resulted in tumor regression in 100% of experimental Sprague–Dawley (SD) rats. One of the possible explanations for the increased tumor regression is that because C6 cells are not syngeneic in SD rats, tumor regression by decorin could be enhanced by host *versus* graft rejection.<sup>25,28–30</sup>

Decorin has the ability to inhibit TGF-β by forming an inactive complex.<sup>4,31,32</sup> Additionally, it has also been shown that decorin inhibits TGF-β mRNA transcription and TGF-β protein synthesis.<sup>13,33</sup> However, these functions appear to be highly cell-type specific. In certain cellular systems, decorin blocks the activity of TGF-β, whereas in others its binding augments the bioactivity of the cytokine.<sup>34</sup> The capacity of CNS-1 cells to produce biologically active decorin and inhibit the action of



**Figure 7** Kaplan–Meier survival curves for CNS-1-implanted Lewis rats treated with RA/GFAP/decorin or RA/hCMV/decorin. (a) At the viral dose of  $8 \times 10^7$ , the survival rate of rats treated with RA/hCMV/decorin was statistically better compared to rats treated with either RA/hCMV/ $\beta$ -gal or RA/GFAP/decorin ( $n=5$ , log-rank test  $P<.005$ ). (b) Kaplan Meier survival curves for CNS-1 implanted Lewis rats treated with either RA/hCMV/decorin or RA/hCMV/HSV1-TK and ganciclovir. Lewis rats implanted with CNS-1 cells and treated with either RA/hCMV/decorin or RA/hCMV/HSV1-TK and ganciclovir survived significantly longer than those in control groups ( $n=5$ ), with RA/hCMV/HSV1-TK and ganciclovir being more effective than RA/hCMV/decorin.

TGF- $\beta$  was assessed *in vitro* using a highly sensitive and specific assay.<sup>27</sup> The specificity and sensitivity of the assay are the result of using a truncated PAI-1 promoter which retains the two regions responsible for maximal response to TGF- $\beta$ . These experiments proved that CNS-1 cells stably transfected with BCMGS/neo/decorin or infected with RAd encoding decorin express and secrete active peptide which can significantly suppress TGF- $\beta$  biological activity. RA/GFAP/decorin only displayed a significant inhibitory effect that was detected at MOI of 300 (Fig 6). This could be explained by the weaker transcriptional activity of the GFAP promoter. Cell-type specific promoters are generally weaker than the widely used strong promoters derived from viruses (e.g., CMV), which are able to drive high levels of transgene expression within many cell types. We have also previously demonstrated that the GFAP promoter driving transgenes encoded within recombinant adenovirus vectors exerts a several fold lower translational activity when compared to the hCMV promoter.<sup>35</sup>



**Figure 8** Representative photomicrographs demonstrating brain histology and immunohistochemical staining for ED1 and CD8 $\beta$ -positive cells in rat CNS-1 gliomas treated with RA/hCMV/decorin, RA/hCMV/HSV1-TK and ganciclovir, RA/hCMV/ $\beta$ -gal or saline. (a) Enlargement of the lateral ventricle observed in an animal treated with RA/hCMV/decorin and that survived for 12 months; this was not seen in animals that had been treated with RA/hCMV/HSV1-TK and ganciclovir which had also survived for 12 months. (b) Rats from control groups (saline and RA/hCMV/ $\beta$ -gal) had high levels of ED1<sup>+</sup> and CD8 $\beta$ <sup>+</sup> cells throughout the tumor mass, with CD8 $\beta$  immunoreactive cells being higher in animals injected with RA/hCMV/ $\beta$ -gal. Rats that were completely tumor free at 12 months post-tumor implantation and gene therapy had infiltration of ED1-positive monocytes/macrophages in the scar remaining at the injection site, and scattered ED1 staining throughout the striatum (RA/hCMV/HSV1-TK and RA/hCMV/decorin). Scale bar represent 200  $\mu$ m.

To test the therapeutic effects of adenovirus-mediated decorin gene therapy in our brain tumor model, 3 days after the implantation of tumor cells, adenovirus vectors bearing the therapeutic gene were stereotactically injected into the tumor. The intratumoral delivery of  $4 \times 10^7$  iu of RA/GFAP/decorin and RA/hCMV/decorin resulted in no significant difference in the survival of CNS-1 tumor-bearing rats as compared with RA/hCMV/ $\beta$ -gal-injected animals (data not shown). However, increasing the dose of inoculated virus two-fold to  $8 \times 10^7$  iu resulted in a statistically significant increase in the survival time of experimental rats treated with RA/hCMV/decorin (Fig 7a). Work from our laboratory has demonstrated that the GFAP promoter has restricted glial cell-type specificity;

however, transgene expression levels are lower than the hCMV promoter, both in cell lines and in primary cultures.<sup>36</sup> Lower expression from the GFAP promoter could explain the failure of RAd/GFAP/decorin to improve the survival of tumor-bearing animals.

To evaluate the reproducibility, the experiment using RAd/hCMV/decorin was repeated on two different occasions (Fig 7b). In these experiments saline-, RAd/hCMV/ $\beta$ -gal- and RAd/hCMV/HSV1-TK-injected animals were evaluated as control groups. We observed significant tumor regression in rats treated with either RAd/hCMV/HSV1-TK or RAd/hCMV/decorin compared with RAd/hCMV/ $\beta$ -gal or saline injected. However, the survival of RAd/hCMV/HSV1-TK and GCV-treated tumor-bearing animals was better than the survival of RAd/hCMV/decorin-treated tumor-bearing animals. A possible explanation is that the rapid growth rate of CNS-1 cells may favor some gene therapy approaches, such as the HSV1-TK/GCV system, that rely on cell division to affect tumor cell killing, while it may be more difficult to see high effectiveness for a strategy like decorin overexpression, that relies on the activation of the immune response and its effector mechanisms.<sup>37</sup>

Decorin is a secreted protein that has been suggested to act as both an autocrine and paracrine regulator of tumor growth. Decorin has been previously shown to bind the EGFR. This interaction results in activation of the mitogen-activated protein (MAP) kinase pathway, induction of p21, a potent inhibitor of cyclin-dependent kinases, and ultimately cell cycle arrest.<sup>8</sup> Recently, it has been determined that decorin causes a sustained down-regulation of the EGFR and the attenuation of EGFR-mediated mobilization of intracellular calcium resulting in a decrease in EGFR kinase activity and inhibition of tumor growth *in vivo*.<sup>10</sup> Further, following adenovirus-mediated transfer of decorin in a mouse lung carcinoma tumor model, tumor volume was decreased and cell apoptosis was revealed in regions surrounding decorin-positive cells in these mice.<sup>38</sup> *In vivo* studies using decorin have also provided evidence that decorin may act through a novel mechanism, where it suppresses vascular endothelial growth factor (VEGF), resulting in inhibition of tumor growth.<sup>3</sup> The differences in effectiveness seen in our studies using CNS-1 cells that were stably transfected with decorin, when compared to gene transfer of decorin using RAds, could be due to the fact that in the former, decorin is expressed from the time of tumor cell implantation and therefore its inhibiting effects are seen very early on, while in the latter model, decorin is being delivered using a RAd, which although efficient, does not infect 100% of the tumor mass. Also, delivery was performed 3 days post-tumor implantation, once the tumor is already established, and this might impair the effectiveness of decorin in inhibiting tumor progression.

Enlargements of the lateral ventricle could be seen in the vicinity of the RAd/hCMV/decorin-treated tumors, which is caused by tumor-induced destruction of striatal tissue. Similar effects can be seen in CNS-1 decorin-implanted animals that were tumor free at day 180, but

not in RAd/hCMV/HSV1-TK and GCV-treated tumors. This finding suggests that RAd/hCMV/HSV1-TK and GCV can kill tumors before they reach a size that causes striatal tissue destruction.

In conclusion, our results indicate that although decorin has significant potential to slow CNS-1 glioma growth *in vivo*; it will be necessary to increase its levels of expression, for example, using the murine CMV promoter,<sup>39</sup> to enhance its effectiveness further. It will also be necessary to express decorin very early on. Combination of decorin overexpression with conditional cytotoxic strategies, and other antitumor agents, may provide added therapeutic advantages.

## Materials and methods

### Recombinant adenoviruses

The RAd vectors used are E1/E3-deleted first-generation adenovirus, in which the cassette containing a recombinant transgene and promoter is inserted in place of the E1 region. Four different vectors were used in this study: (I) RAd/GFAP/decorin; (II) RAd/hCMV/decorin; (III) RAd/hCMV/ $\beta$ -gal; (IV) RAd/hCMV/HSV1-TK. Construction of the RAd/hCMV/ $\beta$ -gal and RAd/hCMV/HSV1-TK has been described in detail elsewhere.<sup>21,40</sup> To create the shuttle vector p $\Delta$ E1/GFAP/decorin, which was used to generate RAd/GFAP/decorin, a blunted *EcoRI* human decorin fragment (1780 bp) was cloned into the blunted *BamHI* site of the p $\Delta$ E1/GFAP.<sup>36</sup> A blunted *EcoRI* human decorin fragment (1780 bp) was cloned into the blunted *BamHI* site of pAL119 (pMV35 in Shering et al<sup>40</sup>) to create the shuttle vector pAL119/decorin which was used to generate RAd/hCMV/decorin. Recombinant adenoviruses encoding the decorin gene were then generated by homologous recombination in 293 cells following cotransfection of the shuttle vector and pJM17 plasmid (Microbix biosystems, Inc.). Restriction enzyme and Southern blot analysis were carried out to confirm the presence of the human decorin sequences in the viral genome. Production of high-titer stocks, purification by double-cesium chloride density-gradient separation, and titration of viruses were carried out as previously described.<sup>41,42</sup> Viral stocks were found to be free of replication-competent adenovirus using a supernatant rescue assay able to detect one replication-competent virus within 10<sup>9</sup> recombinant viruses.<sup>43</sup> Adenovirus preparations were determined to be endotoxin (lipopolysaccharide) free, according to the criteria of Cotton et al<sup>44</sup> using the E-TOXATE assay (Sigma-Aldrich, Dorset, UK).

### Cell culture

The kidney embryonic 293 cell line was obtained from Microbix biosystems (Ontario, Canada). The HeLa cell line was purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). The mink lung epithelial cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct was a gift from

Dr DB Rifkin (Department of Cell Biology, New York University Medical Center, NY). The rat glioma CNS-1 cell line was a generous gift from Professor. W Hickey (Department of Pathology, Dartmouth Medical Centre, Lebanon, NH). The 293 cells were grown in MEME containing 10% FCS and 2 mM glutamine. The HeLa cells, CNS-1 cells and mink lung epithelial cells were grown in DMEM containing 10% FCS and 2 mM glutamine. All growing cells were incubated in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

#### *Generation and evaluation of glioma CNS-1 clonal cell sublines stably expressing decorin*

Human decorin cDNA was cloned into the BCMGS/neo plasmid.<sup>26</sup> BCMGS/neo/decorin or BCMGS/neo without insert, as a control, was then transfected into CNS-1 glioma cells by electroporation.<sup>13</sup> PCR was used to confirm the presence of human decorin sequences in genomic DNA extracted from decorin stably transfected CNS-1 cells. A 944 bp decorin-specific PCR product was amplified using the primers decorin-fwd (5'-CCCA GAAGTTCCTGATGAC-3') and decorin-rev (5'-CA GAGCGCACGTAGACAC-3'). Two control rat  $\beta$ -actin primers,<sup>17</sup>  $\beta$ -actin-fwd (5'-CCAGCCATGTACGTAGC CATCC-3') and  $\beta$ -actin-rev (5'-GCAGCTCATAGCTC TTCTCCAGG-3'), were used to produce a 340 bp  $\beta$ -actin specific PCR product. In a 50- $\mu$ l PCR reaction, 10  $\mu$ l of genomic DNA was used in a solution containing 1  $\times$  PCR buffer (Promega), 200  $\mu$ M dATP, 200  $\mu$ M dTTP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTP, 2 mM MgCl<sub>2</sub>, 50 pM of each primer oligonucleotide, 2 U *Taq* polymerase (Promega). PCR conditions were as follows: 45 seconds denaturation, 1-minute annealing, and 1-minute extension for 15 cycles, followed by another 7 minutes of extension. The annealing temperature for all primers was 60°C. The PCR products were separated by 2% agarose gel electrophoresis and were visualized on a UV transilluminator after being stained with ethidium bromide.

Transgene expression was assessed by immunocytochemical analysis. CNS-1 cells were grown directly on glass coverslips in six-well plates, washed twice with PBS and fixed in 4% paraformaldehyde/0.12 M sucrose for 20 minutes. Cells were then permeabilized with 0.5% Triton X-100 for 10 minutes and blocked with 10% normal horse serum in PBS for 30 minutes. A volume of 60  $\mu$ l of the primary antibody (sheep anti-human decorin, United States Biological) diluted 1:1000 in PBS/1% horse serum, were placed onto coverslips and left at 4°C overnight. The next day, cells were washed three times with PBS and incubated with secondary FITC-labeled antibody (donkey anti sheep), diluted 1:1000 in PBS/1% horse serum, for 2 hours at 4°C. Cells were washed 3 times with PBS, and incubated with 1  $\times$  DAPI solution (Sigma) for 10 minutes. Unreacted DAPI was removed with three successive washes in PBS. The cell-coated coverslips were mounted in Mowiol mounting solution (Calbiochem Nottingham, UK) and left to dry at 4°C in dark. Images were acquired

using Openlab software (Improvision, Coventry, UK) on an Olympus Corp. (Tokyo, Japan) Vanox microscope.

The ability of decorin-transfected CNS-1 cells to express biologically active protein and suppress TGF- $\beta$  bioactivity was evaluated using a mink lung epithelial cell line that was stably transfected with a plasminogen activator inhibitor-1 promoter-Luciferase construct (MLE/PAI/L cells). Exposure of MLE/PAI/L cells to TGF- $\beta$  induces a dose-dependent increase in Luciferase activity.<sup>27</sup> The medium was aspirated from 25 cm<sup>2</sup> flasks containing CNS-1 cells at 40% confluency and replaced with 3 ml of DMEM supplemented with 0.1% bovine serum albumin and 2 mM L-glutamine. The cells were then incubated in 5% CO<sub>2</sub> at 37°C for 48 hours. After 2 days, MLE/PAI/L cells were seeded in a 24-well plate at a density of 8  $\times$  10<sup>4</sup> cells/well and incubated for 3 hours. After 3 hours the medium was aspirated and replaced with 500  $\mu$ l supernatant from CNS-1 cells in triplicate. After incubation in 5% CO<sub>2</sub> at 37°C for 16 hours, the conditioned medium was removed, the cells washed twice with 500  $\mu$ l PBS and lysed by addition of 60  $\mu$ l of cell lysis buffer (1.25 ml 1 M Tris-HCl pH 7.8, 100  $\mu$ l 0.5 M EDTA pH 8, 15% glycerol, 10 mM MgCl<sub>2</sub> and 1% Triton X-100, made up to 50 ml with dH<sub>2</sub>O). The cell debris was pelleted by centrifugation at 13,000 rpm for 2 minutes and the supernatant was transferred to a clean Eppendorf tube. Luciferase assay reagent (Promega) was prepared immediately prior to use by diluting with an equal volume of distilled water. A volume of 20  $\mu$ l of cell lysates were added to 100  $\mu$ l of Luciferase assay reagent (Promega) and Luciferase activity measured over 10 seconds in a luminometer. Luciferase activity was then standardized dividing the RLU by the  $\mu$ g of protein in each sample.

#### *Assessment of the growth rate of decorin-transfected CNS-1 cells in vitro*

The growth rate of CNS-1 cells expressing decorin was assessed *in vitro* and compared with growth rate of wild-type CNS-1 and neomycin control CNS-1 cells. The cells were seeded into 12-well plates at a density of 2500 cells/well in quadruplicates and incubated at 37°C in 5% CO<sub>2</sub>. During 12 days, every 24 hours, the cells were rinsed with 300  $\mu$ l of Dulbecco's PBS and detached using 0.05% Trypsin-EDTA. Then 500  $\mu$ l of growth medium was added, the cells were dispersed by pipetting up and down, and the number of living cells was determined using trypan blue staining. An improved Neubauer haemocytometer was used to count the cells in quadruplicates.

#### *Bioactivity of RAds encoding decorin in vitro*

The medium was aspirated from 25 cm<sup>2</sup> flasks containing HeLa cells at 80% confluency and replaced with 3 ml of DMEM supplemented with 0.1% bovine serum albumin and 2 mM glutamine. The cells were then infected with RAds encoding decorin or  $\beta$ -galactosidase at MOI of 100 and 300. The cells were incubated at 37°C in 5% CO<sub>2</sub> for 72 hours. After 72 hours conditioned medium was removed from 25 cm<sup>2</sup> flasks and TGF- $\beta$ 3 (Promega) was added into the conditioned medium (0.10 ng/ml) and

incubated at 37°C for 3 hours. MLE/PAI/L cells were seeded in a 24-well plate at a density of  $8 \times 10^4$  cells/well and incubated for 3 hours. After incubation in 5% CO<sub>2</sub> at 37°C for 16 hours, the conditioned medium was removed. Luciferase activity was determined as described previously.<sup>27</sup>

### Experimental gliomas

All animal experiments were conducted in accordance with the guidelines of the United Kingdom Animal (Scientific Procedures) Act of 1986. The animals used were adult male Lewis rats with a body weight ranging from 250 to 300 g. All animals were housed at constant temperature and humidity, and 12/12-hours light-dark cycle and had free access to food and water. Following anesthesia with 4% halothane (AstraZeneca), the rats were placed in a stereotaxic frame and connected to an anesthetic machine to deliver 1% halothane in 66% medical oxygen and 33% medical nitrous oxide. After making a hole in the skull, using a Hamilton syringe with a 26 gauge needle,  $5 \times 10^3$  cells (in 3  $\mu$ l PBS) were injected unilaterally into the right striatum at coordinates 1 mm forward and 3 mm lateral from the bregma and 4 mm vertical from the dura. At 3 days post-tumor implantation, viruses were injected into the tumor site. RAD/hCMV/HSV1-TK-injected animals, received 25 mg/kg GCV (Roche products, Welwyn Garden City, UK) intraperitoneally for 7 days. All animals were monitored daily for temperature and well-being. The tumor-implanted animals were monitored twice daily for any observable signs of distress or discomfort and killed when tumors prevented feeding or ambulation. Rats were monitored daily and unwell rats anesthetized, perfused, and fixed, as described previously.<sup>21</sup>

### Histological analysis

Histological analysis of brain tissue and immunohistochemistry were carried out as previously described.<sup>45,46</sup> The primary antibodies and the dilutions at which they were used were anti-decorin (1:1000; US Biological), anti- $\beta$ -galactosidase (1:1000; Promega), anti-ED1 (1:1000; Serotec), anti-CD8 (1:500; Serotec), anti-CD8b (1:1000; Serotec), and anti-CD161 (1:1000; Serotec). All primary antibodies were mouse monoclonal anti-rat, except for anti-decorin which was sheep polyclonal anti-human. Reacted primary antibodies were labeled with biotinylated anti-mouse or anti-sheep antibodies (Dako) at a dilution of 1:200.

### Statistical analysis

Student's *t*-test was used to analyze the *in vitro* experimental results. Survival data were analyzed by Kaplan-Meier estimator analysis, and compared using the log-rank test. Statistical analysis was performed using the SPSS program software (version 10).

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