

# Adenovirus-mediated gene transfer in the ovine pituitary gland is associated with hypophysitis

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## Abstract

Gene therapy for pituitary disease requires evaluation for safety as well as efficacy. We have reported results of adenovirus-mediated gene transfer using the sheep as a large animal model that allows longitudinal evaluation of hormone secretion and have confirmed high levels of transgene expression up to 7 days after direct stereotaxic injection into the pituitary gland. Here we report the results of detailed histological examination of the pituitary glands from animals injected with two recombinant adenoviruses expressing the  $\beta$ -galactosidase marker gene, or with saline vehicle to control for the potential tissue-disruptive effect of the injection volume itself. Pituitaries injected with saline showed no evidence of inflammatory response apart from occasional minor foci of apoptosis. In all other respects they were indistinguishable from normal uninjected control pituitary glands. Glands injected with recombinant adenoviruses containing either

the hCMV- $\beta$ -gal or the hPRL- $\beta$ -gal transgene, on the other hand, displayed variable degrees of inflammatory response, with periglandular fibrosis, lymphocytic infiltrate and venulitis in almost all cases. Focal necrosis and/or apoptosis was noted in six of nine cases.

In summary, we have found evidence of severe inflammatory reaction within the first seven days of adenovirus injection, amounting to significant hypophysitis. The histological extent of this reaction has not previously been recognised by studies of the efficacy of gene transfer in rodents, and was underestimated by immunocytochemical studies of hormone and transgene expression. The findings emphasise the need for careful evaluation of the safety of endocrine gene therapy, and for caution with the dose of vector used.

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## Introduction

Pituitary tumours are conventionally treated by transphenoidal or transfrontal surgery, external beam irradiation, or with long-term suppressive drug therapy. Although these approaches are widely accepted, they have significant shortcomings, such as lifelong hypopituitarism and drug side-effects (Davis *et al.* 1999). For those tumours that are particularly aggressive or the rare malignant cases that have metastasised, existing therapy is often of little or no benefit. For these reasons, interest has grown in the possibility of exploiting gene therapy approaches for pituitary disease (Castro 1999, Davis *et al.* 1999, Barzon *et al.* 2000, Castro *et al.* 2001, Davis & McNeilly 2001). Adenoviral gene transfer is also a potentially attractive approach for studies of pituitary physiology and a variety of

*in vitro* and *in vivo* models are available for evaluation of the effects of therapeutic or interventional gene transfer.

Several studies have reported experience with adenoviruses as highly efficient tools for gene transfer into pituitary cells (Castro *et al.* 1997, Neill *et al.* 1998, Lee *et al.* 1999, 2000, Southgate *et al.* 2000, Windeatt *et al.* 2000). Recombinant adenoviruses infect all pituitary cell types effectively, as judged by monitoring marker genes such as  $\beta$ -galactosidase (Castro *et al.* 1997), and variable degrees of pituitary ablation have been achieved in rodent models using the herpes simplex virus thymidine kinase (HSV-TK) gene (Lee *et al.* 1999, Southgate *et al.* 2000, Windeatt *et al.* 2000). We have recently reported encouraging results using the sheep as a large animal model that allows longitudinal evaluation of hormone secretion, where we can achieve high-level transgene expression for

at least 7 days after direct stereotaxic injection into the pituitary gland without disruption of normal endocrine function or any apparent ill effect (Davis *et al.* 2001).

Any potential application of gene therapy for pituitary disease in man, however, requires evaluation for safety as well as efficacy. Adenoviruses have been associated with significant inflammatory responses in several tissues (Byrnes *et al.* 1995). Work on the pituitary gland to date has suggested little apparent adverse effect (Southgate *et al.* 2000), but a transient inflammatory response in the rat pituitary has been reported, accompanied by a humoral response, both processes peaking at about 14 days after intrapituitary injection (Southgate *et al.* 2001). A significant pituitary inflammatory reaction might be clinically significant even if it was transient, and it is therefore of interest to assess its histological nature and extent. We analysed the pituitary gland sections from our previous work on direct stereotaxic injection in the ovine pituitary (Davis *et al.* 2001), and found surprisingly extensive leucocyte infiltration, despite a lack of any apparent systemic disturbance. We have, therefore, carried out a careful histological survey of these adenovirus-injected pituitary glands, and compared them with normal pituitary and control tissue after stereotaxic injection of the same volumes of saline vehicle. We found evidence of a severe inflammatory reaction associated with injection of the recombinant adenoviruses, with lymphocytic infiltrates, venulitis and periglandular fibrosis in all cases. The extent of this reaction in the pituitary was not suspected from longitudinal assessment of haematological and endocrine parameters. These findings emphasise the need for careful evaluation of possible adverse effects of viral gene transfer into the intact pituitary gland, especially in view of the known variation in immune response among different animal models (Ohmoto *et al.* 1999).

## Materials and Methods

### *Animals and stereotaxic pituitary injection*

The experimental procedure has been described in detail in our earlier report (Davis *et al.* 2001). Briefly, anoestrous Suffolk ewes (2–3 years old, 35–45 kg) were anaesthetised and a stereotaxic frame was set up. A burr-hole was made in the skull anterior to the bregma, and the location of the pituitary gland was deduced from lateral skull radiographs after instillation of radio-opaque dye into the cerebral spinal fluid to visualise the third ventricle (Mori *et al.* 1990, Lignereux *et al.* 1991). For injection of virus particles or of saline control, a fine-bore metal cannula was inserted stereotaxically and lowered to the base of the pituitary fossa. It was withdrawn 2 mm, and 250  $\mu$ l viral vector or saline vehicle were injected into the pituitary gland at each of three levels. The cannula and guide needle were left in place for 1 min, then both were completely withdrawn, reintroduced 2 mm anteriorly, and the

procedure repeated. A third injection procedure was carried out posterior to the initial injection site. A total of nine sites were used to inject virus, using a total volume of approximately 2.2 ml injected over a period of 50–60 min.

### *Adenovirus vectors*

Nine animals were injected with suspensions of recombinant adenovirus in normal saline, and four with saline or with saline containing a dilute suspension of India ink to help confirm the injection sites within the gland. Two alternative recombinant adenovirus vectors were used, based on adenovirus type 5, in which the E1 and part of the E3 regions were deleted. In one of these, RAd-CMV- $\beta$ -gal (also termed RAd-35), the  $\beta$ -galactosidase gene is driven by the short immediate-early human cytomegalovirus ( $\delta$ MIEhCMV) promoter inserted in place of the E1 deletion (Castro *et al.* 1997). In the other vector (RAd-hPRL- $\beta$ -gal) the promoter element comprised a -4429/+14 fragment from the pituitary-specific promoter of the human prolactin gene (Berwaer *et al.* 1991, Takasuka *et al.* 1998), constructed as described (Southgate *et al.* 2000). Virus injections were prepared of RAd-hPRL- $\beta$ -Gal or RAd-hCMV- $\beta$ -Gal, at a concentration that delivered approximately  $1.5 \times 10^8$  pfu per site, giving a total of approximately  $14 \times 10^8$  pfu injected into the gland.

### *Post-operative monitoring*

Blood sampling was carried out before surgery and then daily, via an indwelling jugular catheter as previously reported (Davis *et al.* 2001). Samples were analysed for hormones by radioimmunoassay, and for routine haematology by the Diagnostic Services laboratory, Department of Veterinary Clinical Studies, Easter Bush Veterinary Centre, Roslin, Midlothian, UK. The endocrine data have been reported previously for the virus-injected animals (Davis *et al.* 2001), and data for the saline-injected animals were similar and are not shown in this paper. Haematological data were available for four of the virus-injected animals and for all of the saline-injected animals and controls, and are given in full here for comparison with the histological findings. Animals were killed 4–7 days after virus injection with an overdose of intravenous pentobarbitone. The dura overlying the pituitary gland was examined for puncture holes, and the pituitary glands were dissected, divided sagittally into thirds, and placed into Bouin's fixative within 5 min of death. All experimental procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act 1986 of the United Kingdom.

### *Histopathology*

Tissues were routinely fixed in Bouin's fixative and processed into paraffin. Sections were cut at 4  $\mu$ m and stained with haematoxylin and eosin.

**Table 1** Haematological indices of animals on the day of pituitary collection. Values are shown for four of the adenovirus-injected animals (Ad), for four of the animals whose pituitary glands were injected with saline alone, and for four unoperated controls of the same breed. Laboratory control values (general ovine range, from Diagnostic Service Laboratories, Edinburgh) are shown for reference, and values outside this reference range are asterisked. Note that mild leucocytosis was common regardless of any operative procedure, and that both this and the neutrophilia seen in one virus-injected animal were not reliable markers for the histological evidence of pituitary inflammation (Table 2)

Injection ID#	Haemoglobin (g/dl)	Total leucocytes ( $\times 10^9/l$ )	Neutrophils ( $\times 10^9/l$ )	Lymphocytes ( $\times 10^9/l$ )	Monocytes ( $\times 10^9/l$ )	Eosinophils ( $\times 10^9/l$ )
Ad 11/1639	11.9	7.8	2.34	5.15	0.23	0.07
Ad 12/1640	11.2	11.4*	8.09*	0.23*	2.74*	0.23
Ad 13/1703	10.5	6.7	2.34	3.89	0.47	0
Ad 14/1708	10.3	8.9	4.27	4.45	0.18	0
Saline 15/2294	10.7	9.4	2.54	6.11	0.19	0
Saline 16/2296	11.9	11.8*	2.48	7.55*	0.59	1.06*
Saline 17/2298	11.4	7.8	1.40	5.62	0.55	0.16
Saline 18/2300	11.1	10.7*	3.42	6.42	0.32	0.53
Control	11.5	6.6	1.85	4.42	0.20	0.13
Control	12.1	11.4*	4.90	5.70	0.34	0.34
Control	12.5	10.8*	2.70	7.24	0.11	0.76
Control	13.0	11.3*	3.05	7.34	0.23	0.68
Normal range	8–14	4–10	0.4–5.0	1.6–7.5	<0.60	<1.0

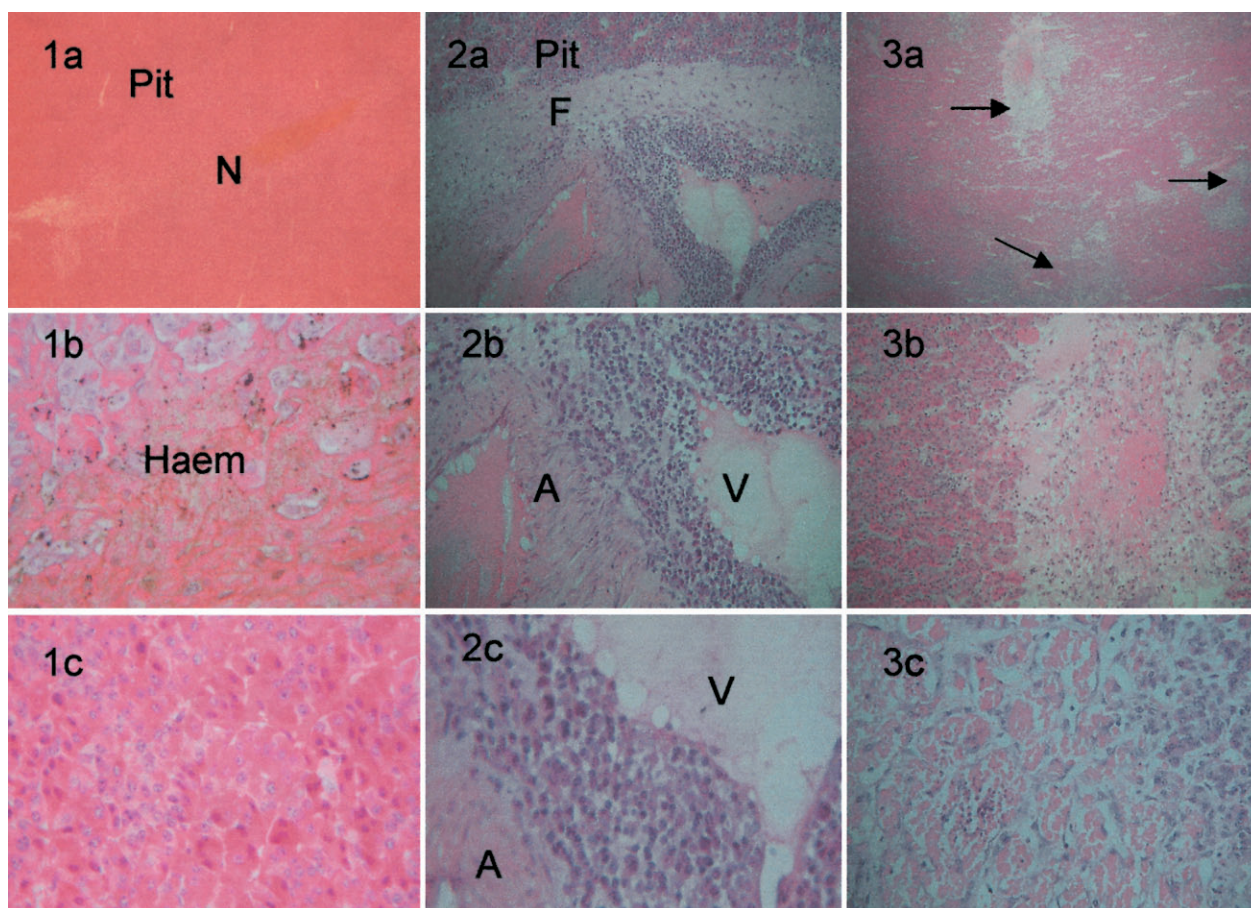
## Results

Animals injected with either adenovirus or with saline remained apparently well. Haematological parameters remained normal in most animals throughout the 7-day period of study after injection. Full details of differential leucocyte counts on the day of pituitary collection for histology are given in Table 1. Mild leucocytosis was seen in many animals, including unoperated controls of this breed, but a mild neutrophilia and relative lymphopaenia was seen in only one animal injected with adenovirus. Measurements of pituitary hormone secretion also remained normal (data not shown, previously reported in Davis *et al.* 2001).

On macroscopic and histological examination of the pituitary glands, needle tracks were clearly visible (Fig. 1, 1a), and in some but not all cases this was associated with microscopic haemorrhage within the parenchyma of the pituitary (Fig. 1, 1b). Pituitaries injected with saline showed no evidence of inflammatory response apart from

two of eight animals where minor foci of apoptosis were noted (Fig. 1, 1c). In all other respects they were indistinguishable from normal uninjected control pituitary glands (Table 2).

Glands injected with recombinant adenoviruses containing either the hCMV- $\beta$ -gal or the hPRL- $\beta$ -gal transgene, on the other hand, displayed variable degrees of inflammatory response. The most prominent findings included marked periglandular lymphocytic infiltrate, oedema and fibrosis (Fig. 1, 2a), with associated venulitis in almost all cases (Fig. 1, 2b and 2c). Within the glandular tissue more minor degrees of inflammatory infiltrate were seen, with focal necrosis in five of the nine virus-injected animals (Fig. 1, 3a-c), and localised apoptosis in two (Table 2). It is noteworthy that the severity of the inflammatory reaction seen in the pituitary glands was not reflected by the relatively unaltered systemic haematological indices in these animals, shown in Table 1, implying that haematological monitoring is not an adequate guide to intra-pituitary inflammatory response.



**Figure 1** (1a) Low power view of pituitary gland injected with saline (#15/2294) in which a needle track (N) associated with an area of haemorrhage is clearly visible. The gland (Pit) is otherwise normal (haematoxylin and eosin; magnification  $\times 43$ ). (1b) Higher power view of the same gland showing haemosiderin-containing macrophages (dark brown granules) at the site of haemorrhage (Haem) (haematoxylin and eosin; magnification  $\times 860$ ). (1c) High power view of a pituitary gland injected with saline (#17/2298) displaying increased numbers of apoptotic cells, characterised by pyknotic, dark staining nuclei and granular, hyper eosinophilic cytoplasm (haematoxylin and eosin; magnification  $\times 430$ ). (2a) Pituitary gland injected with adenovirus (#12/1640) demonstrating periglandular fibrosis (F) with intense lymphoid infiltrate around vessels but also extending into glandular tissue (Pit) (haematoxylin and eosin; magnification  $\times 85$ ). (2b) Higher power view of the same area, showing that the infiltrate mainly involves a venule (V) (to the right) with relative sparing of an adjacent arteriole (A) (to the left) (haematoxylin and eosin; magnification  $\times 210$ ). (2c) Higher magnification shows that this infiltrate consists of lymphocytes, macrophages and plasma cells (haematoxylin and eosin; magnification  $\times 430$ ). (3a) Low power view of a pituitary gland injected with adenovirus (#13/1703) in which there are several foci of necrosis (arrows) (haematoxylin and eosin; magnification  $\times 43$ ). (3b) Medium power view of the largest focus showing necrosis, oedema and inflammatory infiltration (haematoxylin and eosin; magnification  $\times 85$ ). (3c) High power view showing coagulative necrosis (to the left) with occasional neutrophil polymorphs, alongside normal pituitary (to the right) (haematoxylin and eosin; magnification  $\times 210$ ).

## Discussion

A number of previous reports have confirmed the efficacy of adenoviral gene transfer into pituitary cells, both *in vitro* and *in vivo*. This has raised the possibility that gene therapy might be a viable future approach to the treatment of pituitary tumours in man (Castro 1999, Davis *et al.* 1999, Castro *et al.* 2001, Davis & McNeilly 2001). Any future application of gene therapy to pituitary tumours will require careful evaluation of its safety, especially given that pituitary disease is generally

not directly fatal. For this reason we have studied a large animal model that allows multi-parameter longitudinal assessment. Direct intra-pituitary injection of adenovirus appears to be necessary for adequate transgene expression within the pituitary gland (Lee *et al.* 2000). Cell-type specificity can be achieved by the use of pituitary gene promoters such as prolactin and growth hormone (Lee *et al.* 1999, Southgate *et al.* 2000, Davis *et al.* 2001), and this might, in principle, be useful to limit the cytotoxicity of intended ablative transgene expression to the intended target cell type.

**Table 2** Detailed histological findings for pituitary glands injected with adenovirus (Ad) or saline vehicle. The number of days elapsed after injection is shown, and histological details are shown separately for glandular tissue and periglandular tissue

ID#	Vector injected	Days after injection	Glandular tissue					Periglandular tissue			
			Cells	Necrosis	Apoptosis	Inflammation	Haemorrhage	Fibrosis	Inflammatory infiltrate	Venulitis	
Ad5/1572	RAd-CMV-βgal	3	Normal	Focal	Nil	At edge	No	+	++	++	
Ad9/1582	RAd-PRL-βgal	4	Normal	Focal	Nil	Nil	No	+/-	+	Nil	
Ad10/1586	RAd-PRL-βgal	4	Normal	Nil	Nil	Nil	No	+	+/+++	+	
Ad8/1630	RAd-PRL-βgal	6	Normal	Nil	Nil	Nil	No	+	+++	+++	
Ad6/1625	RAd-CMV-βgal	7	Normal	Focal	Focal	Nil	No	+/-	+	+	
Ad11/1639	RAd-PRL-βgal	7	Normal	Nil	Focal	Mild	No	+	++	++	
Ad12/1640	RAd-PRL-βgal	7	Normal	Nil	Nil	At edge	No	++	++	+++	
Ad13/1703	RAd-CMV-βgal	7	Normal	Several foci	Nil	Nil	No	+/-	+	+	
Ad14/1708	RAd-CMV-βgal	7	Normal	Small foci	Nil	Nil	No	+	+	+	
15/2294	Saline	7	Normal	Nil	Nil	Nil	Yes	Nil	Nil	Nil	
16/2296	Saline+ink	7	Normal	Nil	Nil	Nil	Yes	+/-	Nil	Nil	
17/2298	Saline+ink	7	Normal	Nil	Focal	Nil	Yes	Nil	Nil	Nil	
18/2300	Saline	7	Normal	Nil	Focal	Nil	Yes	Nil	Nil	Nil	
Normal pituitary	Uninjected	—	Normal	Nil	Nil	Nil	No	+/-	Nil	Nil	

The histological severity of inflammatory changes was graded: +/- indicates minimal evidence, + indicates mild, ++ moderate, and +++ severe changes.

An inflammatory response to adenovirus injection has been reported in other tissues (Byrnes *et al.* 1995, Wood *et al.* 1996, Chan *et al.* 1999, Dewey *et al.* 1999, Thomas *et al.* 2000) but until recently this had not been considered a major problem until the death of a patient after systemic injection in 1999 (Marshall 1999). The immune response to adenovirus is thought to be primarily a T-lymphocyte-mediated response (Yang *et al.* 1994, Kajiwara *et al.* 1997, Chan *et al.* 1999), although recent evidence in the rat pituitary has implicated activated macrophages and NK cells, and also a neutralizing humoral immune response (Southgate *et al.* 2001). It remains unclear whether the inflammatory response is due to the adenoviral structural proteins or to adenoviral transgene expression (Yang *et al.* 1994, Tripathy *et al.* 1996, Thomas *et al.* 2000). Second generation, helper-virus-dependent ('gutless') vectors deleted of all viral genes may prove safer for use in this respect, and recent reports have suggested that these vectors are associated with substantially attenuated and transient inflammatory responses in the brain (Thomas *et al.* 2000, 2001).

Histological analysis of adenovirus-injected pituitary glands revealed a series of features of hypophysitis. Mild or moderate periglandular fibrosis was seen in six of the nine virus-injected animals, while only a minimal degree was seen in the controls. However, a periglandular inflammatory infiltrate of lymphoid cells was seen only after adenovirus injection and was intense in many of the cases and especially associated with blood vessels. These features are those of severe hypophysitis. The inflammatory infiltrate was composed of lymphoid cells showing

morphological features of mature lymphocytes, macrophages and plasma cells. No polymorphonuclear cells were identified, suggesting that even as early as 7 days after injection the response was predominantly of an histologically chronic type, suggestive of an immune-mediated reaction to viral components. The end result of such inflammation is typically fibrous tissue production as seen here although the timescale is rather more rapid than might normally be expected. The site of needle puncture was confirmed by identification of needle shaped areas of haemorrhage, associated in places with accumulation of haemosiderin within macrophages. These sites of needle puncture did not themselves appear to be associated with significant necrosis, apoptosis or inflammation. Foci of necrosis were identified in the virus-injected animals, showing a typical coagulative necrosis pattern and a focal neutrophil polymorph infiltrate. In two of the animals injected with saline or India ink alone, increased numbers of apoptotic cells were identified, the exact significance of which is uncertain. These apoptotic cells were not associated with inflammatory cell infiltrates or necrosis. Similar foci of apoptosis were noted in two of the virus-treated animals. The finding of apoptotic cells in both groups and in similar numbers may indicate that this is a normal 'house-keeping' phenomenon whereby the pituitary responds to the stress of needle puncture by eliminating damaged cells in a controlled way. Thus apoptosis alone appears not to be a specific feature of virus-mediated damage.

The sheep pituitary is 10–12 mm in antero-posterior diameter, and we reported that a local injection of

recombinant adenovirus can achieve effective transgene expression across substantial regions of the gland (Davis *et al.* 2001). However, the size of the sheep pituitary gland has also allowed us to assess the extent and pattern of inflammatory response in some detail. The extent of the inflammatory reaction that we have described in this report had not previously been recognised by studies of the efficacy of gene transfer in rodents, nor in our system by haematological profiles, immunocytochemical studies of hormone and transgene expression, or hormone secretion studies (Davis *et al.* 2001). Hypophysitis and pituitary infarction ('apoplexy') can present in various ways. Most important, swelling of the gland in the confined space of the pituitary fossa can threaten vision, requiring urgent pituitary surgery to relieve pressure effects on the optic chiasm (Cheung *et al.* 2001). Longer term studies will be needed to assess the time-course of the inflammatory response that we have observed, and it remains to be determined whether there may be important species differences, suggested by strain-specific differences in the response in the mouse brain (Ohmoto *et al.* 1999). The extent of this response is likely to depend on the tissue and on the dose of viral vector used (Gerdes *et al.* 2000), and intratumoral delivery may have different effects to those of injection into the normal pituitary gland. However, our findings emphasize the need for cautious and thorough pre-clinical evaluation before considering the use, for pituitary disease in man, of adenoviruses as vectors (Davis & McNeilly 2001).

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