Journal of Neuro-Oncology (2006) 78: 1–6
DOI 10.1007/s11060-005-9055-3

Laboratory Investigation

Cell type- and region- dependent coxsackie adenovirus receptor expression in the central nervous system

Annette Persson[1], Xiaolong Fan[2], Bengt Widegren[2,3] and Elisabet Englund[1]
[1]*Department of Clinical Science, Lund, Division V, Pathology, University Hospital, SE-221 85, Lund, Sweden;* [2]*Section of Immunology, Lund University, BMCI13SE-221 84, Lund, Sweden;* [3]*Department of Cell and Organism Biology, Lund University, Sölvegatan 35, SE-223 62, Lund, Sweden*

Key words: adenoviral gene therapy, coxsackie adenovirus receptor, immunohistochemistry, normal brain, reactive/inflammatory brain tissue

Summay

Model systems have shown that adenoviral vector mediated transient gene expression can potentially be applied for the treatment of brain tumours, neurodegenerative diseases and brain injuries. Most studies utilized adenovirus serotype 5 (Ad5) based vectors, which as adhesion molecules require the coxsackie adenovirus receptor (CAR) as a critical determinant for cellular infection. In this report, we have systematically characterized CAR expression in the adult human central nervous system (CNS) by using immunohistochemistry. A total of 85 specimens from various CNS regions were investigated for CAR expression in a cell type-dependent context. The most marked staining positivity was found in the choroid plexus and the pituitary gland. The neocortex had scattered positive neurons, while the white matter was mainly negative.We need to consider the possible adverse effects and the possible damage caused by adenoviral gene therapy if the virus–vector *also* binds to normal brain cells.

Introduction

Gene therapy is a promising and potential tool for treatment of human diseases. Among the different viral vector systems, adenoviral vectors can be advantageous because adenoviral vectors infect both dividing and non-dividing cells with high efficiency, thus high levels of gene expression can be achieved in permissive cells [1]. The adenoviral vector genome normally does not integrate into the host cell genome, thus adenoviral vector mediated gene expression is transient in proliferating cells. This can be beneficial when a certain gene is required during a short period and persistent expression of such genes may be deleterious. In addition, it is feasible to generate large quantities of adenoviral vectors.

Most studies have utilized adenoviral serotype 5 (Ad5) based vectors. The infection of host cells by these Ad5 vectors involves two functionally distinct steps. Adenoviral fiber mediated viral attachment to the coxsackie and adenovirus receptor (CAR) is followed by viral internalization via the interaction between the penton base and α_v integrins [2–4]. The relative levels of CAR expression predominantly determine whether a particular cell type is permissive for Ad5 vector infection [5–8].

CAR is expressed ubiquitously on most normal epithelial tissue [9] and is a component of the tight junction [10,11], however the physiological function of CAR is not fully understood.

Adenoviral vectors in cancer gene therapy have become a new treatment form tested, with gratifying results, on several tumours of different origin. The focus has also turned to the application on gliomas, for which the CAR levels are reported to relate to degree of differentiation [12,13].

Presence of CAR in different cell types of the normal human brain is not fully investigated. CAR expression in non-tumour reactive/inflammatory brain tissue is also not established. Presence of viral receptors in different brain regions should have implications for the potential treatment of tumours as well as of reactive/inflammatory and neurodegenerative conditions.

The present study was initiated with the aim to examine different cell types and various regions within the human brain, for possible expression of CAR in normal and in reactive/inflammatory tissue.

Material and methods

Tissue specimens

Formaldehyde-fixed, paraffin-embedded tissue blocks (surgical and autopsy material) were obtained from the Department of Pathology, Lund University Hospital, Sweden. The material represented morphologically normal brain ($n = 52$, 3 surgical and 49 autopsy specimens) as well as reactive/inflammatory tissue from surgery of epilepsy and pseudo-tumoural brain changes ($n = 33$, 31 surgical and 2 autopsy specimens). To confirm the cell contents and morphology of each specimen, all haematoxylin–eosin stained specimen sections were reviewed.

A total of 85 samples from the following brain regions were sampled for the mapping: the cerebral fronto-parietal cortex, the white matter, the basal ganglia,

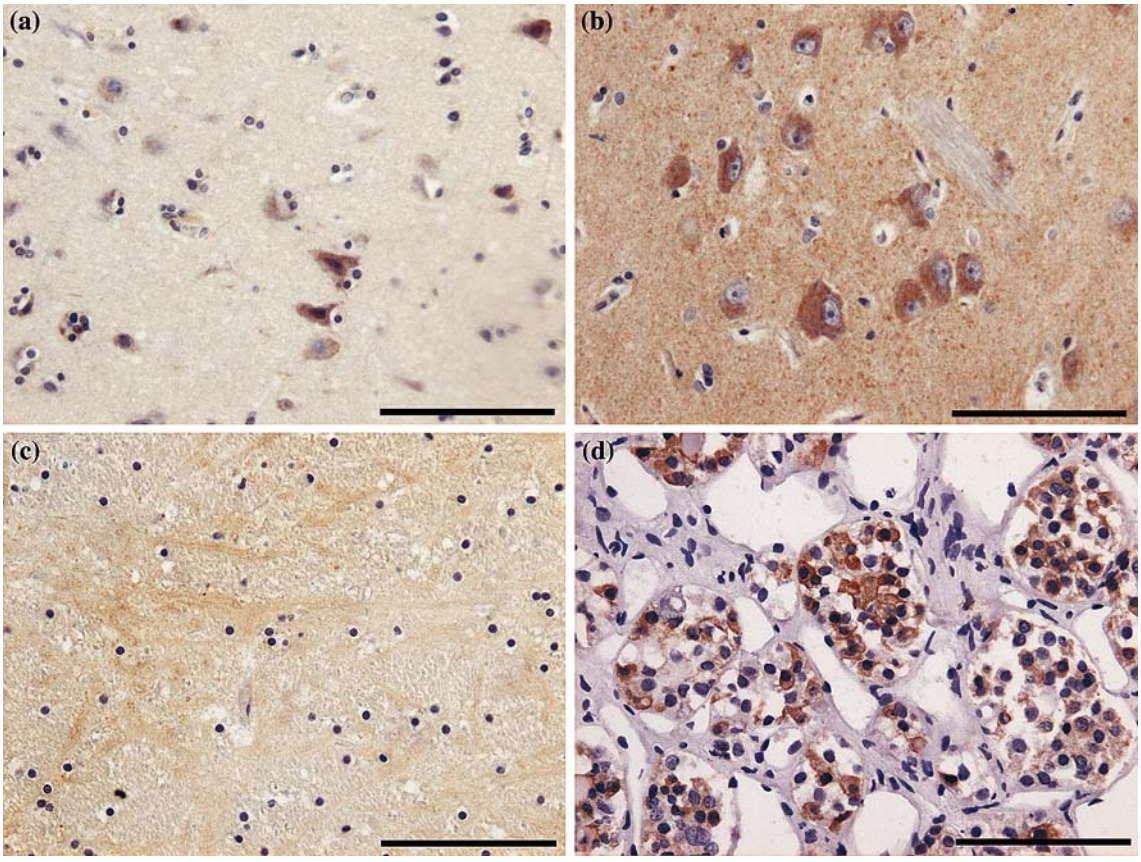

Figure 1. Immunohistochemical staining (positive = brown) for CAR on tissue sections representing; (a) normal cortex; (b) normal brain stem (mid-mesencephalon); (c) normal white matter; (d) normal anterior pituitary gland; (e) normal choroid plexus; (f) cortex with alterations related to epilepsy; (g) normal prostate, short term fixed surgery material, positive control; (h) normal kidney, long term fixed autopsy material, positive control; (i) normal lymph node, short term fixed surgery material, negative control Bars = 0.1 mm.

the limbic system (including the cingulate gyrus, the amygdala and the hippocampus), the cerebellum and brain stem (including mesencephalon, pons and medulla oblongata), the spinal cord, the meninges, the periventricular zone, the choroid plexus, the anterior and posterior pituitary gland and the eye (retina and optic nerve) (Table 1). Of these samples, reactive/inflammatory tissue was represented by cortex and white matter ($n = 30$) and eye/retina ($n = 3$). The samples represented tissue resected due to epilepsy ($n = 14$), tissue with suspected, but not verified tumour ($n = 18$) and one trauma specimen. Data for the specimens are shown in Table 1.

Table 1. Examined CNS tissue regions

Locality	No. samples
Cortex	19
White matter	20
Basal ganglia	11
Limbic system	8
Choroid plexus	4
Brain stem	6
Cerebellum	4
Spinal cord	1
Anterior pituitary gland	4
Posterior pituitary gland	3
The eye/retina	3
Optic nerve	2
Total	85

The study was approved by the local Research Ethical Committee.

Immunohistochemistry

Five μm sections were obtained from the paraffin-embedded blocks, mounted on glass slides (DAKO ChemMate Capillary Gap Microscope Slides, 75 mm, Dako A/S, Glostrup, Denmark) and dried 1 h at 60 °C. All sections were dewaxed, rehydrated and microwave pre-treated in 10 mM citrate buffer (pH 6.0) for 19 min at 750 W to achieve antigen retrieval. An automated immunostainer (TechMate 500 Plus, Dako) was used for the staining procedure with DAKO ChemMate Kit peroxidase/3,3′-diaminobenzidine and a rabbit polyclonal antibody CAR 72 (Onyx Pharmaceuticals, Inc. Richmond, CA, USA) [7] were used as primary antibody, diluted 1:7000. After counterstaining with haematoxylin, the slides were dehydrated in ascending concentrations of alcohol to xylene and mounted.

To ensure specificity of the staining, tissue with known expression/no expression of CAR [normal prostate (Figure 1g) and normal kidney (Figure 1h)/ normal lymph node (Figure 1i)] was used as control. The control tissue was selected to be adequately large and to represent a variety of sample variables with regard to fixation time, autolytic changes and surgery versus autopsy, properties similar to the examination material. The purpose of this was to ensure a specific

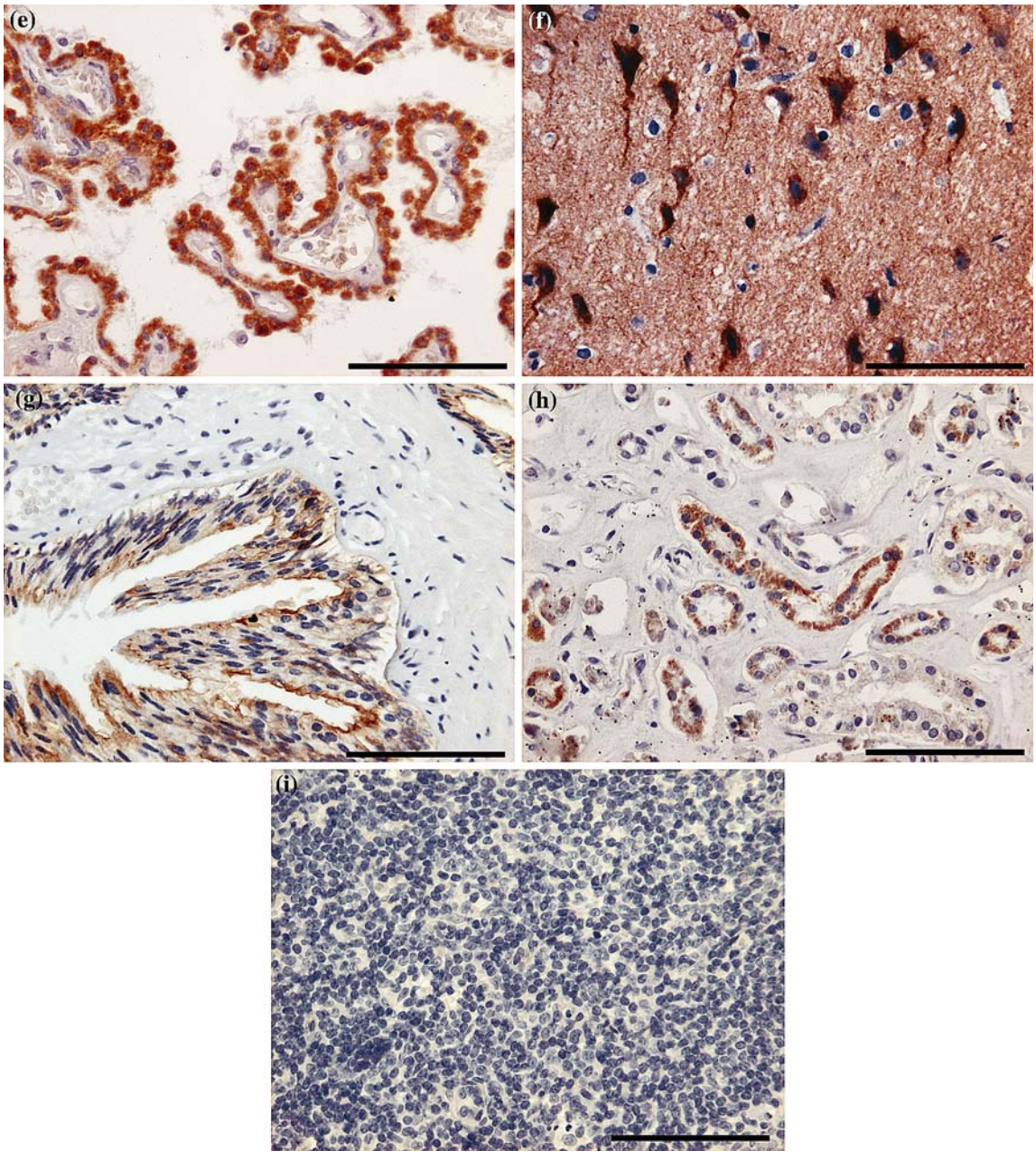

Figure 1. Continued.

and reliable staining and to minimize the risk of including falsely positive or negative staining results.

The examination material was also stained with the primary antibody replaced by buffer, to exclude a conceivable non-specific staining of secondary antibody.

The sections, independently evaluated by two of the investigators (A.P. and E.E.), were analysed with regard to presence/absence (+/−) of positive staining in the cytoplasm and/or cell-membrane. Characterization of staining positivity was made with regard to the amount of positive cells and to regional extent.

Results

A summary of CAR staining positivity/negativity in different normal, non-reactive cell types within the CNS is shown in Table 2. The reactive/inflammatory tissue staining properties are described only in the text below.

Neurons and axons: Some neurons in the cerebral cortex were found CAR-staining positive (CAR+) (Figure 1a), but the vast majority of neurons within the cortical samples were negative. The positive neurons were scattered among negative cells within layer II–VI, without accentuation in any specific layer. Hippocampal neurons were positive in a small portion. Positive neuronal staining was intra-cytoplasmatic and/or membranous: it was noted on the surface of the central cell body and of the dendrites/axons, creating a fine meshwork of microgranular positivity in the neocortex.

Purkinje cells of the cerebellum stained positively in most examined samples, however in only a small subset of cells. Positivities were also seen between the granular

Table 2. CAR staining in normal, non-reactive CNS cell types

Cell type	CAR expression
Cortical neurons	+
Hippocampal neurons	+
Substantia nigra	+
Purkinje cells	+
Axons in white matter	+
Astrocytes	+
Microglia	+
Oligodendrocytes	–
Ependymal cells	–
Choroid plexus	+ +
Anterior pituitary cells	+ +
Posterior pituitary cells	–
Retinal cells	+
Axons in optic nerve	–
Endothelium	–
Meningeal cells	–

+ = Positivity in few or some cells (1–30%), (positive staining in cell-cytoplasm and/or cell-membrane).
+ + = Positivity in most analyzed cells (60–100%), (positive staining in cell-cytoplasm and/or cell-membrane).
– = No positive staining.

cells bodies. The brain stem showed positive neurons/axons in four of six samples (Figure 1b). Strains of CAR + detected in the white matter of some cases, was judged to be axonal positivity (Figure 1c).

The substantia nigra: Mesencephalic samples showed positive staining properties both around and within the pigmented cells. Due to overshadowing of the pigment (neuromelanin), the assessment of CAR + within the pigmented cells was not conclusive.

Glial cells: Positivity was seen in a limited number of glial cells, mainly in scattered cortical and subcortical astrocytes. It also appeared in microglia of the cortex. Staining positivities were intra-cytoplasmatic and/or membranous. Oligodendrocytes showed no positivity for CAR.

Ependymal cells and choroid plexus: The most intense staining positivity of all cell types was noted in the choroid plexus (Figure 1e), which showed a granular and intra-cytoplasmatic staining throughout most cells. The staining positivities were seen in all samples studied. The neighboring ependyma was negative, i.e. showed no CAR + cells.

The eye and optic nerve: There were scattered positivities among the retinal cells, including also the pigmented epithelial cells. The optic nerve was entirely negative.

Pituitary gland: In the anterior pituitary gland distinctly positive cells were seen in all four samples examined (Figure 1d). The posterior pituitary was negative.

Reactive/inflammatory brain tissue: Reactive tissue neurons, astrocytes and microglia, obtained slightly more intense staining positivities than did the normal samples. Furthermore, cortical neurons had a moderately higher ratio of positive/negative cells than in the normal brain, especially in tissue with alterations related to epilepsy (Figure 1f).

Discussion

In this study, various regions and cell types of the normal human brain, not specifically studied earlier, were immunohistochemically CAR + . Also reactive/inflammatory tissue expressed CAR; the number, localization and staining pattern of positive cells however differed.

The choroid plexus and the anterior pituitary gland showed positivities in all examined samples and furthermore, the ratio of positive/negative cells was much higher in these tissue types compared to all other types/regions.

In contrast to the anterior pituitary gland the posterior lobe/neurohypophysis was negative. To summarise the findings among normal CNS cells, the predominating CAR + was found in cylindrical (adenotype) cells.

In reactive/inflammatory brain tissue, the ratio of positive/negative cortical neurons was much higher than in normal brain, especially in epilepsy tissue samples (Figure 1f).

Difficulties to obtain normal human brain tissue generally constitute a limiting factor during selection of examination material. The archival tissue blocks from autopsy, as well as surgical tissue specimens exhibited a difference in antigenicity and variability in the immunohistochemical staining, supposedly corresponding to variations in degree of autolyzation and in fixation time. As described in the Methods section, these variations were taken into account during microscopical evaluation.

Some previous studies present CAR expression in human normal tissue from different organs, including brain. These are few and yet describe partially conflicting results [9,12,14]. With immunohistochemistry on tissue sections, high CAR expression has been demonstrated in the epithelium of normal human prostate [7], normal human bladder [15] and in head and neck squamous cell carcinoma [16], while very low CAR expression has been demonstrated in the normal human heart [17].

In normal fetal mice brain and heart, CAR was strongly expressed until the newborn phase [18]. The expression then decreased postnatally and was absent in normal adult mouse brain tissue. The same expression pattern was seen in rat heart. However, one experimental study showed CAR reexpression in the hearts of adult rats with experimental autoimmune myocarditis, indicating an induction of CAR expression by inflammatory mediators [19]. Furthermore, in human (adult) myocardium, CAR expression was low, whereas strong CAR reexpression was observed in dilated cardiomyopathy [20].

Adenovirus-based gene therapy can be a potential treatment mode for different human disease; neurodegenerative diseases such as Parkinsońs and Alzheimeŕs disease, vascular diseases and retinal diseases [21–23]. Today, focus has mostly been on therapy of tumours of different origin [24]. Of all clinical trials performed with viral vectors in human gene therapy, adenoviral vector represent slightly more than 25% of all vectors. Only a limited number of studies have been performed on other

disease than cancer (Journal of Gene Medicine Website, www.wiley.co.uk/genmed/clinical/).

Virus vector-mediated therapy is a potential tool for glioblastoma multiforme (GBM). The efficiency of adenovirus-based therapy seems to depend on the amount of CAR expression [5]. Studies have shown that many primary malignant cell types, among them glioma cells, express low levels of CAR [7,12,15,16,25,26], a feature that may prevent realization of the full potential of this specific type of therapy.

The choice of therapeutic genes, using adenoviral vectors, most likely has an influence on the effects on normal brain cells. Clinical trials of adenovirus in brain tumours were reviewed by Vecil et al. [27]. The therapy strategies differ between the genes used something which might influence the possible course of events if normal cells "by mistake" become the target cells.

The choroid plexus is responsible for production and circulation of cerebrospinal fluid (CSF). In the choroid plexus samples of our study, a marked staining positivity of CAR was seen in most cells. The possibility that virus-mediated therapy can affect those cells must be considered. Also the pituitary gland showed distinctive positive cells; an influence on the hormone-production must be considered as a possible complication if virus-mediated treatment can affect those cells. CAR+ cells were found in both brain stem and cerebellum. An exposure of virus to cells in these regions may result in neuronal damage and neurological symptoms.

Finally, the CAR reexpression seen in reactive human heart with cardiomyopathy [20] may reflect an expression pattern and mechanism similar to that of the human brain in that reactive brain tissue, such as epileptic centra, express more CAR than the normal brain. This may entail both risks and opportunities in therapeutic attempts.

Acknowledgements

The authors thank Onyx Pharmaceuticals Inc, Richmond, CA, USA for the generous gift of the antibody and Katherine Rauen, Cancer Research Institute, University of California, San Francisco, CA, USA for mediating information. This work was supported by the Märit and Hans Rausing Charitable Foundation, the Swedish Cancer Society and Gunnar Nilsson's Cancer Foundation.

References

1. Benihoud K, Yeh P, Perricaudet M: Adenovirus vectors for gene delivery. Curr Opin Biotechnol 10: 440–447, 1999
2. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW: Isolation of a common receptor for Coxsackie B viruses, adenoviruses 2 and 5. Science 275: 1320–1323, 1997
3. Chretien I, Marcuz A, Courtet M, Katevuo K, Vainio O, Heath JK, White SJ, Du Pasquier L: CTX, a Xenopus thymocyte receptor, defines a molecular family conserved throughout vertebrates. Eur J Immunol 28: 4094–4104, 1998
4. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR: Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73: 309–319, 1993
5. Kim M, Zinn KR, Barnett BG, Sumerel LA, Krasnykh V, Curiel DT, Douglas JT: The therapeutic efficacy of adenoviral vectors for cancer gene therapy is limited by a low level of primary adenovirus receptors on tumour cells. Eur J Cancer 38: 1917–1926, 2002
6. Hemmi S, Geertsen R, Mezzacasa A, Peter I, Dummer R: The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. Hum Gene Ther 9: 2363–2373, 1998
7. Rauen KA, Sudilovsky D, Le JL, Chew KL, Hann B, Weinberg V, Schmitt LD, McCormick F: Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. Cancer Res 62: 3812–3818, 2002
8. Douglas JT, Kim M, Sumerel LA, Carey DE, Curiel DT: Efficient oncolysis by a replicating adenovirus (ad) in vivo is critically dependent on tumor expression of primary ad receptors. Cancer Res 61: 813–817, 2001
9. Tomko RP, Xu R, Philipson L: HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci USA 94: 3352–3356, 1997
10. Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM: The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proc Natl Acad Sci USA 98: 15191–15196, 2001
11. Walters RW, Freimuth P, Moninger TO, Ganske I, Zabner J, Welsh MJ: Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell 110: 789–799, 2002
12. Fuxe J, Liu L, Malin S, Philipson L, Collins VP, Pettersson RF: Expression of the coxsackie and adenovirus receptor in human astrocytic tumors and xenografts. Int J Cancer 103: 723–729, 2003
13. Grill J, Van Beusechem VW, Van Der Valk P, Dirven CM, Leonhart A, Pherai DS, Haisma HJ, Pinedo HM, Curiel DT, Gerritsen WR: Combined targeting of adenoviruses to integrins and epidermal growth factor receptors increases gene transfer into primary glioma cells and spheroids. Clin Cancer Res 7: 641–650, 2001
14. Fechner H, Haack A, Wang H, Wang X, Eizema K, Pauschinger M, Schoemaker R, Veghel R, Houtsmuller A, Schultheiss HP, Lamers J, Poller W: Expression of coxsackie adenovirus receptor and alphav-integrin does not correlate with adenovector targeting in vivo indicating anatomical vector barriers. Gene Ther 6: 1520–1535, 1999
15. Sachs MD, Rauen KA, Ramamurthy M, Dodson JL, De Marzo AM, Putzi MJ, Schoenberg MP, Rodriguez R: Integrin alpha(v) and coxsackie adenovirus receptor expression in clinical bladder cancer. Urology 60: 531–536, 2002
16. Jee YS, Lee SG, Lee JC, Kim MJ, Lee JJ, Kim DY, Park SW, Sung MW, Heo DS: Reduced expression of coxsackievirus and adenovirus receptor (CAR) in tumor tissue compared to normal epithelium in head and neck squamous cell carcinoma patients. Anticancer Res 22: 2629–2634, 2002
17. Noutsias M, Fechner H, de Jonge H, Wang X, Dekkers D, Houtsmuller AB, Pauschinger M, Bergelson J, Warraich R, Yacoub M, Hetzer R, Lamers J, Schultheiss HP, Poller W: Human coxsackie–adenovirus receptor is colocalized with integrins alpha(v)beta(3) and alpha(v)beta(5) on the cardiomyocyte sarcolemma and upregulated in dilated cardiomyopathy: implications for cardiotropic viral infections. Circulation 104: 275–280, 2001
18. Hotta Y, Honda T, Naito M, Kuwano R: Developmental distribution of coxsackie virus and adenovirus receptor localized in the nervous system. Brain Res Dev Brain Res 143: 1–13, 2003
19. Ito M, Kodama M, Masuko M, Yamaura M, Fuse K, Uesugi Y, Hirono S, Okura Y, Kato K, Hotta Y, Honda T, Kuwano R, Aizawa Y: Expression of coxsackievirus and adenovirus receptor in hearts of rats with experimental autoimmune myocarditis. Circ Res 86: 275–280, 2000

20. Fechner H, Noutsias M, Tschoepe C, Hinze K, Wang X, Escher F, Pauschinger M, Dekkers D, Vetter R, Paul M, Lamers J, Schultheiss HP, Poller W: Induction of coxsackievirus–adenovirus-receptor expression during myocardial tissue formation and remodeling: identification of a cell-to-cell contact-dependent regulatory mechanism. Circulation 107: 876–882, 2003
21. Nasuno A, Toba K, Ozawa T, Hanawa H, Osman Y, Hotta Y, Yoshida K, Saigawa T, Kato K, Kuwano R, Watanabe K, Aizawa Y: Expression of coxsackievirus and adenovirus receptor in neointima of the rat carotid artery. Cardiovasc Pathol 13: 79–84, 2004
22. Mallam JN, Hurwitz MY, Mahoney T, Chevez-Barrios P, Hurwitz RL: Efficient gene transfer into retinal cells using adenoviral vectors: dependence on receptor expression. Invest Ophthalmol Vis Sci 45: 1680–1687, 2004
23. Baekelandt V, De Strooper B, Nuttin B, Debyser Z: Gene therapeutic strategies for neurodegenerative diseases. Curr Opin Mol Ther 2: 540–554, 2000
24. Bauerschmitz GJ, Barker SD, Hemminki A: Adenoviral gene therapy for cancer: from vectors to targeted and replication competent agents (review). Int J Oncol 21: 1161–1174, 2002
25. Li Y, Pong RC, Bergelson JM, Hall MC, Sagalowsky AI, Tseng CP, Wang Z, Hsieh JT: Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. Cancer Res 59: 325–330, 1999
26. Cripe TP, Dunphy EJ, Holub AD, Saini A, Vasi NH, Mahller YY, Collins MH, Snyder JD, Krasnykh V, Curiel DT, Wickham TJ, DeGregori J, Bergelson JM, Currier MA: Fiber knob modifications overcome low, heterogeneous expression of the coxsackievirus–adenovirus receptor that limits adenovirus gene transfer and oncolysis for human rhabdomyosarcoma cells. Cancer Res 61: 2953–2960, 2001
27. Vecil GG, Lang FF: Clinical trials of adenoviruses in brain tumors: a review of Ad-p53 and oncolytic adenoviruses. J Neurooncol 65: 237–246, 2003

Address for offprints: Annette Persson, Department of Clinical Science, Lund, Division V, Pathology University Hospital, SE-221 85, Lund, Sweden; Fax: +46-46-143307; E-mail: Annette.Persson@med.lu.se