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Published in:
Journal of Neuro-Oncology

DOI:
[10.1007/s11060-005-9055-3](https://doi.org/10.1007/s11060-005-9055-3)

2006

[Link to publication](#)

Citation for published version (APA):
Persson, A., Fan, X., Widegren, B., & Englund, E. (2006). Cell type- and region- dependent coxsackie adenovirus receptor expression in the central nervous system. *Journal of Neuro-Oncology*, 78(1), 1-6.
<https://doi.org/10.1007/s11060-005-9055-3>

Total number of authors:
4

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This is an author produced version of a paper published in Journal of Neuro-Oncology. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Persson, Annette and Fan, Xiaolong and Widegren, Bengt and Englund, Elisabet
"Cell type- and region- dependent coxsackie adenovirus receptor
expression in the central nervous system."
J Neurooncol. 2005 Nov 29;:1-6 [Epub ahead of print]
<http://dx.doi.org/10.1007/s11060-005-9055-3>

Access to the published version may require journal subscription.
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Cell type- and region- dependent coxsackie adenovirus receptor expression in the central nervous system.

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Keywords: adenoviral gene therapy; coxsackie adenovirus receptor; immunohistochemistry; normal brain; reactive/inflammatory brain tissue

Abstract

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, 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.

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 one hour at 60° C. All sections were dewaxed, rehydrated and microwave pre-treated in 10 mM citrate buffer (pH 6.0) for 19 minutes at 750 W to achieve antigen retrieval. An automated immunostainer (TechMateTM 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 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. Characterisation 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 neurones 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 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 previously specific studied, 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 autolysis 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 Parkinson's and Alzheimer's 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 Fransisco, 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.

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Legends to 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

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

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

















