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Neuroblastomas and medulloblastomas exhibit more coxsackie adenovirus receptor expression than gliomas and other brain tumours.

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Running title: CAR expression in neuronal tumours.

Abstract

Adenoviral vector-mediated treatment is a potential therapy for tumours of the central nervous system. To obtain a significant therapeutic effect by adenoviral vectors, a sufficient infection is required, the power of which predominantly depends on the level of coxsackie adenovirus receptors. We stained surgical biopsies of CNS tumours and neuroblastomas for coxsackie adenovirus receptors. For gliomas, the level of the receptor was low and markedly variable among individual tumours. By contrast, neuroblastoma and medulloblastoma exhibited a higher degree of coxsackie adenovirus receptor expression than gliomas and other brain tumours. We conclude that neuroblastoma end medulloblastoma could be suitable for adenovirus-mediated gene theraphy. Adverse effects of the treatment, however, must be considered because neurons and reactive astrocytes also express a significant amount of the receptor.

Key words: CNS tumours; neuroblastoma; coxsackie adenovirus receptor; gene therapy; immunohistochemistry

Introduction

For malignant gliomas, there is a significant need to improve treatment. The traditional therapies, adjusted and improved only to a limited extent over the last four decades, have failed to successfully defeat the tumour[1]. Gene therapy treatment with the use of viral vectors has been successfully tested on several tumours of different origin and the focus has also turned to the application on gliomas. Important factors which may complicate the effectiveness of such treatment include the innate immune system of the brain and the blood-brain barrier.

Adenoviral vectors are of great interest because of their ability to infect both dividing and non-dividing cells with high efficiency, and they normally do not integrate into the host cell genome[2]. It is also possible to generate large quantities of adenoviral vectors. Most studies have utilized adenoviral serotype 5 (Ad5) based vectors. Host cells are infected by these Ad5 vectors in two functionally distinct steps, viral attachment to the coxsackie adenovirus receptor (CAR) and internalization via the penton base and α_v integrins interaction[3-5]. The relative levels of CAR expression predominantly determine whether a particular cell type is permissive for Ad5 vector infection[6-9].

CAR is expressed ubiquitously on most normal epithelial tissue [10] and is a component of the tight junction [11,12]. The physiological function of CAR, however, is not fully understood.

Since the levels of CAR expression influence the degree of adenoviral infection and consequently determine the effectiveness of associated gene therapy treatment, it is essential to investigate CAR levels in the target cells. The CAR levels for gliomas are reported to relate to degree of differentiation (inversely correlating with malignancy) [13,14] and meningeoma cells are reported to express these receptors to some extent[15]. CAR expression in other CNS tumours has not been fully investigated.

The aim of this study was to map CAR expression in different CNS tumours and in neuroblastoma, in view of the potential use of adenoviral vector-mediated therapy in these tumours.

Material and methods

Tissue specimens

Formaldehyde-fixed, paraffin-embedded tissue blocks (of surgical excision biopsies) were obtained from the Department of Pathology, Lund University Hospital, Sweden. The material represented astrocytoma grade 1, 2, and 3, glioblastoma multiforme, medulloblastoma, neuroblastoma, papilloma of the choroid plexus and adenoma of the pituitary gland. Tissue from 17 glioblastomas and from 10 tumours of each of the other tumour groups was examined, in total 87 specimens. To confirm the tumour diagnosis of each specimen, all haematoxylin-erythrosin stained specimen sections were reviewed. The study was approved by the Regional Ethical Review Board in Lund.

Immunohistochemistry

Five µm thick sections were obtained from each paraffin-embedded block, mounted on glass slides (DAKO ChemMate Capillary Gap Microscope Slides, 75 mm, Dako A/S, Glostrup, Denmark) and dried for one hour at 60 ºC. All sections were dewaxed, rehydrated and microwave pre-treated in 10 mM citrate buffer (pH 6.0) for 19 minutes at 750 W in order to achieve antigen retrieval. An automated immunostainer (TechMate^{TM} 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) were used as primary antibody, in a 1:7000 dilution. After counterstaining with haematoxylin, the slides were dehydrated in ascending concentrations of alcohol to xylene and mounted.

To ensure specificity of the staining, samples from tissue with known expression/lack of expression of CAR (normal prostate and normal lymph node) was used as control[16].

The examination material was also stained with the primary antibody replaced by buffer, to exclude non-specific staining of the 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 tumour cell cytoplasm and/or membrane. Positive staining intensity and extent was also semi-quantitatively evaluated and adjacent non-tumour cells were assessed as well.

Results

Gliomas

For all grades of glioma, CAR expression was generally low (Figure 1a-d). Observed CAR positivity (CAR+) was judged to be both cytoplasmatic and membranous. In general, the vast majority of the tumour cells were negative, but occasional scattered CAR+ tumour cells were seen within in the tumours. Occasional high-grade gliomas showed a larger total CAR expression than did low-grade gliomas, but in relation to tumour cell density the picture was similar, i.e. the ratios of CAR+/- cells were similar. Many of the examined glioma specimens (both low-grade and high-grade gliomas) showed no CAR positivity.

A number of low-grade gliomas showed CAR+ in cortical neurons adjacent to the negative tumour (Figure 1e) and in cases of cerebellar tumours, some of the adjacent Purkinje cells were positive (Figure 1f). Scattered reactive non-tumour cells (mainly astrocytes) also showed CAR+. Some cases showed strains of CAR+ in the white matter adjacent to glioma; these positivities were judged to be axonal.

Medulloblastoma and neuroblastoma

CAR expression was seen in all medulloblastomas and neuroblastomas (Figure 1g-h). The amount of CAR+ cells varied, but was generally high and judged to be confined to tumour cells as opposed to in gliomas. The staining pattern was more markedly membranous than in the glioma cells, but still with some cytoplasmatic contribution. Five of the examined medulloblastomas exhibited sharply marked islands of CAR+ cells surrounded by negative tumour cells, the other five being more homogenously positive. Neuroblastoma showed a generally homogenous CAR positivity in all 10

samples. Staining intensity was higher in 8 of 10 medulloblastomas compared to neuroblastomas and equal with the latter in the remaining two.

Papilloma of the choroid plexus and adenoma of the pituitary gland

Both tumour types were predominantly negative among convincing tumour cells (Figure 1i-j). In zones of borderline-like appearance (cells between clear-cut tumour and original-benign epithelium), there was a diffuse and attenuated CAR positivity. Positive staining was both cytoplasmatic and membranous. CAR+ was even stronger and more extensive in neighbouring epithelial (non-tumour) cells.

Discussion

In contrast to other investigations in this field, this study concerns CAR expression solely in primary brain tumour biopsies and neither cell cultures nor xenograft tissue was produced. The highest expression of CAR was thus found in medulloblastomas and neuroblastomas and also in tumour adjacent non-tumour cells including neurons and reactive astrocytes.

CAR is a transmembranous receptor with one extracellular part and a small tail reaching into the cell cytoplasm [3,10]; therefore one may anticipate to find most of the staining positivity within the cell-membrane. However, variations in degree of autolyzation and perhaps also fixation time might have influenced the staining pattern, i.e. the appearance of intracytoplasmatic staining.

In an earlier study, we investigated CAR expression in different non-neoplastic cell types and regions of the CNS[16]. The main findings in that report was that the normal choroid plexus and the pituitary gland showed the most marked staining positivity within all investigated areas of the brain and that there were CAR+ neurons in the neocortex. We also found that in reactive /inflammatory brain tissue, the ratio of positive /negative cortical neurons was higher than in normal brain and especially high in epilepsy tissue samples. This might depend on a CAR reexpression similar to that seen in reactive human heart with cardiomyopathy[17]. In the tumour tissue material from gliomas cases investigated in this study, some specimens also included reactive/inflammatory areas, such as epileptic centres. These areas presented a CAR expression clearly higher than did the tumour itself. The findings of the present study indicate that CAR expression within CNS cells may appear/reappear in reactive brain tissue and thus confirm the findings in our earlier study[16].

The invasive growth seen in gliomas with scattered tumour cells in the normal brain, often far from the tumour origin, is difficult to reach with established treatments. One hypothetical advantage with gene therapy would be the possibility to act at long distance from the solid tumour. Considering the abundance of CAR+ normal and reactive CNS cells found, therapeutic attempts may leave entail an undesired binding of vectors to normal and/or reactive cells as well as to the target cells. The risk of inserting high doses of a viral vector into the brain has been discussed earlier[18]. Furthermore, if the treatment needs to be recurrent, non-target cells also run the risk to be exposed repeated times.

Several different therapeutic genes can be inserted in the adenoviral vector [19,20]. By choosing a therapeutic gene that can act for long-time after introduction, repeated administration of the viral vector may be avoided. This might also be a way to partly target the treatment. Also, the transgene used should ideally be made specific to properties in target cells.

Since adenovirus vectors seem to be suitable for gene delivery in human tumour therapy, different options are tested to get a sufficient infection in target cells that do not express CAR. Identifying alternative cellular receptors on target cells might be a good strategy [15,21,22], but if the vector remains unaltered it could probably still bind to CAR-expressing non-target cells. A modified, retargeted vector would therefore be essential for further improvement of therapy [23].

Adenovirus mediated gene therapy has attained increased actuality in glioblastomas, since established treatment such as surgery, radiation and chemotherapy have prolonged survival only to a marginal extent. The efficiency of gene therapy with adenoviral vectors seems to depend on the amount of coxsackie adenovirus receptors [6,24], which are expressed in very low levels in gliomas. Our findings of higher CAR

levels in medulloblastomas and neuroblastomas compared to in gliomas imply that the former tumour forms may be more suitable for adenovirus mediated gene therapy. We need to be aware, however, of possible adverse effects and potential damage caused by an undesired binding to non-target cells.

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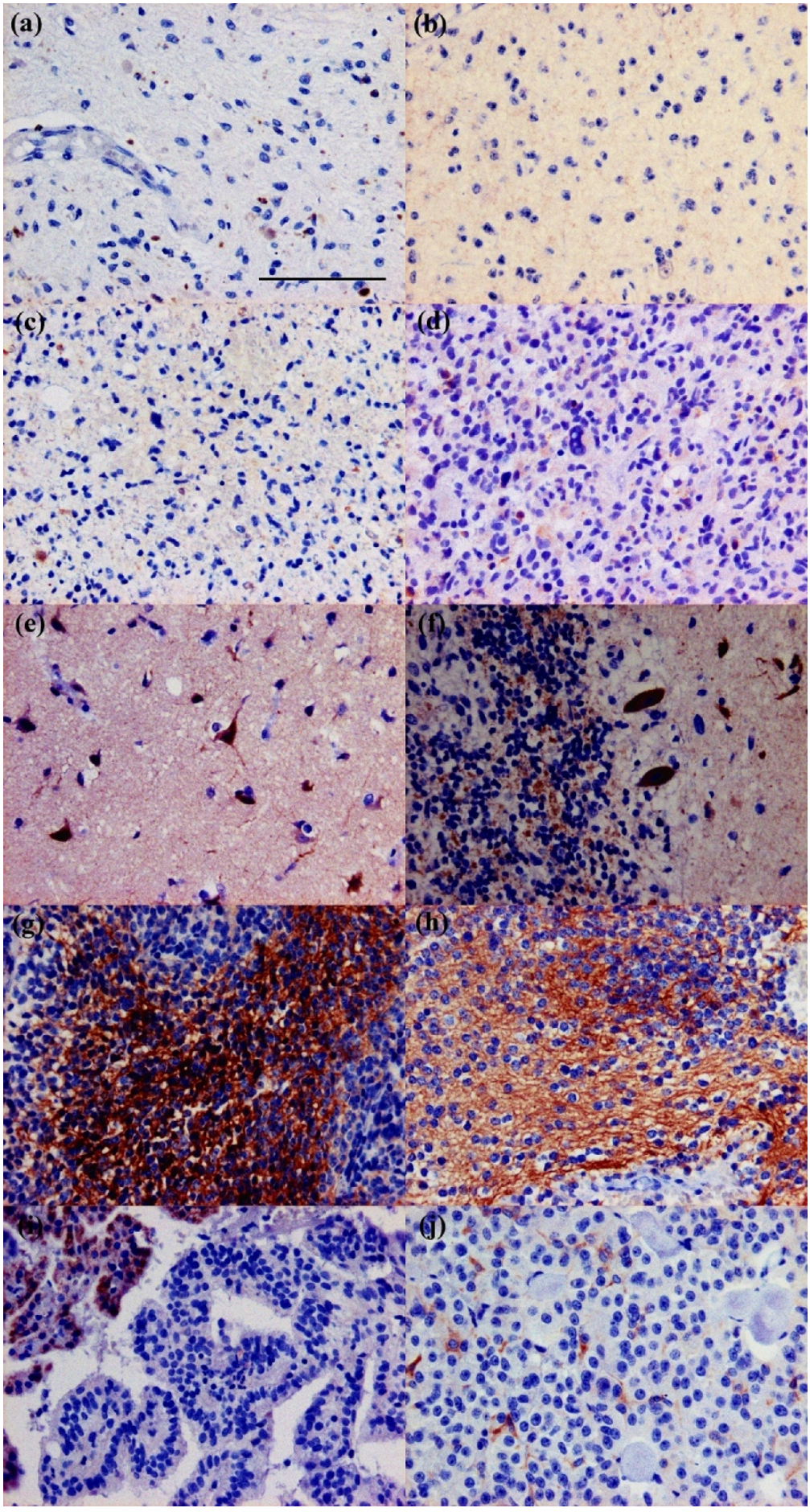
(a)
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Figure1

Immunohistochemical staining for coxsackie adenovirus receptor expression in brain tumours, neuroblastoma and reactive non-tumour tissue. Positive = brown. Bar = 0,1 mm.

a) Astrocytoma grade I
b) Astrocytoma grade II
c) Astrocytoma grade III
d) Glioblastoma multiforme
e) Reactive cortical neurons adjacent to astrocytoma
f) Purkinje cells adjacent to medulloblastoma
g) Medulloblastoma
h) Neuroblastoma
i) Papilloma of the choroid plexus
j) Adenoma of the pituitary gland