Coxsackievirus and adenovirus receptor expression on primary osteosarcoma specimens and implications for gene therapy with recombinant adenoviruses.
Letter to the editor

We have read with great interest the paper by Gu and coworkers reporting the coxsackievirus and adenovirus receptor (CAR) status on primary musculoskeletal tumors (1). We are particularly interested in their findings on osteosarcomas (OS), since we are developing gene therapy strategies for this cancer. CAR is the high-affinity viral receptor for the majority of human adenovirus serotypes including serotype 5, which is the most commonly used serotype for gene therapy applications. CAR expression, which is often low on primary human tumors (2-5), is an important determining factor for adenoviral gene transfer efficiency. Gu and coworkers report highly variable CAR mRNA expression in a panel of 20 primary OS tumor samples, as measured by real-time quantitative RT-PCR (Q-PCR). Of these specimens, 5 were found to lack detectable CAR expression, while 11 expressed high levels of CAR mRNA. In an earlier report, the same research group found 2 OS tumor samples expressing high levels of CAR mRNA among 5 tested (6). Based on the average CAR mRNA level in primary OS, Gu and coworkers concluded that adenoviral vectors are potentially useful for the treatment of OS (1). We share the opinion that gene therapy with recombinant adenoviruses could be considered as a treatment modality for OS. However, based on our results discussed below we consider it preferred to retarget adenovirus entry via receptors other than CAR.

Previously, we studied CAR expression in 7 OS tumor specimens by immunohistochemistry (IHC) and found that 4 samples were CAR negative and the remaining 3 expressed CAR in less than 10% of the cells. FACS analysis of OS short-term cultures confirmed low CAR expression (3). In addition, primary OS samples were resistant to cell kill by a conditionally replicative adenovirus with native tropism. Retargeting adenovirus infection by incorporation of an Arg-Gly-Asp (RGD-4C) integrin binding-motif into the adenovirus fiber knob markedly enhanced primary OS cell kill (7). Our observations thus seem to contrast with those of Gu and coworkers. Because our experiments were performed on a smaller panel of OS specimens and CAR expression was determined using IHC or FACS analysis instead of Q-PCR, we decided to extent our investigation to a larger panel of OS short-term cultures in which we measured CAR expression by both FACS and Q-PCR.

Eleven primary osteosarcoma tumor samples were obtained from six patients through open biopsy before chemotherapy treatment was started (OS-2, 6, 8, 13,
CAR expression on osteosarcoma

15 and 16), from one patient after chemotherapy (OS-1A), and from two patients before (OS-11 and 12) and after chemotherapy (OS-11A and 12A) and short-term cultures were established as previously reported (3). OS tumor cell morphology was confirmed on all short-term cultures by histopathology. For comparison, three OS cell lines, i.e., SaOs-2, U2OS and MG-63, of which the former two are known to express high levels of CAR and the latter low or absent levels of CAR, were included (3, 6). FACS analysis was performed as reported by us previously (3) and Q-PCR was done as described by Gu and coworkers (1). FACS analysis revealed high level CAR expression in SaOs-2 and U2OS cells, at 9 and 11 times the fluorescence intensity of second antibody-stained control cells, respectively. In contrast, MG-63 and all short-term cultured OS samples expressed low levels of CAR, not exceeding 1.5 times the fluorescence expression level of controls (figure 2.1A). Q-PCR analysis, normalized by expression of housekeeping gene glucose-6-phosphate dehydrogenase (G6PD) and given relative to the value for HeLa cells, revealed high expression of CAR mRNA in SaOs-2 and U2OS exceeding CAR expression in HeLa cells by 5 and 3 times, respectively. In contrast, in MG-63 and in all primary OS cells CAR mRNA levels were low, with a median expression of 0.02 times HeLa CAR mRNA expression (range 0.002-0.4) in short-term cultured OS specimens. Hence, flow cytometry and Q-PCR analysis correlated very well, showing that all tested primary OS specimens were low in CAR.

We also evaluated susceptibility of the panel of primary OS cells to adenovirus infection. For this purpose, we used a recombinant E1-deleted adenovirus expressing luciferase under the control of the cytomegalovirus promoter (AdCMVluc) and a similar adenovirus that carries a cyclic RGD epitope in the HI-loop of the fiber (AdCMVlucRGD) to allow cell entry through binding to integrins (8), which are highly expressed on OS cells (7). OS cell lines and primary cells were subjected to a 1-hour incubation with AdCMVluc or AdCMVlucRGD and luciferase expression was measured 40 hours later. As depicted in figure 2.1B, luciferase expression following transduction with AdCMVluc was negligible in the majority of primary OS specimens. In contrast, transduction with AdCMVlucRGD led to increased luciferase expression in all CAR negative primary OS specimens, at levels comparable to or even exceeding those in CAR positive cell lines SaOs-2 and U2OS (figure 2.1C). Hence, integrin-targeting overcame resistance to adenovirus infection through lack of CAR expression.
Fig. 2.1 CAR expression and adenovirus vector transduction of OS cell lines and primary specimens. (A) OS cell lines SaOs-2, U2OS and MG-63, eleven short-term cultures derived from OS biopsies and HeLa cells were analyzed for CAR expression by flow cytometry (left axis and bars) and CAR mRNA expression by Q-PCR (right axis and black line). CAR protein expression on the cell surface is given as the median fluorescence intensity of RmC6 anti-CAR antibody-stained cells divided by the median fluorescence of second antibody-stained control cells. Flow cytometry data are mean of three independent experiments with standard deviation. CAR mRNA expression was normalized for G6PD expression and is given relative to the value obtained for HeLa cells which is set at 1. (B, C) Transduction efficiency of AdCMVluc (B) and AdCMVlucRGD (C) on OS cells. Cells were subjected to 100 plaque-forming units adenovirus vector per cell for 1 hour and luciferase expression in relative light units (RLU) was measured forty hours later. Data shown in panels B and C are from the same representative experiment performed in triplicate and are given as mean RLU per cell + SD.
A possible explanation for the discrepancy between our findings and those of Gu and coworkers could be that we analyzed short-term cell cultures and they used tissue pieces. We could establish short-term cultures from all biopsies, suggesting that we did not select for a certain subset of OS specimens. Obviously, tumor tissues are more heterogeneous than short-term cultures, because non-malignant cells are mostly lost upon culture initiation. As CAR expression levels have been found to inversely correlate with cancer grade and disease stage (9-11), preferential outgrowth of more malignant cells from heterogeneous tumors could perhaps yield lower CAR expression values in short-term cultures than in tumor tissues. However, our previous observation that CAR is also low or absent in OS tumor tissue analyzed by IHC (3) argues against this explanation. In any event, our observations show that OS tumors contain OS cells with low CAR expression. Interestingly, Gu and coworkers excluded tumors with a high degree of necrosis from their analysis, because preliminary examination had shown that necrosis decreased CAR expression (1). Necrosis creates a hypoxic microenvironment, which may select for tumor cell subpopulations with increased metastatic potential (12).

In addition, hypoxia induces cancer cells to alter the expression of many genes, including genes involved in cell adhesion (13). Since it has been suggested that CAR mediates homotypic cell adhesion as part of adherens junctions (14), it is tempting to speculate that hypoxia might also affect CAR expression. This would be in line with the proposed modulation of CAR expression during cancer progression (9). Hence, selecting tumors with a low degree of necrosis could perhaps introduce a bias towards higher CAR expressing tumors. Additional studies into a possible relation between tumor hypoxia and CAR expression are therefore warranted.

Taken together, the data from Gu and coworkers and our laboratory demonstrate that a substantial proportion of OS tumors contain malignant cells expressing low levels of CAR. This creates a hurdle for efficient gene transfer with adenoviral vectors that can be alleviated by retargeting cell entry via an alternative receptor. Therefore, in our opinion effective treatment of osteosarcoma with recombinant adenoviruses will require the use of retargeted vectors in many cases.
Acknowledgments

We like to thank Adhiambo Witlox for retrieving and processing the primary osteosarcoma tumor specimens and short-term cultures. Victor W. van Beusechem is supported by a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW) and Jan E. Carette by the VU University Stimulation Fund (USF).

References