Letters to the Editor

Coxsackievirus and Adenovirus Receptor Expression on Primary Osteosarcoma Specimens and Implications for Gene Therapy with Recombinant Adenoviruses

To the Editor: We have read with great interest the article 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 because we are developing gene therapy strategies for this cancer. CAR is the high-affinity viral receptor for most 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 osteosarcoma tumor samples, as measured by real-time quantitative reverse transcription–PCR (Q-PCR). Of these specimens, 5 were found to lack detectable CAR expression, whereas 11 expressed high levels of CAR mRNA. In an earlier report, the same research group found 2 osteosarcoma tumor samples expressing high levels of CAR mRNA among 5 tested (6). Based on the average CAR mRNA level in primary osteosarcoma, Gu and coworkers concluded that adenoviral vectors are potentially useful for the treatment of osteosarcoma (1). We share the opinion that gene therapy with recombinant adenoviruses could be considered as a treatment modality for osteosarcoma. 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 seven osteosarcoma tumor specimens by immunohistochemistry and found that four samples were CAR negative and the remaining three expressed CAR in <10% of the cells. Fluorescence-activated cell-sorting (FACS) analysis of osteosarcoma short-term cultures confirmed low CAR expression (3). In addition, primary osteosarcoma 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 osteosarcoma cell kill (7). Our observations thus seem to contrast with those of Gu and coworkers. Because our experiments were done on a smaller panel of osteosarcoma specimens and CAR expression was determined using immunohistochemistry or FACS analysis instead of Q-PCR, we decided to extend our investigation to a larger panel of osteosarcoma 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, 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). Osteosarcoma tumor cell morphology was confirmed on all short-term cultures by histopathology. For comparison, three osteosarcoma cell lines (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 done 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 osteosarcoma samples expressed low levels of CAR, not exceeding 1.5 times the fluorescence expression level of controls (Fig. 1A). Q-PCR analysis, normalized by expression of housekeeping gene glucose-6-phosphate dehydrogenase 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 five and three times, respectively. In contrast, in MG-63 and in all primary osteosarcoma 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 osteosarcoma specimens. Hence, flow cytometry and Q-PCR analysis correlated very well, showing that all tested primary osteosarcoma specimens were low in CAR.

We also evaluated susceptibility of the panel of primary osteosarcoma 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 osteosarcoma cells (7). Osteosarcoma 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 Fig. 1B, luciferase expression after transduction with AdCMVluc was negligible in the majority of primary osteosarcoma specimens. In contrast, transduction with AdCMVlucRGD led to increased luciferase expression in all CAR-negative primary osteosarcoma specimens, at levels comparable to or even exceeding those in CAR-positive cell lines SaOs-2 and U2OS (Fig. 1C). Hence, integrin-targeting overcome resistance to adenovirus infection through lack of CAR expression.

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 osteosarcoma specimens. Obviously, tumor tissues are more heterogeneous than short-term cultures because nonmalignant 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
osteosarcoma tumor tissue analyzed by immunohistochemistry
(3) argues against this explanation. In any event, our observations
show that osteosarcoma tumors contain osteosarcoma 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). Because 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 toward 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 show that a substantial proportion of osteosarcoma
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.

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Fig. 1  CAR expression and adenovirus vector transduction of osteosarcoma cell lines and primary specimens. A, osteosarcoma cell lines SaOs-2, U2OS, and MG-63, 11 short-term cultures derived from osteosarcoma 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 Rmcb 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 SD. CAR mRNA expression was normalized for glucose-6-phosphate
dehydrogenase (G6PD) expression and is given relative to the value obtained for HeLa cells which is set at 1. B and C, transduction efficiency of
AdCMVluc (B) and AdCMVlucRGD (C) on osteosarcoma 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 40 hours later. Data shown in B and C are from the same representative
experiment done in triplicate and are given as mean RLU per cell + SD.
REFERENCES


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