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 re-target 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 killing 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, 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 overcame 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.
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In Response: We appreciate the valuable comments from Dr. Graat et al. regarding our recent reports (1).

In several human tumors, coxsackievirus and adenovirus receptor is down-regulated during progression to malignancy. Reduced expression of adenovirus receptor in tumor tissue compared to normal epithelium has been reported in some epithelial tumors (2, 3). On the other hand, adenovirus receptor expression is very low or absent in normal condition in bone tissue (1, 4, 5). Only undifferentiated osteoblasts in fractured bone express adenovirus receptor (4). We have demonstrated that undifferentiated osteoblasts and a subset of human osteosarcoma cell lines or osteosarcoma tissues expressed high levels of mRNA and protein of adenovirus receptor (1, 4, 5).

We consider that their conclusion of low adenovirus receptor expression in osteosarcomas is still controversial because their analysis of adenovirus receptor in tumor specimens was carried out by immunohistochemistry, not by quantitative PCR (6). Their analyses by quantitative PCR were applied for primary short-term culture cells, which may be modulated by the culture conditions and may contain many normal mesenchymal cells.

We agree with Dr. Graat et al. that a substantial proportion of osteosarcomas expresses low or absent level of adenovirus receptor. Indeed, 25% cases of osteosarcomas in our study expressed no detectable level of adenovirus receptor by reverse transcription–PCR methods. However, our studies and their letter have demonstrated that subsets of osteosarcoma cell lines, such as SaOs-2 and U2OS, expressed high-level expression of adenovirus receptor compared to HeLa cells (5).

We also agree that a possible explanation of different results may be contributed by tumor sampling. Our tissues of osteosarcoma were obtained mainly by surgical resection. We sampled vivid tumor tissue and excluded necrotic ones. Unfortunately, at present, we have no data regarding correlation between hypoxia and adenovirus receptor expression.

Effective treatment of osteosarcoma with recombinant adenoviruses will require the use of retarget vectors in many tumors without expression of adenovirus receptor (6, 7). Because there are still too many patients with osteosarcoma who cannot benefit from modern treatment, further study should be needed to make new therapeutic approaches.

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