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Isolated Limb Perfusion Based Anti-p21*ras* Gene Therapy in a Rat Rhabdomyosarcoma

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Abstract. *Background: Inhibition of ras oncogene is a promising new strategy. Gene therapy against ras proved successful in human and murine tumour cell lines. Previously we demonstrated effective targeted transfection of tumour in a rat model by using an isolated limb perfusion (ILP) for the delivery of adenoviral vectors. Materials and Methods: This study explores the anti-tumour activity of an adenoviral construct encoding an intracellular single-chain antibody (scFv) against p21ras (Y28). In order to determine the influence of the ras status on the efficacy of the scFv, we used a wild-type rat rhabdomyosarcoma and its ras–oncogene transfectant, for in vitro studies. In vivo we used the ILP delivery method to study anti-tumour activity on established limb tumours. Results: In vitro studies demonstrated an inhibition of growth caused by the Y28 construct. No significant difference between transfected and wild-type cell lines could be demonstrated. Upon ILP, homogeneous transduction was observed in 5% of tumour cells. Perfusion with the Y28 construct, however, did not result in any additional anti-tumour activity compared to controls. Conclusion: Despite in vitro activity and in vivo transfection, no significant tumour response could be detected using anti-p21ras gene therapy in this ILP-tumour model.*

The wild-type ras gene leads to the production of p21–ras, a protein that catalyses the hydrolysis of guanosine diphosphate to guanosine triphosphate. Through this pathway cell proliferation is controlled by interfering with signal transduction (1). Inhibition of ras oncogene expression is a promising new strategy in anti-tumour therapy. Gene therapy against ras using ribozymes, anti-sense RNA or single chain antibodies (scFv) proved successful in causing growth inhibition and apoptosis in human and murine tumour cell lines (2-13).

However, data obtained in *in vitro* gene therapy experiments often do not correlate with *in vivo* results. *In vivo* transfection of established tumours remains a major problem and, as a consequence, limits the possibilities of clinical cancer gene therapy. Isolated limb perfusion (ILP) proved to be a successful method for the administration of chemotherapeutics and cytokines in patients with locally advanced soft tissue sarcomas and in-transit melanoma metastases (14-17). We were able to replicate this method of administration in our rat ILP model (18-21). High concentrations of anti-tumour agents can be reached at the tumour site with negligible systemic exposure. Recently others and ourselves demonstrated effective targeted transfection of tumour in a rat model by using an ILP for the delivery of adenoviral vector (22, 23).

We now report on a study that explores the anti-tumour activity of an adenoviral construct encoding an intracellular scFv against p21-ras. Delivery of viral vectors *via* intratumoral injections is an established method to reach transfection but its clinical applicability is limited. A transvascular approach could increase the therapeutic window for gene therapy in clinical trials. Considering this advantage, we used the ILP delivery method to study anti-tumour activity of an anti-ras construct on established tumours in the hind limb of a rat.

In order to study the influence of the ras status on the efficacy of the scFv, we used a wild-type rat rhabdomyosarcoma and its ras–oncogene transfectant, expressing six copies of the ras gene, during *in vitro* studies. In experiments performed *in vivo* we only used the ras oncogene transfected rhabdomyosarcoma.

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Key Words: Isolated perfusion, ras-oncogene, rhabdomyosarcoma, gene therapy, adenovirus.

Table I. *In vitro transfection rate by means of X-gal staining on R2, R2T24 and R2-neo cells after 48-hour incubation with increasing concentrations of AV1.0CMV.LacZ. Wild-type R2 is more sensitive to transfection in vitro by the LacZ construct then the transfected R2T24 and R2-neo cell line at densities from 2.0 x 10^5 to 2.0 x 10^7 vp/ml ($p<0.05$). A clear dose-transfection relationship is present in all cell lines ($p<0.05$).*

Dose (vp/ml)	R2 % pos (±SD)	R2T24 % pos (±SD)	R2-neo % pos (±SD)
$2x10^8$	100 (28)	100 (19)	86 (36)
$1x10^8$	100 (33)	100 (21)	75 (26)
$2x10^7$	93 (19)	72 (39)	65 (30)
$1x10^7$	82 (27)	66 (29)	59 (38)
$2x10^6$	59 (17)	29 (11)	33 (16)
$1x10^6$	45 (30)	24 (18)	20 (7)
$2x10^5$	18 (7)	7 (12)	8 (9)

Materials and Methods

Recombinant adenovirus constructs. AV1.0CMV.Y28 is a recombinant, replication-deficient adenoviral vector expressing the Y28 gene. It encodes for the hypervariable regions of an anti-p21-ras single chain antibody driven by the human cytomegalovirus (CMV) promoter. It is derived from the rat Y13-259 monoclonal antibody to p21-ras (3, 24, 25). The Y28 expression unit, which also contains the bovine Growth Hormone polyadenylation signal (bGH polyA), replaces the E1 adenovirus region. The AV1.0CMV.Y28 backbone is an E1-deleted and E3-partially-deleted human adenovirus serotype 5. This construct was subjected to multiple plaque purification and produced in the 293 cell line (human transformed primary embryonal kidney cell line) trans-complementing for E1 gene products.

AV1.0CMV.LacZ and AV1.0CMV are also recombinant replication-deficient adenovirus vectors constructed on the same basis of an E1 and partially E3 deleted human adenovirus type 5 backbone and produced in 293 packaging cell line. The former express the *E. coli* derived β-galactosidase protein that can be detected by histochemistry in order to access the transduction efficacy of the vector. The latter contains only the CMV promoter and bGH polyA signal without any transgene inserted. This "empty" construct has been used as the control vector in all experiments. A pilot study revealed a Maximum Tolerated Dose (M.T.D.) of 2.5 x 10^{11} vp for systemic treatment *in vivo* for each of the constructs.

Tumour. The rat rhabdomyosarcoma cell line R2 and the transfectants R2T24 and R2-neo, which can be maintained in tissue culture, have been described previously (26, 27). The *in vivo* tumour was produced by subcutaneous implantation of cells. The tumour is transplantable in syngeneic WAG/RIJ rats and was subsequently passaged serially. The R2T24 cell line was cotransfected with the plasmid pT24 carrying the c-H-ras oncogene (28) and the plasmid carrying the neo gene (29). The R2-neo cell line was transfected by the neo gene only. The R2T24 contains six copies of the 6.6 kb *Bam*HI fragment of the pT24 plasmid revealed by Southern blot analysis and exhibits constitutive expression of the c-H-ras oncogene determined by Northern blot assay (26). In the parental R2 cell line no H-ras oncogene could be detected (26).

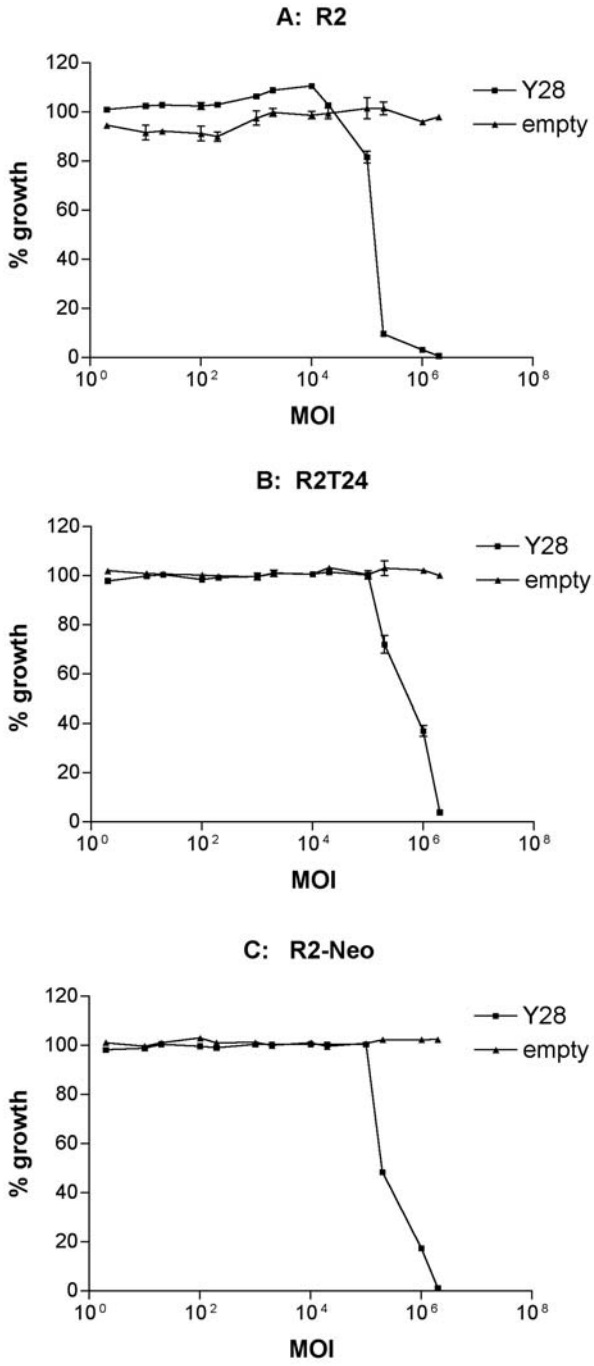

Figure 1. *In vitro bioassay of A: wild-type R2 cells, B: R2T24 cells transfected with six copies of H-ras, C: R2-neo cells transfected with the neo gene only. Cells were incubated for 48 hours with increasing concentrations of AV.1.0CMV.Y28 (■) or AV1.0CMV (empty) (▲). Four independent bioassays were performed in duplicate for each point of the line. Mean values ± SEM are shown.*

In vitro transfection efficacy study and anti-tumour activity study. R2T24, R2 and R2-neo cells were grown in Dulbecco's modified culture medium (Gibco BRL, Paisley, GB) supplemented with 10% fetal calf serum (Harlan/Sera-Lab, GB), 1% penicillin (5000 IU/ml), 1%

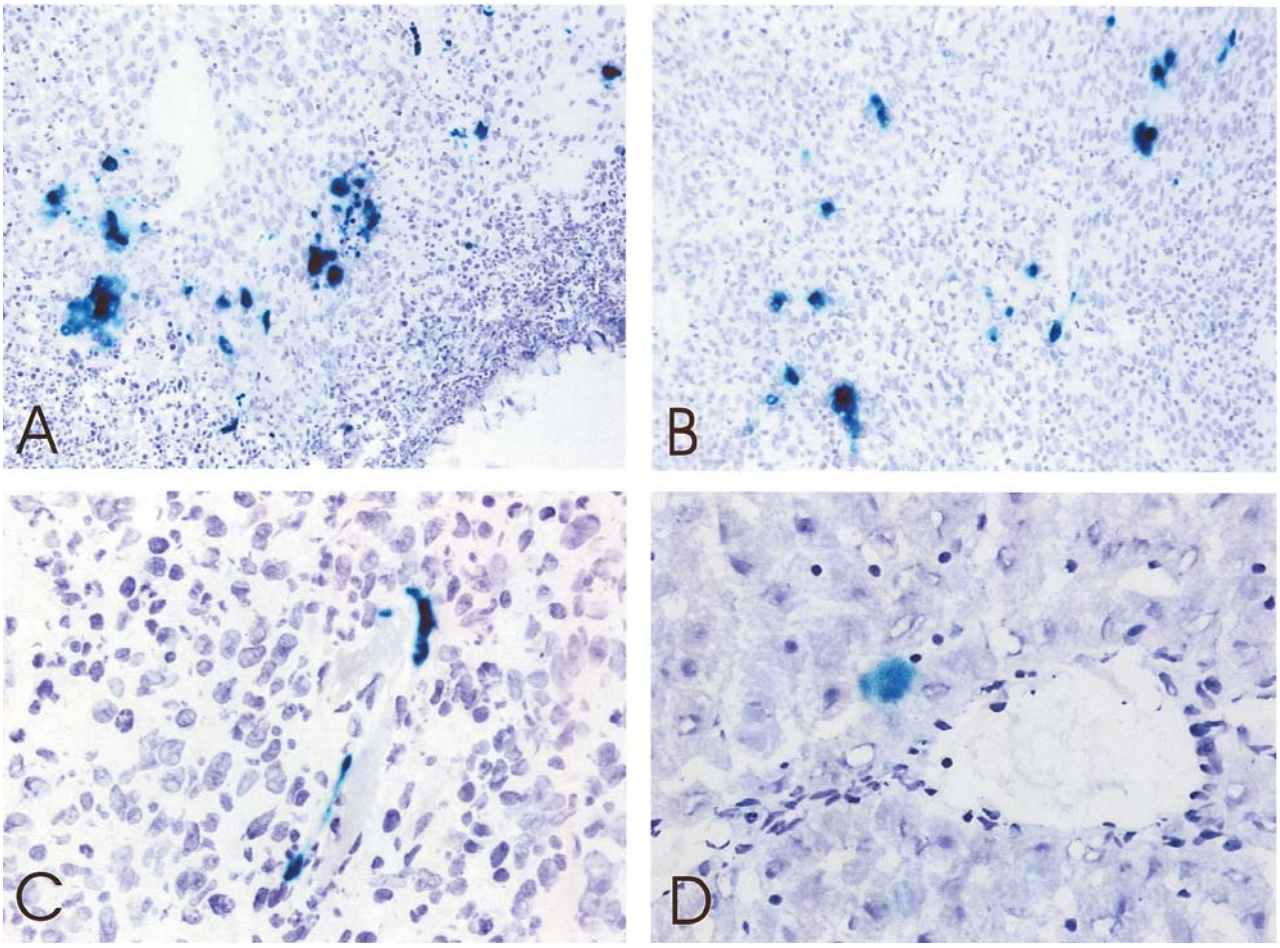

Figure 2. *Light microscopy of X-gal-stained cryosections of tumour and liver 24 hours after in vivo treatment by ILP with 2.5 x 10^{11} vp AV1.0.CMV.LacZ. Showing:*
A: R2T24 tumour, viable rim area
B: R2T24 tumour, central area
C: R2T24 tumour, detail: perivascular transduction
D: liver
Original magnification: A, B and C: 160 x, D: 400x

streptomycin (5000 IU/ml) and 1% L-glutamine (200mM) (all Gibco BRL) in a humidified incubator at 37˚C and 5 % CO_2. Before use, the cells were trypsinized (1 min, 37˚C), centrifuged (5 min, 700 g), resuspended and viability was measured by trypan blue exclusion. Viability always exceeded 85%. For *in vitro* tests, cells were seeded in flat-bottomed 96-wells microtiter plates (Costar, USA), in a final volume of 200 ml and 1.0 x 10^4 cells per well. After 24 hours the cells were incubated with different densities of adenovirus for 48 hours ranging from 1.0 x 10^4 virus particles (vp) /ml to 2.0 x 10^{10} vp/ml in a final volume of 200 µl per well (corresponding with a multiplicity of infection (M.O.I.) of 5 to 100,000).

Transfection efficacy study: After a 48-hour incubation with the LacZ construct, the cells were washed with PBS and fixed for 30 minutes with 4% paraformaldehyde at 4˚C. Then the cells were washed three times with PBS and stained with X-gal staining solution overnight at 37 ˚C. This solution is a mixture of solution A: $K_4Fe(CN)_6.3H_20$ 5 mM, $K_3Fe(CN)_6$ 5mM in wash buffer ($MgCl_2$ 2mM, deoxychlate 0.01%, NP-40 0.02% in 0.1 M sodium phosphate buffer pH 7.8) and solution B: 5-bromo, 4-chloro, 3-indolyl β-d-galactopyranoside 50 mg/ml in dimethyl formamide) in a ratio of 50 : 1. The cells were then washed once with PBS and stored at 4˚C until further analysis.

Anti-tumour activity study: After incubation with Y28 or empty construct, the cells were washed with PBS and fixed for 30 minutes with 10% trichloro-acetic acid at 4˚C. Growth of tumour cells was measured using the sulpharhodamine-B assay according to the method of Shekan *et al.* (30). Tumour cell proliferation was measured using the formula: tumour growth= (test well/control well) x 100%. At least five independent tests were performed for each time point.

Animals. Male inbred WAG/RIJ strain rats, weighing 250-300 g, obtained from Harlan-CPB (Austerlitz, The Netherlands) were used. The rats were fed a standard laboratory diet. All animals were housed under standard conditions of light and accommodation. The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act 1977 and the published Guidelines on the Protection of Experimental Animals by the Council of the European Community 1986. The protocol was approved by the committee on animal research of the Erasmus University Medical Centre, Rotterdam, The Netherlands.

Isolated limb perfusion (ILP) model. The perfusion technique was performed as described previously (18, 19). Briefly, small fragments (3-5 mm) of the rhabdomyosarcoma were implanted subcutaneously into the right hind limb. Perfusion was performed at a tumour diameter between 12 and 15 mm. Time between implantation and perfusion was about 14-16 days. Animals were anaesthetized with Hypnorm (Janssen Pharmaceutica, Tilburg, The Netherlands) and Ketamine (Apharmo B.V., Arnhem, The Netherlands). Heparin (50 IU) was injected intravenously to prevent coagulation. To keep the rat's hind limb at a constant temperature, a warm water mattress was applied. Temperature was measured with a temperature probe on the skin covering the tumour and was maintained between 38.0 and 39.0˚C. The femoral artery and vein were cannulated with silastic tubing (0.012 inch inner diameter (ID), 0.025 inch outer diameter (OD); 0.025 inch ID, 0.047 inch OD, respectively, Dow Corning, Michigan, USA). Collaterals were occluded by a groin tourniquet and isolation time started when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion solution consisted of 5 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands) and the adenoviruses were added as boluses to the oxygenation reservoir. A roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/ min. A washout with 5 ml oxygenated Haemaccel was performed at the end of the perfusion. After the perfusion, the cannulas were removed and the femoral vessels of the perfused limb were ligated. The extensive collateral circulation restored the blood supply of the perfused leg.

In vivo transfection efficacy study. In order to determine transfection efficacy, tumour-bearing rats (n=3) were treated with 2.5 x 10^{11} vp AV1.0CMV.LacZ construct by ILP. The animals were sacrificed 24 hours after treatment. Tumour, quadriceps muscle of the perfused limb, quadriceps muscle of the other hind limb, liver and spleen were taken out, snap-frozen in liquid nitrogen and stored at -80˚C until further analysis. Before X-gal staining, cryosections were fixed in 4% paraformaldehyde for 30 minutes at 4˚C. After three washings with phosphate-buffered saline (PBS) pH 7.4, sections were incubated with X-gal staining solution overnight at 37˚C. Thereafter sections were washed twice in PBS, counter-stained with haematoxylin, dehydrated with ethanol and xylene and coverslipped with entalan.

In vivo anti-tumour efficacy study. Four rats were left untreated to determine the normal growth pattern of the R2T24 tumour *in vivo*. Another four rats were sham perfused with Haemaccel only. ILP with 2.5 x 10^{11} vp of AV1.0CMV.Y28 (n=6) or the control vector AV1.0CMV (n=5) were performed. Tumour size was determined daily by calliper measurement in a standardised way. Tumour volume was calculated by using the formula: tumour volume = A^2 x B x 0.4, in which A is the smallest diameter and B the diameter perpendicular to A.

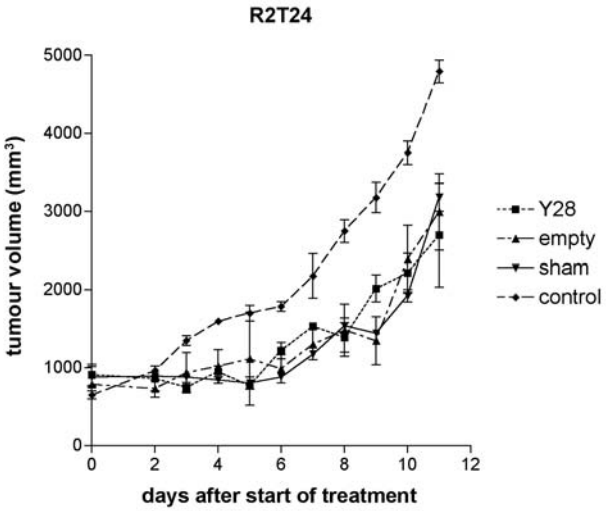

Figure 3. *Growth curve of R2T24 tumour in vivo: without treatment (◆, n=4), after sham ILP with perfusion medium only (▼, n=4) after ILP with 2.5 x 10^{11} vp AV1.0CMV(empty) (▲, n=6) or 2.5 x 10^{11} vp AV.1.0CMV.Y28 (■, n=6). Mean values ± SEM are shown.*

Statistical analysis. In vitro and *in vivo* results were evaluated for statistical significance with the Kruskal-Wallis test and Mann-Whitney test with SPSS v10.0 for Windows 2000. A significance level of $p=0.05$ was used.

Results

In vitro transfection efficacy is dose-dependent. After X-gal staining the percentage of transfected cells was calculated by means of light microscopic scoring. Positive cells were counted in four different fields with magnification of 100 X. The results for the different cell lines are shown in Table I. The wild-type R2 is more sensitive to transfection by the LacZ construct then the transfected R2T24 and R2-neo cell line at densities from 2.0 x 10^5 to 2.0 x 10^7 vp/ml ($p<0.05$). There are no significant differences between R2T24 and R2-neo. A clear dose-transfection relationship is present in all cell lines ($p<0.05$).

In vitro anti-tumour activity induced by the Y28 construct. The bioassay of R2, R2T24 and R2-neo incubated with the viral constructs demonstrated an inhibition of growth caused by the Y28 construct (Figure 1, panel A-C). Inhibition of growth occurred at a density of 1.0 x 10^8 vp/ml and higher for all cell lines. The dose causing 50% growth density (ID_{50}) was 3.9 x 10^8 vp/ml in the wild-type R2 cell line, 1.5 x 10^9 vp/ml in the R2T24 cell line and 8.9 x 10^9 vp/ml in the R2-neo cell line. Statistical analysis showed no significant difference between the three cell lines ($p=0.17$)

Homogeneous in vivo transduction by ILP. X-gal staining of cryosections of tumours and organs (3 slides per tumour or organ) treated with AV.1.0CMV.LacZ were scored by light microscopy. Upon ILP, homogenous transduction was observed in 5% of tumour cells (Figure 2). There was a homogeneous distribution of transduction in the tumour (Figure 2A,B). Moreover, around tumour-associated vessels transduced cells could often be detected (Figure 2C). Muscle tissue of the perfused limb showed almost no transduced cells. In liver tissue a few positive cells could be detected, which can be caused by adenoviruses left in the limb after the ILP or by some leakage during the ILP (Figure 2D). No transduced cells could be detected in spleen, kidney, heart, lung or intestine.

Lack of in vivo anti-tumour efficacy. All rats survived the surgical procedure and no systemic toxic side-effects were observed as measured by weight changes. However, in each of the virus-treated group one rat suffered impaired limb function and oedema. So this local toxicity determined the dosage of 2.5×10^{11} vp as the M.T.D. Sham ILP resulted in a temporary growth inhibition of R2T24 tumours compared to untreated tumours (Figure 3). We previously observed similar perfusion-related growth inhibitory effect after sham ILP in the ROS-1 osteosarcoma that also grows in WAG-RIJ rats (31). We hypothesize this may be a rat strain-dependent effect, rather than a tumour-dependent effect. Perfusion with the Y28 anti-ras construct, however, did not result in any additional anti-tumour activity. The control vectors showed comparable results (Figure 3). In all groups rats demonstrated progressive disease 7 days after ILP.

Discussion

In the present study we explored *in vitro* and *in vivo* anti-tumour activity of an anti-ras adenovirus construct. Significant growth inhibition of the Y28 scFv could be demonstrated *in vitro*, but was only noted when high concentrations were used indicating only modest activity at best. The empty control virus did not have any anti-tumour activity. Administration of an adenoviral LacZ construct in the ILP model resulted in homogeneous transfection of the tumour without significant systemic transfection or systemic toxicity. Despite these *in vitro* and *in vivo* results, the Y28 construct lacks anti-tumour efficacy using ILP in this tumour model.

The *in vitro* results suggest that the status of the ras gene has no influence on the response of tumour cells after AV1.0CMV.Y28 transfection. The R2T24 cell line expressing six copies of H-ras did not show an increased or reduced sensitivity to the anti-p21ras scFv. Russell *et al.* found Y28-mediated enhancement of radiosensitivity in various tumour cell lines with N-ras, K-ras and wild-type ras *in vitro* (31), confirming that the mechanism of Y28 is independent of the status of the ras-gene. Y28 scFv has been derived from the neutralizing antibody Y13-259. This antibody recognizes the H-, N- and K-ras proteins (24, 25). It seems that this aspecific inhibition of ras expression in tumour cells influences the cell function in a broad spectrum from enhancement of radiosensitivity to induction of apoptosis (4, 31).

In soft-tissue sarcoma, osteosarcoma and colon carcinoma rat tumour models, we have already demonstrated the transfection efficacy of adenoviral vectors by ILP and isolated hepatic perfusion (IHP) (13, 22). Moreover, ILP for adenovirus-mediated IL-3β gene therapy resulted in a significant tumour response in soft tissue sarcoma (BN-175) as well as osteosarcoma (ROS-1) (32). We currently performed ILP with an adenoviral vector harbouring the LacZ gene in order to determine the transduction efficacy in R2 rhabdomyosarcoma. Remarkable perivascular transduction was observed in 5% of tumour cells. On the basis of these results, we studied the *in vivo* anti-tumour activity of the Y28 construct. We only investigated the R2T24 tumour because it has the fastest growth *in vivo* compared to the R2 and R2-neo (26). This is of special importance because vectors driven by a CMV promoter are preferably active in dividing cells, since the promoter is activated by factors in the nucleus (33). Nevertheless, AV1.0CMV.Y28 could not induce a significant anti-tumour effect in this tumour by using the ILP. In spite of the fact that the R2T24 was a fast growing tumour, it took about 2 weeks after implantation in the limb to obtain a tumour with an adequate size for ILP. The above-mentioned BN-175 and ROS-1 tumours grew twice as fast. We previously demonstrated that fast growing tumours were well vascularized (34). We speculate that R2T24 may be less vascularized and, therefore, individual tumour cells may be reached with difficulty by a transvascular delivery method.

Cochet *et al.* described significant growth inhibition of the HCT116 human colon carcinoma cell line after intratumoural injection of AV1.0CMV.Y28 (4). They reported similar transduction efficacy after intratumoural injection. As a consequence some bystander effect of Y28 transduction *in vivo* in the HCT116 cell line was suggested. However, upon Y28-based ILP no additional anti-tumour activity was present, indicating that Y28 transduction lacks a bystander effect in this tumour. We hypothesize that a higher transduction level is needed to achieve anti-tumour efficacy in this tumour model. Higher transduction rates can be obtained by using a higher virus dose or repeated administration, as previously demonstrated in our liver metastases model in which we achieved tumour response only upon five-times-repeated hepatic artery infusion (13). Since there is minimal systemic exposure in ILP of the vector, we anticipated a dose escalation. However, in this tumour model we were confronted with local toxicity of the

limb in 2 out of the 11 perfusions. This result prohibited usage of higher dosages of the vector.

Furthermore, internalisation of adenoviral vectors is receptor-mediated. Predominantly this occurs *via* the Coxsackie- and adenovirus receptor (CAR) (35). Absence of CAR on the surface of R2, R2-neo and R2T24 cells might be responsible for the relatively high M.O.I. necessary for inducing proliferation inhibition *in vitro* as well as growth inhibition *in vivo*.

For successful cancer gene therapy tumour targeting is essential. Adjusting vector delivery methods has a major influence on transduction efficacy (36). ILP proved to be effective in reaching the tumour, however, next to vector delivery, also tumour type, extent of tumour vascularisation, presence of receptors and type of gene to be delivered are factors crucial in the process of achieving a tumour response *in vivo*.

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