Pergamon

European Journal of Cancer Vol. 32A, No. 10, pp. 1774–1780, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

PII: S0959-8049(96)00175-X

Original Paper

Improved Efficacy of Chemotherapy by Parvovirus-mediated Sensitisation of Human Tumour Cells

P. Klein-Bauernschmitt,[1,2] M. von Knebel Doeberitz,[2] M. Ehrbar,[1] K. Geletneky,[3] J. Kleinschmidt[1] and J.R. Schlehofer[1,4]

[1]German Cancer Research Center, Applied Tumour Virology, INF 242, 69120 Heidelberg; [2]Section of Molecular Diagnosis and Therapy, Department of Surgery, INF 120, University of Heidelberg; [3]Department of Neurosurgery, INF 400, University of Heidelberg, Germany; and [4]CRNS URA 1160, Institut Pasteur de Lille, 1, Rue Calmette, Lille, France

Increasing resistance of tumour cells towards the cytotoxic action of chemotherapeutic drugs is a major limitation in the treatment of cancer patients. The non-pathogenic human adeno-associated viruses (AAV) have been reported to sensitise HeLa cervical cancer cells to gamma irradiation *in vivo* and *in vitro*. To test whether these parvoviruses might render other human tumour cells more sensitive towards chemotherapeutic drugs, we analysed the effects of AAV type 2 (AAV-2) infection on established cancer cell lines and freshly explanted tumour biopsies treated with chemotherapeutic agents (e.g. cisplatin). AAV-2 infection significantly increased the cytotoxic activity of chemotherapeutic drugs compared with uninfected controls. AAV-2 infection without concomitant chemotherapeutic treatment had no significant effect on viability of the cells. In nude mice, combined application of AAV-2 infection and chemotherapeutic treatment significantly increased the therapeutic activity on tumours arising from subcutaneously injected tumour cells compared with tumours treated by chemotherapeutics only. These results indicate that AAV-2 infection sensitises human cancer cells towards the cytotoxic action of chemotherapeutic drugs.

Key words: AAV, chemotherapy, human tumour cells, parvovirus
Eur J Cancer, Vol. 32A, No. 10, pp. 1774–1780, 1996

INTRODUCTION

The therapeutic efficacy of cytotoxic drugs in the chemotherapy of human cancers is mainly limited by the increasing resistance of tumour cells in the dose range which can be applied to patients. Dose escalation studies, performed previously for many cancers, have demonstrated that the therapeutic efficacy of cytotoxic drugs can be significantly enhanced by increased doses [1]. However, this approach is limited by severe toxic side-effects. Therefore, specific sensitisation of human cancer cells towards the cytotoxic action of chemotherapeutic drugs without increasing the side-effects on normal tissues is a great challenge in the chemotherapy of neoplastic diseases.

Adeno-associated viruses (AAVs) are small single stranded parvoviruses which cause no known pathological condition and depend for their replication on helper functions provided by co-infecting helper viruses. AAVs have been shown to inhibit the transformation of cells induced by viral [2–4] or activated cellular oncogenes [5, 6]. AAV type 2 (AAV-2) infection interferes with carcinogen- or virus-induced mutagenicity [7] and selective DNA-amplification [8–12]. In contrast to normal tissues, tumour cells treated with chemical or physical carcinogens have been shown to support low-level AAV-2 replication even in the absence of a helper virus [13]. Moreover, it has been shown that HeLa cervical carcinoma cells, which persistently harbour AAV-2 DNA integrated into the cellular genome, are more sensitive to gamma irradiation *in vitro* and *in vivo* [14, 15]. All these observations point to an efficient AAV-2-associated antineoplastic activity which might also enhance the cytotoxic efficacy of drugs used in the chemotherapy of human neoplasia. To test this hypothesis, we analysed the cytotoxic activity of cisplatin *in vitro* and *in vivo* either in the presence or absence of AAV in three different established human cancer cell lines and in cells derived from freshly explanted human cancer biopsies. Our results indicate

Correspondence to P. Klein-Bauernschmitt.
Received 11 Dec. 1995; revised 26 Feb. 1996; accepted 28 Feb. 1996.

that infection of human tumour cells of various origins with AAV enhances significantly the sensitivity towards chemotherapeutic drugs such as cisplatin.

MATERIALS AND METHODS

Cells

The human glioblastoma cell line, A172; the lung adenocarcinoma cell line, A-549; the cervical carcinoma cell line, HeLa; the melanoma cell line, Colo38; the melanoblastoma cell line, FO1; the breast carcinoma cell line, MDA-MB-435S; the kidney carcinoma cell line, MRI-H-121 and the kidney carcinoma cell line, KTCTL-26, were provided by Tumourbank, DKFZ, Heidelberg, Germany. All cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) plus antibiotics and grown in 5% CO_2 in a humidified atmosphere. Biopsy samples derived from a brain tumour histologically and immunologically confirmed as a glioblastoma (KG2) and from a primary squamous cell lung cancer (P693) were dissected, trypsinised and cultured in DMEM (P693) or RPMI 1640 (KG2).

Virus

AAV-2 was propagated in HeLa cells using adenovirus type 2 as a helper. Cells were lysed by three rounds of freezing and thawing, and purification of AAV-2 virions by CsCl gradient centrifugation was performed as described by Berns and colleagues [16] and Senepathy and Carter [17].

Infection protocol

Infection with AAV-2 was performed by inoculating PBS-washed cells at a multiplicity of tissue culture infectious units (MOI) per cell as indicated in the legends to Figures 1–3 and Table 1 for 30 min at 37°C. Unabsorbed virus was removed by washing infected cells twice with medium.

To exclude effects of adenovirus possibly still present in the purified AAV-2 virus stocks (AAV-2 was heated at 56°C for 30 min prior to use), we infected tumour cells with UV- or heat-inactivated adenovirus type 2. At high virus inputs, infection with inactivated adenovirus type 2 had no effect on the plating efficiencies.

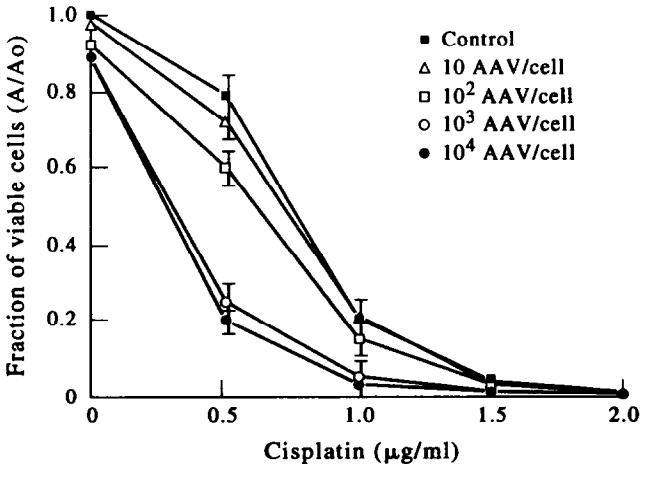

Figure 1. Response of human lung carcinoma cell lines (A549 cells) to treatment with cisplatin with and without infection with AAV-2. Cytotoxicity, as evidenced by less viable cells, was determined by the MTT assay. The index (A/Ao) was calculated by the ratio of the colorimetric values of viable control versus viable infected and/or treated cells. A549 cells were treated with increasing concentrations of cisplatin and infected with different multiplicities of infectious AAV-2 particles (10^1–10^4).

Treatment with chemotherapeutic agents

Cells were grown in plastic flasks, trypsinised and washed with PBS. After centrifugation (5000*g*), cells were cultured in medium containing chemotherapeutics at concentrations indicated for 24 h and grown at 37°C in a humidified atmosphere with 5% CO_2. One day after treatment, cells were washed twice with PBS and plated in growth medium without chemotherapeutics.

MTT-assay

Cells (10^4–10^5) were grown in microtitre plates and treated with chemotherapeutic agents and infected at MOI as indicated in the legends to the figures. After 3 days, cells were washed in PBS, and incubated with 0.5 μg/ml MTT-solution for 2 h. After washing with PBS, 200 μl isopropanol was added to the cell pellet. After 1 h, supernatant was transferred on to a microtitre plate and measured in a "Titertek Multiscan Plus" at 540 nm.

Colony forming ability

Plating efficiency was determined by plating 1000 cells of treated or untreated or infected cultures and counting the numbers of outgrowing clones after fixation with 4% formaldehyde and staining with crystal violet (Fluka, Buchs, Switzerland), one week after treatment. All experiments were performed in triplicate and reproduced at least once.

Soft-agar cloning

A 1% stock solution of agar (Bacto Agar, Difco, Hamburg, Germany) in water was autoclaved for 15 min at 121°C. Agar was cooled to 45°C. DMEM or RPMI 1640 (2-fold concentrated) and FCS were warmed to 45°C. A base layer was prepared by adding 30 ml of 2× DMEM or 2× RPMI to 30 ml of agar-stock solution (1%). Of this base layer, 5 ml was poured into 5 cm petri dishes. The dishes were cooled for at least 1 h to 4°C. Suspensions of 1000 cells in 0.3% agar were carefully placed on to the basal layer. Prior to incubation at 37°C, dishes were allowed to cool. Size and number of colonies were detected after 3 weeks. All experiments were performed in triplicate and reproduced at least once.

Animal experiments

Nude mice (CD1-nu/nu) were purchased from Charles River WIGA (Sulzfeld, Germany). Animals were housed (four per cage) in isolators, and received food and water *ad libitum*. Exponentially growing P693 cells or A549 cells were injected subcutaneously into the flanks of 7-week-old female nude mice (2×10^6 cells). Four (P693 cells) or five (A549 cells) weeks after inoculation of the tumour cells, when tumours reached a volume of 400 mm^3, animals were infected with AAV-2 and treated with cisplatin. For 5 weeks (P693 cells) or 6 weeks (A549 cells), 10^8 infective units of AAV-2 (in 100 μl of phosphate buffered saline) were weekly injected intratumourly (i.t.) into the animals. Cisplatin was administered weekly by injecting 5 mg/kg body weight (low dosage) intraperitoneally, followed by AAV-2 infection. In each group (control, infection, cisplatin, combination of infection and cisplatin), eight animals were included. Tumour volume was determined weekly by measuring three diameters of the developing tumour.

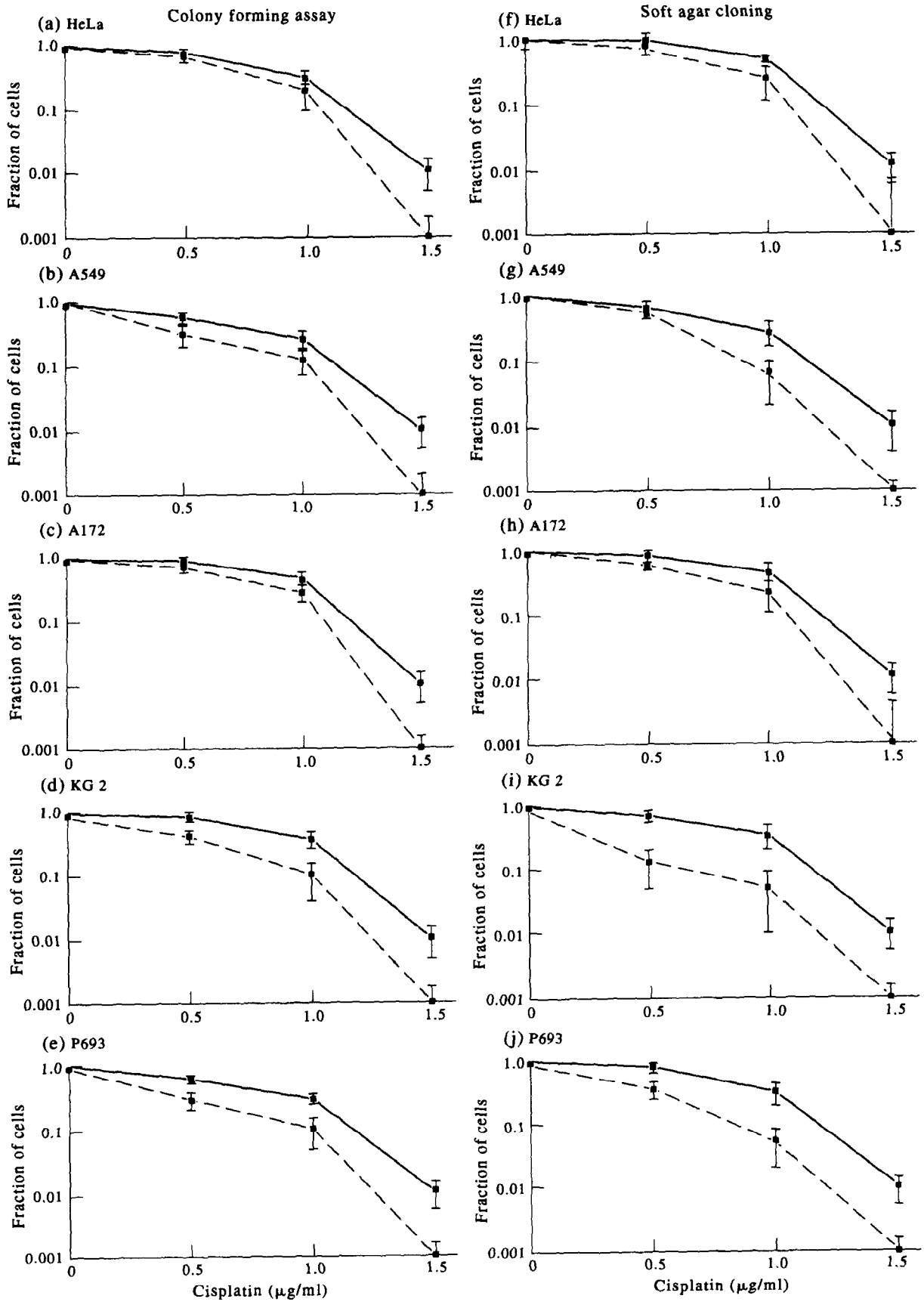

Figure 2. Influence of AAV-2 infection on the survival of cisplatin-treated established tumour cell lines *in vitro*. Survival of infected (10^3 AAV-2/cell) and cisplatin-treated cells was measured by ability to form colonies (a–e) and grow in soft-agar (f–j) as described in Materials and Methods. Survival was calculated by the ratio of control colonies versus infected and/or treated colonies. Cisplatin-treated cells: —; cisplatin-treated and AAV-infected cells: -----. *P*-value determined by statistical Student's *t*-test are as follows: HeLa: $P = 0.05$ in the soft-agar cloning assay at 1.0 μg/ml cisplatin. A549: $P = 0.01$ in the soft-agar cloning at 1.0 μg/ml cisplatin. A172: $P = 0.07$ in the soft-agar cloning assay at 1.0 μg/ml cisplatin. KG2: $P = 0.001$ in the soft-agar cloning assay at 0.5 μg/ml cisplatin. P693: $P = 0.003$ in the soft-agar cloning assay at 0.5 μg/ml cisplatin.

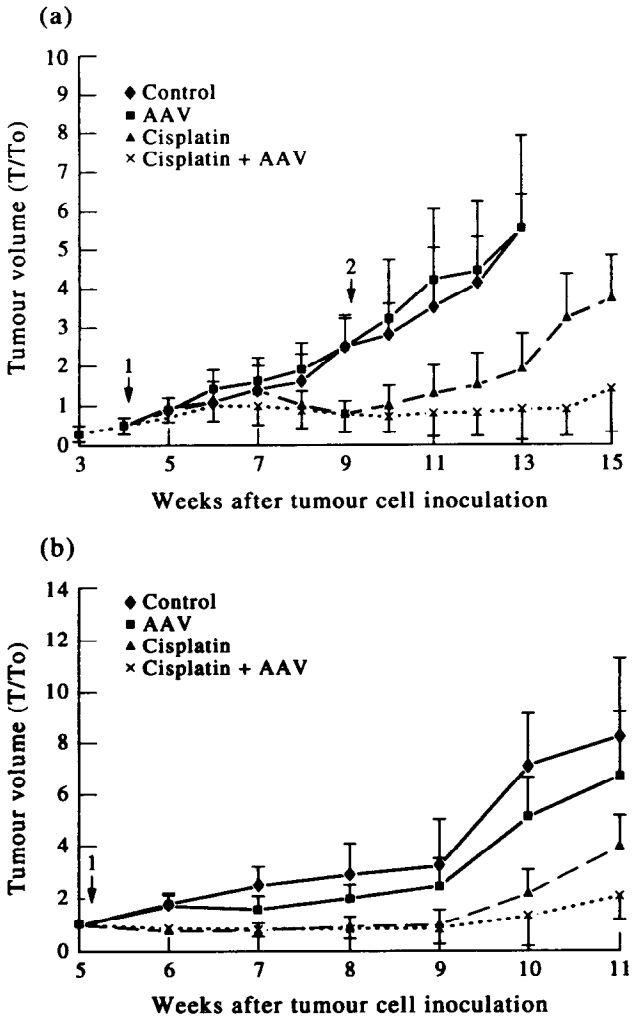

Figure 3. Growth of (a) P693 and (b) A549 tumour cells inoculated into nude mice. Eight animals were included in each group. For P693, therapy was arrested 9 weeks after tumour cell inoculation (as indicated by arrow 2). For A549, therapy was stopped 11 weeks after tumour inoculation. Arrow 1 indicates start of therapy. For both P693 ($P = 0.033$) and A549 ($P = 0.04$) tumour growth of AAV-2-infected and cisplatin-treated animals was significantly reduced compared with cisplatin-treated controls. Control, animals without treatment or infection; AAV, AAV-2-infected animals; cisplatin, cisplatin-treated animals, cisplatin + AAV, animals treated with cisplatin and infected with AAV. Index (T/To) was calculated by the ratio of the tumour volume of infected and/or treated animals versus the tumour volume of animals at the beginning of the experiment.

Statistical analysis

To evaluate the results of the animal experiments, the multivariate rank statistic test, a distribution-free statistical methology for the comparison of tumour growth curves, was used [18]. Statistical analyses of data in the colony forming and soft-agar cloning assays were performed by Student's *t*-test.

Detection of AAV protein expression

AAV protein expression (early proteins (rep) and capsid proteins (cap)) were analysed by Western blot and indirect immunofluorescence using monoclonal antibodies as described by Wistuba and coworkers [19].

Southern blot analysis

Genomic DNA was prepared from AAV-2 infected and control cells as described earlier [20]. Ten microlitre aliquots of restriction enzyme-digested high-molecular-weight DNA were electrophoresed on a 0.8% agarose gel, blotted on to GeneScreen Plus filters (DuPont, NEN, Boston, U.S.A.) as described by Southern [21], and hybridised with ^{32}P-labelled (α-[^{32}P]dCTP; Amersham, Braunschweig, Germany), randomly primed, cloned AAV-2 DNA (pAV-2 or ptAV-28) [8] for 16 h at 65°C in 0.5 M Na_2HPO_4/7% sodium dodecyl sulphate (SDS)/1 mM EDTA. Hybridised filters were washed at 65°C three times with 1% SDS/50 mM Na_2HPO_4 and autoradiographed.

RESULTS

Influence of AAV infection on the survival of cisplatin-treated tumour cells in vitro

To test whether AAV-2 infection affects the sensitivity of tumour cells to chemotherapy treatment, A549 lung cancer cells were infected with increasing multiplicities of infectious AAV-2 particles and treated subsequently with increasing concentrations of cisplatin as a representative example of chemotherapeutic drugs (Figure 1). The fraction of viable cells was subsequently determined using a colorimetric assay as described in Materials and Methods [22]. Increased doses of AAV-2 (MOI up to 10^3 AAV infectious units per cell) rendered the cells more susceptible to cisplatin, but this effect could not be enhanced by further increased AAV-2 doses.

After identification of the efficient sensitising dosage of AAV-2 for cisplatin cytotoxicity in A549 cells, the effects of AAV-2 infection (10^3 AAV/cell) and subsequent cisplatin treatment on the survival and colony forming ability of tumour cells of different histogenetic origin were determined. Effects of AAV-2 infection on cisplatin sensitivity were analysed in three established cell lines, namely HeLa cervical carcinoma cells, A549 cells derived from an adenocarcinoma of the lung, and A172 glioblastoma cells. In addition, freshly explanted tumour cells derived from a squamous cell carcinoma of the lung (P693) and a glioblastoma multiforme (KG2) were investigated (Figure 2). Cells were infected with AAV-2 (10^3 AAV/cell), treated with cisplatin (0.5–1.5 μg/ml, 3 h after infection) and seeded at a density of 10^3 cells/dish into culture dishes or semisolid medium (soft-agar). AAV-2 infection prior to cisplatin treatment resulted in a significantly reduced number of outgrowing colonies compared with cisplatin treatment without AAV-2 infection ($P = 0.05$–0.001, depending on the cell line, cf. Figure 2). Infection with AAV-2 without subsequent cisplatin treatment did not decrease the number of colony forming cells. These results suggest that AAV-2 infection renders these tumour cells significantly more sensitive to genotoxic damage induced by cisplatin. A similar sensitising effect of AAV-2 infection was observed in experiments with other chemotherapeutic drugs as BCNU, doxorubicin or 5-fluorouracil in cells of breast, kidney and skin tumour cell lines (Table 1).

Effect of AAV-2 infection on cisplatin-treated tumour cells inoculated into nude mice

P693 squamous cell bronchial carcinoma cells were subcutaneously injected into the flanks of nude mice. Four weeks after *in vivo* tumour cell inoculation, therapy with cisplatin and infection with AAV-2 was started (Figure 3a). Tumour volumes were determined for up to 15 weeks after tumour cell

Table 1. *Cell growth of different human tumour cell lines after infection with AAV and treatment with chemotherapeutic agents*

Treatment	Colo38 G4/G0	FO1 G4/G0	MDA-MB435 G4/G0	MRI-H-121 G4/G0	KTCTL-26 G4/G0
Control	8.8 (±0.8)	4.5 (±0.5)	7.2 (±0.9)	4.4 (±0.3)	16 (±1.5)
AAV	8.3 (±0.6)	4.3 (±0.5)	6.9 (±0.8)	4.3 (±0.3)	14.5 (±1)
1 μg/ml BCNU	6.6 (±0.5)	3.1 (±0.3)	ND	ND	ND
BCNU + AAV	5.7 (±0.4)	2.4 (±0.2)	ND	ND	ND
0.033 μg/ml DOX	ND	ND	3.4 (±0.4)	ND	ND
Dox + AAV	ND	ND	2.4 (±0.3)	ND	ND
2 μg/ml 5-FU	ND	ND	ND	2.1 (±0.2)	5.4 (±0.4)
5-FU + AAV	ND	ND	ND	1.2 (±0.2)	4.6 (±0.4)

Four days after infection and/or treatment with chemotherapeutic agents cells were harvested and cell numbers were determined in a haemocytometer by Trypan blue exclusion method. Cell proliferation was calculated by the ratio of living treated and/or infected cells at day 4 (G4) versus untreated/mock-infected controls at the beginning of the experiment (G0). Control, mock-infected cells; AAV, cells infected with AAV-2 (MOI = 100); treatment with chemotherapeutic drugs and infection with AAV; 5-FU, 5-fluorouracil; Dox, doxorubicin; BCNU, carmustine. ND, not done.

inoculation. In animals infected with AAV and treated with cisplatin, tumour growth was significantly reduced compared with controls treated only with cisplatin ($P = 0.033$). In two animals of the cisplatin-treated and AAV-2-infected group, tumours totally disappeared in contrast to no complete regression in the group treated only with cisplatin. Similar results were obtained with A549 cells inoculated into nude mice (Figure 3b; $P = 0.04$).

Analysis of AAV-2 DNA replication

Earlier studies have shown that adeno-associated virus can replicate in the absence of a helper virus in cells treated with cytotoxic or genotoxic agents [23, 24]. To elucidate whether AAV-2 DNA was synthesised in cells treated with cisplatin, a Southern blot analysis of infected and cisplatin-treated cells was performed. Total genomic DNA was extracted from cells 3 days after infection. Helper-independent viral DNA replication was detected exclusively in A549 cells (Figure 4). No AAV-2 DNA replication nor AAV-2-specific protein synthesis (Western blot analysis or indirect immunofluorescence of rep- and cap-proteins) was observed in all other cell lines tested (HeLa, A172, KG2, P693) (data not shown). These results suggest that the sensitising effect of AAV-2 infection on the cytotoxic activity of cisplatin observed here is not dependent on AAV-2 DNA replication nor expression of specific viral proteins.

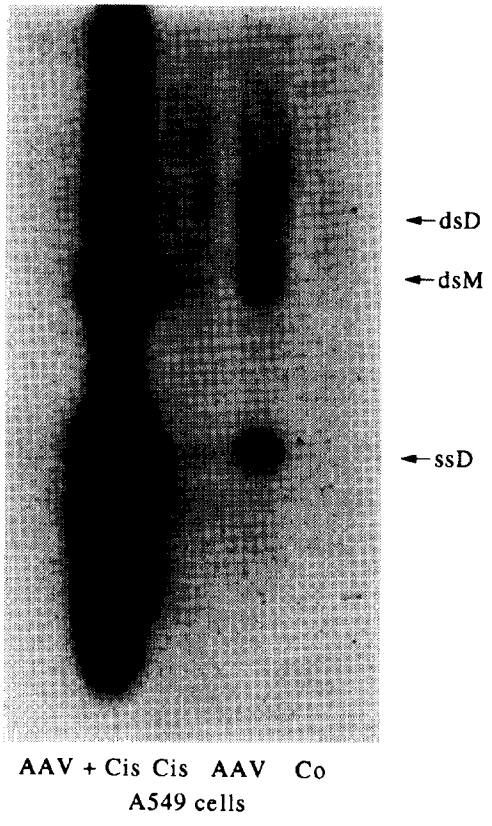

Figure 4. Southern blot analysis of AAV-DNA replication in A549 cells. Cellular DNA digested with *Bgl* II; Co, mock-infected cells; AAV, cells infected with AAV-2; Cis, cisplatin-treated cells; AAV + Cis, cells infected with AAV and treated with cisplatin. The different forms of AAV-DNA detected are indicated: ssD, single-stranded DNA; dsM, double-stranded DNA monomer; dsD, double-stranded DNA dimer.

DISCUSSION

Adeno-associated viruses are known to have tumour suppressive properties [25, 26]. In addition, infection with AAV-2 has been reported to enhance cell killing of carcinogen-treated cells [27] and to confer increased irradiation sensitivity to tumour cells [15]. This prompted us to analyse the potential use of this non-pathogenic human parvovirus to improve the efficiency of chemotherapy of human tumours. In cell culture assays, different cell lines (A172, HeLa, A549) were found to be sensitised to chemotherapeutic treatment after infection with the parvovirus. In colony forming, as well as in soft-agar cloning assays, AAV-2-infected and cisplatin-treated cell lines and freshly established tumour cells (KG2, P693) displayed a reduced number of colony forming units.

In animal experiments, we confirmed the *in vitro* data, demonstrating a significantly reduced tumour growth in animals infected with AAV-2 and treated with cisplatin in comparison with tumour growth in control animals which received cisplatin alone. Although AAV can infect murine cells, no side-effects pointing to damage of non-neoplastic mouse tissues were observed. In contrast, mice infected with the virus and treated with cisplatin remained in a better physical condition and underwent less extensive or no tumour cachexia, compared with cisplatin-treated controls (data not shown). Thus, AAV-2 infection combined with chemotherapy appears

to specifically sensitise the tumour cells to cytotoxic drugs. In our experiments, infection with AAV-2 allowed the dosage of chemotherapeutic agents to be decreased considerably to achieve the same cytotoxic efficacy.

Despite the fact that studies on enhanced cell killing of tumour cells after infection with AAV were published many years ago [27], the mechanisms by which AAV-2 exerts its sensitising effects on chemotherapeutic treatment in tumour cells are not yet understood. Since DNA replication of AAV-2 was observed in only one of five cancer cell cultures infected and treated with cisplatin, replication of the virus is unlikely to contribute to the observed sensitising activity. Moreover, in none of the replication-inactive infected and cisplatin-treated cell lines could viral protein expression (rep- or cap-proteins) be detected, suggesting that the sensitising activity may be rather due to host cell factors activated in response to AAV-2 infection. This is in agreement with previous observations, indicating that sensitisation of HeLa cells towards gamma irradiation does not depend on AAV-2 DNA replication nor gene expression [15]. Further studies are required to unravel the mechanisms of AAV-mediated tumour cell sensitisation. Cellular factors and components of the virus, which are involved in the sensitising effects described here need to be characterised. Such studies should also clarify whether the virions (or parts thereof) might account for the sensitising effects and whether these can be even more conveniently administered as whole virus preparations. In addition, it remains to be tested whether cellular functions involved in the AAV-mediated sensitising pathways are retained in cancer cell clones, and whether AAV-resistant cancer cell clones might emerge under respective treatments protocols.

Hillgenberg [28] has shown that a modulation of cellular genes such as *BCL-2, P53* and *RB* is not observed in AAV-2-infected and chemotherapeutically treated small cell lung cancer cell lines. It cannot be excluded that integration of viral genes into the cellular genome triggers the sensitising effect of AAV, but this seems to be unlikely since viral DNA could not be detected in most of the cell lines by Southern blot analysis a few days after infection.

AAV-infection has been shown to prevent DNA amplification [26], frequently involved in the development of chemotherapy resistance in cancer cells. However, since the sensitising activity of AAV-infection was instantly observed on large tumour cell numbers, which were not previously treated with cytotoxic drugs, prevention of DNA amplification could not account for the sensitising effects described here. Under clinical conditions, however, prevention of DNA amplification-associated tumour cell resistance towards cytotoxic drugs might provide additional benefits for the therapeutic outcome. Further studies on tumour cells which develop drug resistance under chemotherapy will show how significantly AAV-2, in addition to the sensitising effecs to radio- and chemotherapy described here, might prevent the outgrowth of therapy refractory clones. However, our data clearly demonstrate the potent sensitising activity of a non-pathogenic virus infection on cancer cells treated with cytotoxic drugs.

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Acknowledgements—We thank Dr H. zur Hausen for constant support and helpful discussions. We thank Dr D. Komitowski for histological investigations, Dr L. Edler for statistical analysis (DKFZ, Heidelberg) and Dr A. Bürkle for critical reading of the manuscript (DKFZ, Heidelberg). Brain cancer biopsies were kindly provided by Dr F. Albert (Department of Neurosurgery, Heidelberg). Glioblastoma-specific antibodies were a generous gift from Dr D. Atassi (Houston, U.S.A.), BCNU and glioma-specific antibodies were kindly provided by Dr J. Eisenbarth (DKFZ, Heidelberg) and Dr J. Kartenbeck (DKFZ, Heidelberg). This work was supported by Deutsche Krebshilfe, Dr Mildred Scheel Stiftung (M31/93/KL2) to PK-B and a BMFT grant of the Verbund Klinisch-Biomedizinische Forschung to MvKD.