Chlorpromazine and apigenin reduce adenovirus replication and decrease replication associated toxicity

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Abstract

Background Adenoviruses can cause severe toxicity in immunocompromised individuals. Although clinical trials have confirmed the potency and safety of selectively oncolytic adenoviruses for treatment of advanced cancers, increasingly effective agents could result in more toxicity and therefore it would be useful if replication could be abrogated if necessary.

Methods We analyzed the effect of chlorpromazine, an inhibitor of clathrin-dependent endocytosis and apigenin, a cell cycle regulator, on adenovirus replication and toxicity. First, we evaluated the in vitro replication of a tumor targeted Rb-p16 pathway selective oncolytic adenovirus (Ad5/3-Da24) and a wild-type adenovirus in normal cells, fresh liver samples and in ovarian cancer cell lines. Further, we analyzed the in vitro cell killing efficacy of adenoviruses in the presence and absence of the substances. Moreover, the effect on in vivo efficacy, replication and liver toxicity of the adenoviruses was evaluated.

Results We demonstrate in vitro and in vivo reduction of adenovirus replication and associated toxicity with chlorpromazine and apigenin. Effective doses were well within what would be predicted safe in humans.

Conclusions Chlorpromazine and apigenin might reduce the replication of adenovirus, which could provide a safety switch in case replication-associated side effects are encountered in patients. In addition, these substances could be useful for the treatment of systemic adenoviral infections in immunosuppressed patients. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords adenovirus; gene therapy; virus replication; reduced toxicity

Introduction

The efficacy and safety of adenoviral gene therapy as an antitumor approach has been validated in recent randomized [1–3] and non-randomized [4] trials. However, when faced with bulky advanced disease, effective tumor transduction continues to be the limiting step for achieving clinical benefits. Replication-competent viruses such as oncolytic adenoviruses might prove useful in this regard [5]. The replicative life cycle of the virus results in cancer cell destruction (‘oncolysis’), while modifications in the viral genome reduce replication in normal tissues.
An increasing understanding of adenovirus biology and interactions with cellular proteins has allowed creation of more effective viruses for cancer gene therapy. However, with more potent agents an increase in side effects is also possible [6]. The potential for adenovirus-associated toxicity is corroborated by reports of severe toxicity in immunocompromised individuals [7] and a fatality reported in an adenoviral gene therapy trial [8]. We hypothesized that we may be able to reduce adenovirus replication with pharmacological intervention.

Initial binding of adenovirus occurs via interaction of the fiber knob domain with the primary receptor. Binding is followed by internalization, which triggers endocytosis via clathrin-coated pits, viral disassembly, endosomal lysis and transport into the nucleus. Endosomal uptake into clathrin-coated pits seems a universal feature regardless of the virus receptor [9]. The antipsychotic agent chlorpromazine inhibits the assembly of clathrin adapter protein AP2 on clathrin-coated pits, which causes the pits to disappear from the cell surface and reappear on endosomal membranes [10]. Another general requirement for effective DNA virus replication is induction of S-phase, which can be inhibited with G2/M arrest mediated by the natural bioflavonoid apigenin [11].

Materials and methods

Cell lines and agents

293 cells were obtained from Microbix (Toronto, Canada), while lung adenocarcinoma cell line A549 was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). Ovarian adenocarcinoma cell lines SKOV3.ip1, Hey and OV-4 were obtained from Dr. Price, Dr. Wolf (both M. D. Anderson Cancer Center, Houston, TX, USA) and Dr. Eberlein (Harvard Medical School, Boston, MA, USA), respectively. All cell lines were cultured in recommended conditions. Chloropromazine and apigenin were purchased from Sigma-Aldrich Finland (Helsinki, Finland). Gemcitabine (Gemzar®) was obtained from Eli Lilly and Co. (Indianapolis, IN, USA).

Recombinant adenoviruses

Ad5/3-Δ24 was previously described [12]. Ad300wt, a wild-type human Ad5, was obtained from ATCC. Both viruses were propagated on A549 cells, and purified on cesium chloride gradients. The viral particle (vp) concentration was determined at 260 nm, and standard plaque assay on 293 cells was performed to determine infectious particles. The ratio of vp/infectious particles was 10.3 and 10 for Ad5/3-Δ24 and Ad300wt, respectively.

Replication in fresh human liver tissue and cell monolayers

Fresh liver samples were obtained with the signed informed consent and ethical committee permission from patients undergoing surgery at the Helsinki University Central Hospital. Precision-cut (250 µm) slices were cut with a Vibratome 1000 Plus sectioning system (Vibratome, St. Louis, MO, USA), and preincubated with chlorpromazine (5 µg/ml), apigenin (2.5 µg/ml) or growth medium (mock) for 1 h before adding 10^7 vp of wild-type or Ad5/3-Δ24. Liver sections do not adhere and thus washing was not performed. At indicated time points, liver slices and supernatant were frozen separately. After gradual thaw on ice, the liver slices were minced into small pieces, combined with supernatant and subsequently freeze-thawed three times. After centrifugation, the supernatant was titered on 293 cells by TCID_{50} assay. Further, released liver aspartate transaminase (AST) level was measured from supernatant after 24 h infection with a photometric method (Roche Hitachi MODULAR, Hitachi Ltd., Tokyo, Japan). Cell line monolayers were preincubated with chlorpromazine (5 µg/ml), apigenin (2.5 µg/ml) or growth medium (mock) for 1 h. The viruses (10 vp/cell) were added on supernatant, which was replaced by fresh growth medium ± reagents 1.5 h later. Replication was analyzed after three freeze/thaw cycles as above. Time points for analysis were selected based on assumptions on maximal replication on one hand and limitations on liver tissue availability on the other.

Cell killing assay

Cell monolayers were preincubated with chlorpromazine (5 µg/ml) or apigenin (2.5 µg/ml) for 1 h. Thereafter, cells in quadruplicate were infected with Ad300wt, Ad5/3-Δ24 or no virus for 1.5 h at 37°C, and fresh growth medium ± reagents was added. Cell viability was measured using the CellTiter 96 AQueous One Solution cell proliferation assay (MTS assay, Promega, Madison, WI, USA), when any combination at 100 vp/cell displayed complete cell killing.

In vivo models of efficacy, replication and toxicity

All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice were obtained from Taconic (Ejby, Denmark) at 4 weeks of age and quarantined at least for 1 week prior to the study. Subcutaneous (s.c.) human ovarian cancer (Hey cell) tumors were established in female NMRI CD-1 nude (n = 10 tumors/group), and treated with intratumoral (i.t.) injections of 3 × 10^8 vp of Ad5/3-Δ24 or no virus on days 0, 2 and 4. Mice received...
intrapertitoneal (i.p.) injections of phosphate-buffered saline (PBS), chlorpromazine (200 µg) or apigenin (250 µg) every day. Tumor growth and body weight were followed. Tumor volume is calculated from the formula 0.5 × length × width², and the data is expressed as % of the tumor volume at the initiation of therapy (32–88 mm³) to allow correction for variation in initial tumor volume.

In replication assay, the s.c. Hey cell tumors were established as above, and treated with a single i.t. injection of 3 × 10⁸ vp of Ad5/3-Δ24 on day 0. Mice received i.p. injections of PBS, chlorpromazine (200 µg) or apigenin (250 µg) every day (n = 12 tumors/group). Four tumors/group were harvested at indicated time points, homogenized, and the virions were released by three freeze/thaw cycles. The amount of infectious particles was analyzed by TCID₅₀.

Liver toxicity data was analyzed using the x² test. Only preplanned comparison was made.

Results and discussion

We analyzed the in vitro replication of a tumor targeted Rb-p16 pathway selective oncolytic adenovirus (Ad5/3-Δ24) [12] and a wild-type adenovirus in fresh human liver explants. Wild-type virus displayed replication over 48 h. Interestingly, chlorpromazine reduced the titer 8-fold (Figure 1A, P = 0.0271 at 48 h). Further, lower liver aspartate transaminase (AST) levels were measured from chlorpromazine-treated livers suggesting reduced hepatocyte damage (Figure 1E). Importantly, as Ad5/3-Δ24 is in development for human trials, it did not replicate productively in the liver explants (Figure 1B). Nevertheless, even marginal replication was further attenuated by chlorpromazine as evidenced by decreasing titer at later time points. In non-malignant 293 cells both viruses replicated avidly as expected, and chlorpromazine reduced replication up to 1960-fold (Figures 1C and 1D, wild-type: P = 0.0278, 0.0056 and 0.0433; Ad5/3-Δ24: P = 0.0248, 0.0040 and 0.0259 at 24, 36 and 48 h, respectively).

In ovarian adenocarcinoma Hey cells apigenin reduced replication 100-fold (Figures 2A and 2B, wild-type: P = 0.0156; Ad5/3-Δ24: P = 0.0478 at 72 h), but the effect was less pronounced in another ovarian cancer cell line OV-4 (Figures 2C and 2D, P = 0.0428 at 24 h). Presumably, genetic heterogeneity between OV-4 and Hey resulted in variation in replication attenuation with the drugs. The close association of replication to cell killing was corroborated in a longitudinal assay where apigenin reduced the activity of Ad5/3-Δ24 (Figure 2E, P = 0.0680). Carette et al. reported 3 to 4 orders of magnitude reduction in oncolytic adenovirus DNA copy number with apigenin treatment in a quantitative polymerase chain reaction (PCR) assay, and also the expression of transgene was reduced up to 4 logs in various cancer cell lines [13]. Of note, in those experiments the apigenin concentration was 8 times higher than the concentration we utilized. In our experiments higher concentrations displayed cell toxicity (data not shown).

Liver toxicity was assessed in vivo on the toxicity of CPZ and apigenin. As human adenoviruses do not replicate productively in murine normal tissues [14], human xenografts in mice were utilized for in vivo studies. Both apigenin and chlorpromazine were found to reduce the antitumor efficacy of Ad5/3-Δ24 (both P < 0.0001, Figure 3A), due to reduced replication (Figure 3B). Production of new viruses was reduced 36-fold with chlorpromazine (P = 0.0286 and 0.0312 on days 3 and 7, respectively). An 11-fold decrease was seen also with apigenin (P = 0.1380). Neither apigenin nor chlorpromazine had antitumor activity per se. We do not fully understand the reason for the discrepancy in the effect of chlorpromazine on Hey cells with Ad5/3-Δ24 in vitro versus in vivo. However, because this virus replicates very rapidly in vitro, the speed of replication may be the limiting factor in vivo. It is likely that the differential effects observed in vivo and in vitro are due to the different environments and time periods involved.
of replication may actually become counterproductive to packaging of functional virions. Theoretically, cells might be lysed before producing the maximum number of virions. Thus, slowing of the speed of replication may not necessarily be sensitively seen as reduction of in vitro virion production. Nevertheless, tumor penetration and intratumoral dissemination might be improved by rapid replication and release, and these aspects cannot be assessed in vitro. Therefore, we feel that the in vivo data gives a more complete and relevant picture of the actions of the drugs.

To assess the general toxicity of the treatment regimens and the overall health of the mice, their body weight was followed (Figure 4A). There was no weight loss in any treatment group, and the lowest body weights were in groups not treated with virus.

The liver is the most relevant organ for adenovirus-associated toxicity [8]. Previously, we developed a murine model of adenovirus replication associated liver toxicity [6]. Peritoneally disseminated human ovarian cancer is first inoculated and then cured by injecting Ad5/3-Δ24 and gemcitabine. However, the increase in cancer-free survival uncovered treatment-related toxicity. Many mice died due to fulminant liver necrosis caused by persistent virus replication and subsequent sustained liver damage that was exacerbated by gemcitabine. Here, this model was repeated and mice succumbed with liver necrosis, foamy degeneration and steatosis. Further,
Reduction of Adenovirus Replication

Figure 2. Reduction of replication of wild-type adenovirus (A, C) and tumor-selective oncolytic Ad5/3-Δ24 (B, D) in vitro in cancer tissues. Infectious particles were titrated on 293 cells by TCID50 assay. (A, B) Ovarian adenocarcinoma cell line Hey. (C, D) Ovarian adenocarcinoma cell line OV-4. (E) To corroborate infectious virus production with a longitudinal cell killing assay (MTS), Hey cell monolayers were preincubated with the reagents and infected with Ad5/3-Δ24.

the few surviving hepatocytes displayed large nuclei (Figure 4B). Of note, apigenin- or chlorpromazine-treated mice displayed less liver damage (Figure 4C and 4D). When all evaluable livers were analyzed in a blinded manner, liver toxicity was present more often in the PBS group compared to mice that received apigenin or chlorpromazine ($P = 0.0213$).

Heretofore, oncolytic adenoviruses have been well tolerated in cancer trials at doses up to $2 \times 10^{13}$ vp [5]. However, the most widely studied oncolytic agent is dl1520 (a.k.a. ONYX-015), which is rather attenuated even in tumor cells, when compared to wild-type adenovirus [5]. Newer generation agents replicate similarly or even more rapidly than wild-type adenovirus [5,12]. More potent oncolytic adenoviruses could mean more side effects.

The data presented here suggest that apigenin and chlorpromazine can reduce the replication of adenoviruses in vitro and in vivo which could thereby provide a safety switch in case of side effects. Compounding the need for an antidote, virus replication may persist for weeks [4], as opposed to most conventional medicines that have short halflives. Importantly, chlorpromazine has been used for decades in millions of humans and the safety and side effect profile are well understood [15]. Apigenin is present in many vegetables, such as parsley and onion, which suggests that patients participating in oncolytic adenovirus trials might benefit from dietary advice to avoid attenuation of antitumor activity. Also apigenin has been studied in clinical trials with good safety data [16]. In this study, both substances were used at concentrations previously reported to translate into...
Figure 3. Reduction of replication in vivo. (A) Human ovarian cancer (Hey) tumors (n = 10) were established in female nude mice, and treated with intratumoral (i.t.) injections of $3 \times 10^8$ viral particles (vp) of Ad5/3-Δ24 or no virus on days 0, 2 and 4. Mice received intraperitoneal (i.p.) injections of PBS, chlorpromazine (200 µg) or apigenin (250 µg) every day. Tumor volume is expressed as % of day 0 volume. (B) To evaluate virus replication, Hey tumors (n = 12) were established and treated with a single i.t. injection of $3 \times 10^8$ vp of Ad5/3-Δ24 on day 0. Mice received daily i.p. injections of PBS, chlorpromazine or apigenin. Four tumors/group were harvested at indicated time points, homogenized, and freeze-thawed three times. Infectious particles were determined by TCID$_{50}$ (n = 8). Data present mean ± SE, *$P < 0.05$ (ANOVA), ***$P < 0.0001$ (PROC MIXED, Tukey-Kramer adjusted).

Figure 4. Reduction of replication-associated toxicity in vivo. (A) Lack of weight loss suggested good tolerability. (B–D) In a murine model of adenovirus replication associated toxicity [6], reduced toxicity was seen with chlorpromazine or apigenin ($P = 0.0213$, $\chi^2$ test). $10^7$ SKOV3.ip1 ovarian cancer cells were injected i.p. into CB17 SCID mice. Ten days later, mice (n = 7–8/group) were injected i.p. with $3 \times 10^7$ vp of Ad5/3-Δ24. PBS, chlorpromazine and apigenin were administered daily. Typical histopathological appearance of the livers of (B) PBS-, (C) chlorpromazine- and (D) apigenin-treated mice (H&E, magnification ×20).

well-tolerated and active human serum concentrations [15,16]. In addition, these substances could be useful for the treatment of systemic adenoviral infections in immunosuppressed patients, particularly pediatric bone marrow transplant recipients. Intriguingly, the mechanisms targeted by these drugs are central for replication of many other types of viruses, including herpes- and parvoviruses. Therefore, this approach may have applicability beyond adenoviruses.

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