ORIGINAL ARTICLE

In vivo and in vitro distribution of type 5 and fiber-modified oncolytic adenoviruses in human blood compartments

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Abstract

Background. Successful tumor targeting of systemically administered oncolytic adenoviruses may be hindered by interactions with blood components.

Materials and methods. Blood distribution of oncolytic adenoviruses featuring type 5 adenovirus fiber, 5/3 capsid chimerism, or RGD-4C in the fiber knob was investigated in vitro and in patients with refractory solid tumors.

Results. Virus titers and prevalence in serum of patients increased over the first post-treatment week, suggesting replication. Detection of low virus loads was more sensitive in blood clots than in serum, although viral levels did not differ significantly between both sample types. While adenovirus bound to erythrocytes, platelets, granulocytes, and peripheral blood mononuclear cells in vitro, the virus was mainly detectable in erythrocytes and granulocytes in cancer patients. Taken together with a temporary post-treatment decrease in thrombocyte counts, platelet activation by adenovirus and subsequent clearance seem likely to occur in humans. Fiber modifications had limited observed effect on virus distribution in blood cell compartments. Neutrophils, monocytes and cytotoxic T lymphocytes were the major leukocyte subpopulations interacting with adenoviruses.

Conclusion. Serum and blood clots are relevant to estimate oncolytic adenovirus replication. Insight into viral interactions with blood cells may contribute to the development of new strategies for tumor delivery.

Key words: Blood, gene therapy, oncolytic viruses, pharmacokinetics

Introduction

Oncolytic adenoviruses have therapeutic potential for the treatment of solid and hematological malignancies (1,2). However, targeting the viruses to specific disease sites may be challenging because of interaction with blood components (3–5). Adenovirus may interact with blood proteins such as vitamin K-dependent coagulation factors and specific antibodies, blood cells, the vascular endothelium and is subjected to clearance by Kupffer cells in the liver (6,7). Interactions with human blood cells are particularly poorly understood, even though they may affect the bioavailability of the virus and influence adverse side-effects, including activation of the coagulation
cascade which can lead to disseminated intravascular coagulation in the most severe cases (5,8).

Few studies have been published on the pharmacokinetics of adenoviruses in human blood, and most have focused on wild-type viruses, providing sometimes contradictory results. In a pediatric population, group C type 5 adenovirus (Ad5) DNA was similarly detected in whole blood and plasma (9). In another study, group B adenovirus present in the blood of a pediatric patient was mainly extracellular (10). In contrast, more than 98% of Ad5 DNA in the blood of cancer patients was cell associated (3).

Adenoviruses have been reported to interact with all blood cell compartments. In an assessment of relative binding of Ad5 to different types of human blood cells (erythrocytes, neutrophils, and peripheral blood mononuclear cells (PBMCs)), 70%–95% of input virus remained associated with erythrocytes (3). Furthermore, binding of viral particles to these cells resulted in reduced infectivity of susceptible cancer cell lines. In another report, 90% of Ad5 incubated with human blood was retrieved from erythrocytes, and authors identified Coxsackie adenovirus receptor (CAR) and complement receptor 1 (CR1) as the cellular binding sites (4).

Thrombocytopenia has been documented following intravenous administration of adenovirus (11–16). Ad5 administered intravascularly to mice was shown to induce platelet-leukocyte aggregates mediated by von Willebrand factor (VWF) and P-selectin, resulting in platelet activation and subsequent clearance by macrophages, including Kupffer cells (5). CAR and $\alpha_{v}\beta_{3}$ integrins have been suggested to mediate attachment of adenovirus to platelets (5,8).

Group C adenovirus DNA has been identified in peripheral blood lymphocytes during fatal acute infection (17) and in immunosuppressed patients (18). In addition, adenovirus transgene expression detectable 2 to 4 hours after infection suggested that PBMCs may become infected after intravenous administration before the virus is cleared from the blood stream (3). Immune cells may also protect the virus from both neutralizing antibodies and complement, and release it in the tumor environment (19–21).

In clinical settings, kinetics of adenovirus in blood may be important determinants of virus bioavailability, treatment efficacy, and occurrence of side-effects. In this study, we investigated the blood kinetics of six different oncolytic adenoviruses in cancer patients. Viral loads and prevalence of infection were determined. Also, we compared the sensitivity of viral detection in serum versus blood clot, since this might have relevance for optimal diagnosis of adenovirus infections and obtaining reliable correlative data in adenoviral gene therapy trials. Based on blood material from cancer patients and healthy donors, we assessed viral binding to blood cells and identified leukocyte subpopulations interacting with the different adenoviruses.

Materials and methods

Viruses

The study was based on six different oncolytic viruses featuring a 24 bp deletion which abrogates the retinoblastoma (Rb)-binding site of E1A, thus
enabling tumor specificity (Table I). The E1A gene of Ad5/3-D24-Cox2L is additionally controlled by the Cox2 tumor-specific promoter, while Icovir-7 features insertion of an E2F1 tumor-specific promoter preceded by the myotonic dystrophy locus (DM-1) insulator sequence to reduce transcriptional leakage. A Kozak sequence further ensures optimized transcription. In Ad5-D24-GMCSF, Ad5/3-D24-GMCSF, and Ad5-RGD-D24-GMCSF the viral gp19k and 6.7k sequences in E3 were replaced with granulocyte-macrophage colony-stimulating factor (GMCSF) encoding sequence. Incorporation of the RGD-4C motif in the HI loop of the fiber and 5/3 chimerism retarget virus entry via CAR-independent pathways.

Virus construction, production, and titers, assessment of efficacy and toxicity in vitro and in vivo, and initial clinical results in patients have been described previously (11,15,16,22,23).

**Patients and viral treatment**

The analysis is based on 109 patients with progressive metastatic solid tumors and treated with one of the six oncolytic adenoviruses. Blood samples were collected after a single round of viral treatment at doses of \(2 \times 10^9\) to \(6 \times 10^{11}\) viral particles (VP) (11,15,16,22,23). Samples consisted of serum and blood clot collected in serum tubes, and whole blood collected in ethylenediamine tetra-acetic acid (EDTA) tubes. Written informed consent was obtained, and the study was completed according to good clinical practice and the Helsinki Declaration of the World Medical Association. Treatments were evaluated and approved by the Medicolegal Department of the Finnish Ministry of Social Affairs and Health and the Gene Technology Board, and are regulated by Finnish Medicines Agency Fimea. Viruses were produced according to the principles of good manufacturing practices.

**Incubation of viruses with blood from healthy donors**

Whole blood was collected from Red Cross donors who had approved their samples to be used for research purpose. Blood was drawn by venipuncture into EDTA or acid citrate dextrose (ACD) tubes. Ad5-, Ad5/3-, or Ad5-RGD-virus armed with GMCSF was incubated with 5.5 mL blood at 37°C for 30 minutes. Three different viral doses \(10^7, 10^8,\) and \(10^9\) VP/mL were used.

**Separation of blood cells**

Cells were isolated from 5.5 mL EDTA- or ACD-treated blood. Blood was centrifuged (180 g, 10 minutes), and plasma containing platelets was collected and further centrifuged (1750 g, 5 minutes). Plasma was removed and platelets were suspended in phosphate-buffered saline (PBS), applied on an equal volume of 44% Percoll (Sigma, Saint Louis, MO, USA) and centrifuged (400 g, 5 minutes followed by 800 g, 15 minutes). The interface between PBS and 44% Percoll was collected, resuspended in PBS, and centrifuged (1400 g, 10 minutes). Platelets were washed twice and resuspended in 800 μL PBS. The blood pellet containing erythrocytes, polymorphonuclear cells (PMNs), and PBMCs was diluted in PBS and applied on a three-layered Percoll solution (44%, 62%, and 75% Percoll). After centrifugation (2830 g, 40 minutes), PBMCs were collected at the interface between 44% and 62% Percoll and PMNs between 62% and 75% Percoll. Erythrocytes were recovered from the lowest Percoll layer. Cells were washed twice and resuspended in 800 μL PBS.

**Table I. Structure of the oncolytic adenoviruses used in cancer treatment.**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Insulator</th>
<th>E1A promoter</th>
<th>E1A</th>
<th>E3</th>
<th>Fiber feature</th>
<th>Targeted receptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5-D24-GMCSF</td>
<td>–</td>
<td>wt</td>
<td>Δ24</td>
<td>Δ3 gp19k/6.7k (\rightarrow) GMCSF</td>
<td>wt</td>
<td>CAR</td>
<td>(11)</td>
</tr>
<tr>
<td>Ad5/3-D24-GMCSF</td>
<td>–</td>
<td>wt</td>
<td>Δ24</td>
<td>Δ3 gp19k/6.7k (\rightarrow) GMCSF</td>
<td>Ad3 knob</td>
<td>Ad3 receptor</td>
<td>(23)</td>
</tr>
<tr>
<td>Ad5/3-D24-Cox2L</td>
<td>–</td>
<td>Cox2L</td>
<td>Δ24</td>
<td>wt</td>
<td>Ad3 knob</td>
<td>Ad3 receptor</td>
<td>(26)</td>
</tr>
<tr>
<td>Ad5-D24-RGD</td>
<td>–</td>
<td>wt</td>
<td>Δ24</td>
<td>wt</td>
<td>RGD in HI loop of the knob</td>
<td>αV integrins</td>
<td>(25)</td>
</tr>
<tr>
<td>Ad5-RGD-D24-GMCSF</td>
<td>–</td>
<td>wt</td>
<td>Δ24</td>
<td>Δ3 gp19k/6.7k (\rightarrow) GMCSF</td>
<td>RGD in HI loop of the knob</td>
<td>αV integrins</td>
<td>(22)</td>
</tr>
<tr>
<td>Icovir-7</td>
<td>DM-1</td>
<td>E2F1-1</td>
<td>Kozak leading sequence, Δ24</td>
<td>wt</td>
<td>RGD in HI loop of the knob</td>
<td>αV integrins</td>
<td>(27)</td>
</tr>
</tbody>
</table>

wt = wild-type; GMCSF = granulocyte-macrophage colony-stimulating factor; CAR = Coxsackie adenovirus receptor; DM = myotonic dystrophy.
Lymphocytes, monocytes, and neutrophils separations

Percoll-isolated PBMCs were separated into CD4+, CD8+, CD19+, and CD14+ subpopulations using MACS magnetic beads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Neutrophils were purified from the Percoll-separated PMNs population by means of CD16 MACS microbeads. Accuracy of cell purification was assessed by flow cytometry analysis using the following antibodies: CD4-FITC, CD8-FITC, CD19-FITC, CD14-FITC, and CD16-FITC (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Purity percentages of the isolated cells were: 94% (CD4+), 97% (CD8+), 92% (CD19+), 96% (CD14+), and 89% (CD16+).

Quantitative real-time PCR for viral genome detection

Serum, plasma, blood clots, and suspended cells were assayed for viral DNA. Blood clots were dispersed before DNA extraction using Clotspin Baskets (Qiagen GmbH, Hilden, Germany). Quantitative real-time PCR (qPCR) was based on primers and TaqMan probes targeting the E1A gene region presenting the 24 bp deletion in the oncolytic adenoviruses, thus enabling the specific detection of these viruses versus a wild-type Ad5 infection (15). Positive samples were subsequently confirmed by independent real-time PCR reactions using LightCycler480 SYBR Green I Master mix (Roche, Mannheim, Germany) and primers specific for each oncolytic adenovirus (Cox2L FW: CACGTCAG GAACTCCTCAG; Cox2L RV: CCGCCATTTCT TCGTATAA; Icovir FW: GCGGGAAGACTGA ATAAGAGG; Icovir RV: CCGGACCGGTGTGAA CTG; RGD FW: AAACACGCTGGTGGATT TATGC; RGD RV: GATGCGGCAAGAACAGTCC; GMCSF FW: AACACCAACCCTCCT TACCTG; GMCSF RV: TCATTCACTTCA CGACAGTG; Fiber3 FW: AGCGTATCCATT TGCTTCC; Fiber3 RV: GTTATAGGGTGT GCCCTAGT; Fiber5 FW: CACAAATCCCTCC TAAAACAAA; Fiber5 RV: GCCAAAACCTGAAA CTGTAGCAA). The melting curve analysis enabled viruses to be identified in the samples. In all cases, viruses detected in patient samples were the same as those used for treatment. Human beta-actin primers and probe were used for normalization to human genomic DNA in PBMCs and PMNs samples (24). DNA extraction and qPCR conditions have been described previously (15). For blood clots and blood cells, 8 μL of DNA were used as template for the qPCR.

Standard curves for viral DNA quantification in serum or in dispersed blood clot were based on DNA extracted from Ad5/3-D24-Cox2L serially diluted (10^8–10^1 VP/mL) in the respective sample type. Viral loads in blood cells were calculated using a standard curve based on pAd5easy plasmid (Stratagene, La Jolla, CA, USA) serially diluted in PBS (10^9–10^0 copies). Known amounts of human genomic DNA (800 ng-0.08 ng) were used to generate a standard curve for the beta-actin gene.

Neutralizing antibody titers

293 cells were seeded at 1 × 10^3 cells/well on 96-well plates and cultured overnight. Twenty-four hours later, cells were washed with Dulbecco’s modified Eagle’s medium (DMEM) without fetal calf serum (FCS). Serum samples were incubated at 56°C for 90 minutes to inactivate the complement, and a 4-fold dilution series (1:1 to 1:16384) was performed in serum-free DMEM. Ad5luc1, Ad5/3luc1, or Ad5luc-RGD was mixed with serum dilutions and incubated at room temperature for 30 minutes. Thereafter, cells in triplicates were infected with 100 VP/cell in 50 μL of mix, and 100 μL of growth medium with 10% FCS added 1 h later. Twenty-four hours post-infection, cells were lysed, and luciferase activity was measured with Luciferase Assay System (Promega, Madison, WI, USA) utilizing TopCount luminometer (PerkinElmer, Waltham, MA, USA). To evaluate the effect of neutralizing antibodies in serum, luciferase readings were plotted relative to gene transfer achieved with the virus alone. The neutralizing antibody titer was determined as the lowest degree of dilution that blocked gene transfer more than 80%.

Statistical analysis

Statistical significance for comparisons of continuous measures was assessed using t tests. Least-squares regression analysis was performed to examine the relationship between the levels of viral DNA detected in serum and blood clot samples presenting a viral load above the limit of quantification (≥ 500 VP/mL). McNemar’s test was used to assess the concordance of viral loads in serum and blood clot.

Results

Kinetics of circulating oncolytic adenoviruses in cancer patients

Viral shedding in blood was assessed by qPCR in 109 patients treated with one of the six oncolytic adenoviruses listed in Table I. No wild-type Ad5 was detected. Between 78.9% and 100% of patients presented circulating oncolytic virus at least at one time point after treatment. Viral loads in the serum of positive patients were highest between day 2 and day
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8 after administration, thus suggesting a peak of viral replication followed by decreased shedding into the blood stream (Figure 1). The overall prevalence of virus in patients also peaked during the first week after treatment (Figure 2). During this time-span, there was a trend for lower levels of shedding of adenoviruses armed with GMCSF in comparison to the other viruses ($P = 0.11$) (Figure 1). Ad5/3-D24-GMCSF

Figure 1. Oncolytic adenovirus loads in serum of cancer patients. Analysis is based on loads in patients tested positive for viral DNA. Samples were obtained from patients treated with a single round of virus at doses of $2 \times 10^9$ to $6 \times 10^{11}$ viral particles (VP). Viral loads were determined by quantitative real-time PCR. Loads under the limit of quantification of 500 VP/mL were extrapolated. The box plot represents the lowest load, the lower quartile, the median, the upper quartile and the highest load. All samples tested before treatment on day 0 were negative; ($n$) represents the number of positive patients in each time range. Last day of sample collection: 267 (Ad5-D24-GMCSF); 91 (Ad5/3-D24-GMCSF); 136 (Ad5/3-D24-Cox2L); 70 (Ad5-D24-RGD); 64 (Ad5-RGD-D24-GMCSF); 77 (Icovir-7). Latest day of viral detection: 35 (Ad5-D24-GMCSF); 60 (Ad5/3-D24-GMCSF); 38 (Ad5/3-D24-Cox2L); 48 (Ad5-D24-RGD); 64 (Ad5-RGD-D24-GMCSF); 28 (Icovir-7).
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and Ad5-RGD-D24-GMCSF nevertheless presented the longest duration of viremia, with viral detection up to 60 and 64 days, respectively.

Comparison of viral detection in serum versus blood clot

A total of 90 paired samples of serum and blood clot originating from patients treated with one of the six oncolytic viruses were analyzed for the presence of viral DNA. Positive or negative results in both sample types were obtained in 80 of 90 (88.9%) pairs (Table II). Among the ten discordant sample pairs, nine were positive only in the blood clot. However, the viral load in seven of these nine samples was < 500 VP/mL (lower limit of quantification). Based on McNemar’s test, detection of viral DNA was more sensitive in blood clot than in serum ($P = 0.02$).

A regression analysis applied to samples presenting a viral load greater than the quantification threshold of 500 VP/mL showed a correlation ($r = 0.93$; $P < 0.01$) between the viral loads in serum and blood clot (Figure 3A). Based on the $y$-intercept,

Table II. Serum versus blood clot detection of viral DNA in cancer patients. The analysis is based on pooled data from patients treated with Ad5/3-D24-Cox2L, Ad5-D24-RGD, Icovir-7, Ad5-D24-GMCSF, Ad5/3-D24-GMCSF, or Ad5-RGD-D24-GMCSF. The limits of detection and quantification of the real-time PCR were 500 VP/mL both in serum and blood clot.

<table>
<thead>
<tr>
<th>Viral load, VP/mL</th>
<th>Serum</th>
<th>Blood clot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood clot</td>
<td>0</td>
<td>&lt;500</td>
</tr>
<tr>
<td>0</td>
<td>16$^a$</td>
<td>–</td>
</tr>
<tr>
<td>&lt;500</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>&gt;500</td>
<td>2 ($1.1 \times 10^3$)</td>
<td>24 ($2.2 \times 10^3$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Number of samples. Values in parenthesis are mean viral loads when above the limit of quantification.
Relative distribution of oncolytic adenoviruses in blood cells from cancer patients

After adenoviruses had been extensively detected in blood clots of cancer patients, further analysis was conducted to assess the relative distribution of the viruses in the different blood cell compartments. Eight sets of samples originating from seven patients presented positive qPCR data (Table III). In four sets, the highest viral load evaluated by qPCR was found in erythrocytes, accounting for 84%–99% of the total amount of viral DNA recovered in blood cells. In three other sets, viral loads in erythrocytes were under the limit of quantification, and more than 60% of the virus was recovered from granulocytes. The discrepancy in viral distribution seemed to be independent of the fiber modifications of the viruses and of the neutralizing antibody titers against the respective viral capsid. PBMCs were less frequently positive and presented lower viral loads than granulocytes (Table III; Supplementary Figure 1). In all but one set, viral loads retrieved from platelets were negative or under the limit of quantification. The only viral load in platelets higher than the limit of quantification did not exceed $5.5 \times 10^2$ copies. The sample, collected on day 3 post-treatment, originated from a patient also tested on day 1 when most of the virus was associated with granulocytes (Table III). Thus, in cancer patients, viruses were mainly retrieved from erythrocytes and granulocytes in the cellular compartment. In all seven patients, platelet counts were decreased compared to pretreatment values (Supplementary Table I). Erythrocyte levels were also lower than base-line in the five patients tested on day 1 after treatment. In addition, the three patients treated with Ad5/3-D24-GMCSF showed a marked decrease in circulating leukocytes.

In vitro interaction of adenoviruses with blood cells

To further investigate binding properties of the oncolytic viruses according to their fiber characteristics, blood from healthy donors was incubated with Ad5-, Ad5/3-, or Ad5-RGD-virus armed with GMCSF, and viral distribution in the cell compartments was quantified by qPCR. Despite a variation in patterns of distribution between donors, the relative amount of virus associated with platelets in vitro (Figure 4A and Supplementary Figure 2) was higher than in cancer patients (Table III). With the exception of one sample set, less than 5% of virus was recovered in platelets in patients, whereas 22.2% to 47.9% of the input virus was retrieved from those cells in vitro. When results based on the three viral doses were pooled, platelet binding was significantly higher with Ad5/3-virus than with Ad5-RGD-virus ($P = 0.02$) (Supplementary Figure 2). In contrast, the proportion of viral DNA copies recovered in erythrocytes was significantly lower ($P < 0.05$) with Ad5/3-virus (8.2%) than with Ad5- (22.5%) and Ad5-RGD- (19.1%) viruses.
tubes was incubated with Ad5-, Ad5/3-, or Ad5-RGD-virus but not with Ad5-virus (Supplementary Figure 4B).

Identification of the leukocyte subpopulations interacting with adenoviruses

To assess which PBMCs subpopulation(s) are potentially relevant for virus binding, CD14+, CD4+, CD8+, and CD19+ cell fractions from the blood of healthy donors were separated after incubation of the blood with one of the three viruses armed with GMCSF. Between 67% and 76% of viral DNA was associated with the CD14+ monocytes (Figure 5A). To determine whether this association was also observed in vivo, further analysis was conducted with separated PBMCs from four cancer patients treated with the same viruses. Similarly to donors, 55% to 95% of the virus was retrieved in the CD14+ cell compartment (Figure 5B). Interestingly, high virus loads were also found in CD8+ cells in two patients (Figure 5B). To illustrate the relative affinity of adenoviruses for the different types of PBMCs, viral loads normalized to genomic DNA are presented in Figure 5C and 5D, respectively.

In the granulocyte compartment, Ad5-, Ad5/3-, and Ad5-RGD-viruses were found to interact mainly with CD16+ cells, i.e. neutrophils. Incubation of the viruses with blood from three donors showed 87%–90% of the virus associated with the CD16+ cells after normalization to genomic DNA (Supplementary Figure 5).
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Discussion

This study provides insight into the kinetics of shedding into blood of six different oncolytic adenoviruses used for the treatment of patients with solid tumors refractory to chemotherapy. Whereas one virus features the fiber of Ad5, the others present an RGD-4C motif in the fiber knob or 5/3 capsid chimerism (11,22,23,25 – 27). Three viruses also express GMCSF which is a potent inducer of anti-tumor immunity. Circulating virus was detected in 78.9% to 100% of the patients. Levels between day 2 and day 8 were up to 10^6-fold higher than 24 hours after infusion, thus suggesting replication of the virus during this time-span. Similarly, oncolytic adenovirus loads measured in previous clinical studies were highest during the first week after infusion (28 – 30).

Assessing the presence of infectious viruses in the circulation would nevertheless require further analysis, including testing samples by tissue culture infectious dose 50 (TCID_{50}) assay. Insertion of the GMCSF sequence in the genome of three viruses seemed to be associated with lower viral shedding but prolonged detectable levels up to 60–64 days post-treatment. It could be hypothesized that a lower replication rate of these viruses in the early stage of infection limits the extent of immune clearance, thus enabling prolonged replication.

Figure 4. Binding of Ad5-, Ad5/3-, and Ad5-RGD-viruses to blood cells in vitro. Viruses were incubated 30 minutes with EDTA-treated whole blood at three different viral doses (10^7, 10^8, and 10^9 VP/mL). After Percoll separation, the amount of viral DNA in blood cells was quantified by real-time PCR. The analysis is based on samples from five different healthy blood donors for each virus and dose. A: Number of viral DNA copies retrieved in each blood cell compartment. B: Amounts of viral DNA copies in granulocytes and PBMCs after normalization to genomic DNA. Error bars are mean ± SEM.
Available data on the relative distribution of Ad5 in blood cells versus plasma are not unequivocal (3,4,9). In our study, despite a high concordance of qPCR results between serum and blood clots in patients, viral DNA detection was more sensitive in blood clots. Discrepancies were seen mainly in samples with low viral loads in blood clots and undetectable viral DNA in serum. At the quantitative level, viral loads did not differ significantly between both sample types even after stratification according to the fiber features of the viruses. Although serum seems to be acceptable for the estimation of replication of oncolytic adenoviruses, analysis of clots might be preferable for sensitive detection of low titers.

Analysis of interactions between oncolytic adenoviruses and blood cells was performed both in vitro and in vivo. In cancer patients, viruses were mainly associated with erythrocytes or granulocytes. There was no apparent relationship between distribution patterns and neutralizing antibody titers or the fiber characteristics of the administered viruses. In vitro, Ad5- and Ad5-RGD-viruses were more often associated with erythrocytes than Ad5/3-virus. These observations seem to be supported by a previous report of Ad5 binding to erythrocytes via CAR and CR1 (4). Mature erythrocytes lack expression of αv integrins (31,32), known to interact with the RGD-4C motif. Nevertheless, the incorporation of this sequence in the fiber knob of Ad5-RGD-virus does not seem to prevent CAR binding (33). In contrast, retargeting via 5/3 fiber chimerism abrogates CAR interactions (34,35), thus possibly explaining the lower affinity of Ad5/3-virus for erythrocytes. In contrast with cancer patients, a high amount of virus was retrieved from platelets in vitro. The proportion of Ad5/3-virus recovered in this cell compartment was higher than that of Ad5-RGD. Whether this feature reflects indeed a higher affinity of Ad5/3 for platelets via still unidentified receptor(s) would require further investigation. In cancer patients, the absence or low amount of virus in the platelet compartment 24 hours after treatment was concomitant with a decreased thrombocyte and leukocyte count. These results might correlate with a previous observation in mice of platelet-leukocyte aggregates after intravenous delivery of Ad5, which resulted in platelet activation and subsequent clearance from the blood stream (5). Decreased leukocyte levels could also be related to recruitment of these cells to the tumor site.

Our in vitro data of virus distribution contrast with a previous report of Ad5 mainly binding erythrocytes and interacting to a minor extent with platelets (4). As data from that study were based on citrate-phosphate-dextrose (CPT)-treated blood and we used EDTA as anticoagulant, we repeated the incubation of Ad5-, Ad5/3-, and Ad5-RGD-viruses with citrate-treated blood. Less than 1% of viral DNA was retrieved from platelets, and results obtained with Ad5-virus were in agreement with the
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Identifying white blood cell subpopulations interacting with oncolytic adenoviruses is of particular interest as recent approaches of virus delivery to tumor sites have been using for example dendritic cells or T lymphocytes as carriers (20,36). We found in cancer patients that adenoviruses were mainly identified in granulocytes compared with PBMCs, which might reflect a difference in affinity for these cells. In the case of Ad5, lower binding to lymphocytes might result from low CAR expression (37), whereas interaction with neutrophils could be mediated via antibody-dependent uptake or occur in an opsonin-dependent manner via CR1 (38). In the in vitro experiment, a shift in affinity was observed at higher doses (10⁹ VP/mL) where viral loads tended to be higher in PBMCs than in granulocytes. The difference in loads between both cell types was higher in the case of Ad5/3- and Ad5-RGD-viruses than with Ad5. Our data are in parallel with a previous report of more efficient transduction of dendritic cells by Ad5/3Luc1 than Ad5Luc1 (39), which could be in agreement with reports of CD80 and CD86 as possible co-receptors for Ad3 (40,41).

In vitro, neutrophils and monocytes were identified as the major subpopulations binding oncolytic adenoviruses. Viruses retrieved from PBMCs of cancer patients were mainly associated with monocytes and, in two cases, with cytotoxic T lymphocytes. Our results are in agreement with a previous report where transgene expression by Ad5GFP was mainly observed in monocytes with less than 2% expression in CD14-negative cells of the PBMCs compartment (3). Additionally, although T lymphocytes are rarely infectable by Ad5 in vitro, Lavery et al. previously suggested that specific lymphocyte subpopulations would be more susceptible to infection and would serve in vivo as reservoir for the virus (42). Interaction of adenoviruses with these cells might therefore explain our and recently published results of viral DNA recovery in the T cell compartment (43).

In this pharmacokinetics study of oncolytic adenoviruses in cancer patients, blood clots were more sensitive than serum for detecting low virus titers. While adenovirus bound to erythrocytes, platelets, granulocytes, and PBMCs in vitro, most of the virus was found in erythrocytes and granulocytes in cancer patients. Finally, although interaction with cytotoxic T lymphocytes could be relevant in cancer patients, neutrophils and monocytes were identified as the main leukocyte subpopulations responsible for binding adenoviruses.

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Declaration of interest: Akseli Hemminki is co-founder and shareholder in Oncos Therapeutics, Inc. Timo Joensuu is joint owner of Docrates clinic. Elina Haavisto, Aila Karioja-Kallio, and Lotta Kangasniemi are employed by Oncos Therapeutics. The other authors declare no competing financial interests.

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Supplementary material available online
Supplementary Figure 1.
Supplementary Figure 2.
Supplementary Figure 3.
Supplementary Figure 4.
Supplementary Figure 5.
Supplementary Table I.