Suppression of adenoviral-induced host immune response by TGFß1 expression

Research Article

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Abbreviations: Ad, adenovirus; ELISA, standard enzyme-linked immunosorbent assay; i.t., intratumoral; i.v., intravenous; lacZ, ß-galactosidase; moi, multiplicity of infection; pfu, plaque forming units; s.c., subcutaneously; TGFß1, transforming growth factor ß 1; X-gal, ß-galactosidase substrate:5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

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Summary

Adenoviral vector (Ad) is the most commonly used viral vector in gene therapy because of its high transduction efficiency. However, Ad has its limitation due to its transient gene expression and reduced efficacy of repeated vector administration, thanks to host cellular and humoral immune responses. Although it is well known that Ad causes immune response to secondary Ad administration and thus hampers the repeated Ad-mediated gene transfer, the systemic comparison between different viral delivery routes and the optimal elapsed time between repetitive viral deliveries has yet to be extensively studied. In this study, in order to determine (i) to what extent the host immune system attenuates the function of the secondary-administered Ad; and (ii) whether transforming growth factor-ß 1 (TGFß1), an immune suppressor, would reduce this immune response, immune-competent C57BL/6 mice carrying subcutaneous prostate tumors were administrated by either intratumoral (i.t.) or intravenous (i.v.) injection of primary Ad (namely, the 1st viral administration: with either Ad expressing tumor suppressor gene p16, AdRSVp16, or Ad expressing reporter gene ß-galactosidase, AdRSVlacZ), then followed by i.t. injection of secondary Ad (AdRSVlacZ) in the presence or absence of co-delivery of Ad expressing TGFß1 (AdRSVTGFß1). Sera were purified from blood samples collected at various time points. Anti-adenoviral (anti-Ad) antibody in serum and its neutralizing ability to secondary Ad infection were evaluated. No immune response was observed in mice within 3 days after first viral injection regardless of i.t. or i.v. injection. After 7 days, mice by i.v. viral injection developed a strong immune response and this immune potency was increased over the time up to 8 weeks. In contrast, mice by i.t. viral injection had only a minor immune response at day 7, and this response waned in 14 days after viral injection. The immune response was mainly caused by native Ad proteins rather than by transgenes. Moreover, expression of TGFß1 by co-delivery of AdRSVTGFß1 with secondary AdRSVlacZ reduced anti-Ad antibody in sera and prolonged transgene lacZ expression. These results suggest that repeated administration of therapeutic Ad for solid-tumor (such as gene therapy for prostate cancer) by directly i.t. injection with reasonable intervals may provide a rational approach in a clinical setting. The addition of immune suppressor like TGFß1 may be useful to minimize the immune response to the secondary Ad challenge. These results suggest that with reasonable and justified intervals between repeated viral administration, plus the aid of immune response suppressor, local delivery of Ad vector for solid tumor gene therapy should be efficacious with no or minimal immune response.

I. Introduction

Human adenoviruses (Ad) are nonenveloped DNA viruses. There are close to 50 serotypes of Ad that have been identified, but only Ad serotypes 5 and 2 have been tested extensively in strategies for gene therapy (Berkner 1988; Graham and Prevec, 1991). Ad represents the most widely used gene transfer vehicle in gene therapy clinical trials so far because of the following reasons: (1) Ad can be easily rendered replication deficient by deletion of critical viral replication gene such as E1 (Haj-Ahmad and Graham, 1986). (2) Ad can transfer and express therapeutic genes efficiently into a wide variety of
dividing and non-dividing cells in vivo (Bett et al., 1994; Bett et al., 1993; Graham, 1990). (3) Ad has a relatively large carrying capacity for one or more therapeutic transgenes (Bett et al., 1993). (4) Ad can be grown to high titers and purified with relative ease (Graham and Prevec, 1991). However, Ad does not integrate its genome into the chromosomes of the host cells (Graham and Prevec, 1992), thus, Ad-mediated gene expression is transient and repetitive administration of the Ad may be required for effective expression of the therapeutic gene and treatment of diseases (Korst et al., 1995; Rosenfeld et al., 1992; Walter and High, 1997; Lu, 2001). The consequence of this approach is that the secondary (and the thereafter repeated viral administration) Ad-mediated gene transfer is significantly hampered. Because Ad is highly immunogenic (Yang et al., 1994), the development of Ad-specific neutralizing antibodies (i.e., anti-Ad antibody or neutralizing anti-Ad antibody) following primary administration by host immune system has a big immunological limitation for the repeated administration of the Ad (Yang et al., 1996a). Studies showed that delivery of Ad to immune-competent mice by intravenous (i.v.) (Barr et al., 1995), interperitoneal (Yang et al., 1996b), intratracheal (Bout et al., 1994), or via direct injection into the pancreas (McClane et al., 1997) resulted in the production of neutralizing antibodies and a block to repeated Ad administration.

Several strategies have been developed in attempt to overcome this problem, including modification of Ad to decrease its immunogenicity and modulating host immune responses. Modification of the Ad capsid or genome is an alternative approach that may enable circumvention of the humoral immune response against the vector (Krasnykh et al., 1996). Expression of the Ad E3 region in the Gunn rats reduced their anti-Ad humoral immune response and enabled successful repeated administration of the Ad vector (Ilan et al., 1997). Efforts have also been made to suppress host immune system when repeated Ad administration is desired. For example, partial immune ablation using cytokines or CTLA4Ig leads to persistent Ad-mediated gene expression in mouse lung and liver (Kay et al., 1995; Kay et al., 1997). Certain chemotherapy agents commonly used to treat cancer patients can suppress the host immune responses to Ad and enable repeated Ad-mediated cancer gene therapy. For example, etoposide (Bouvet et al., 1998) and cyclophosphamide (Jooss et al., 1996) have the abilities to suppress the host humoral and cellular immune responses to Ad in immune competent mice. TGFβ1 is a most important immunoregulatory cytokine (Rubtsov and Rudensky, 2007). It has an immune suppressive effect in general (Li et al., 2006) and dampens the susceptibility of dendritic cells to environmental stimulation (Ohtani et al., 2009); but its role in the regulation of T or B cell responses remains perplexing, probably due to its dependence on the type of T or B cells being regulated and their cytokine microenvironment (Banu and Meyers, 1999; Prud’homme and Piccirillo, 2000). Although TGFβ1 was reported to be able to suppress the immune response induced by virus (Lu et al., 1999a; Reinhold et al., 1999), no study has been done by systemic comparison for TGFβ1 suppression of the immune response specifically induced by Ad via i.t. and i.v. administration in a time-course manner.

In this study, the immune response to Ad in immune-competent mice by different administrations (i.t or i.v.), repeated viral administration, and the effect of TGFβ1 on attenuation of this immune response were systematically evaluated. More specifically, this study has compared immune responses against secondary Ad challenge from both i.t. and i.v. administration of primary Ad, respectively. The best administration route and optimal elapsed time to achieve the repeated Ad administration with the minimal immune response was provided. In addition, the effect of TGFβ1 on suppression of anti-Ad antibody and sustaining of transgene in secondary Ad challenge was also evaluated.

II. Materials and methods

A. Cell culture and medium

Human prostate cancer cell line PPC-1 (ATCC, Rockville, MD) were grown in PRMI 1640 medium (Cellgro, Herndon, VA) with 10% Fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT). Mouse prostate tumor cell line TRAMP-C2, which was derived from a primary tumor in the prostate of the transgenic adenocarcinoma mouse prostate (TRAMP) model (Greenberg et al., 1995), was a generous gift from Dr. N. Greenberg (Baylor College of Medicine, Houston, TX). TRAMP-C2 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Gaithersburg, MD) with 10% FBS. Human embryonic kidney 293 cells was grown in DMEM with 10% heat inactivated FBS. All cultures were grown in medium with 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco BRL) at 37°C in 5% CO2.

B. Construction of recombinant adenoviral vectors

Constructions of AdRSVlacZ (Lu et al., 1999b) and AdRSVp16 (Steiner et al., 2000) were described before. By the similar way, AdRSVTGFβ1 has been generated in which TGFβ1 cDNA gene is under the control of a Rous sarcoma virus (RSV) promoter and the inserted TGFβ1 gene has been modified so the resultant TGFβ1 protein product is an active form (Pierce et al., 1993). In addition, a control Ad carrying an unrelated gene coding for a cytochrome p450-2C9 enzyme (AdRSV2C9) was also generated. Each recombinant Ad was propagated in 293 cells and purified by twice CsCl gradient ultracentrifugation. The viral titration was performed as described before (Graham and Prevec, 1991). Viral transduction was performed at various moi in a volume of 1ml culture medium and incubated at 37°C with gentle mixing every 15 min. After 90 min incubation, the infectious supernatant was then replaced with fresh medium.

C. Viral delivery routes and repeated Ad administration

TRAMP-C2 prostate cancer cells (5x10⁶) were subcutaneously (s.c.) injected into the flank of the syngeneic C57BL/6 mice (Harlan Sprague-Dawley, Indianapolis, IN). When the tumors reached the volume of about 50 mm³, the mice were injected with 1x10⁹ plaque forming units (pfu) indicated recombinant Ad (AdRSVp16 or AdRSVlacZ) by either i.v. or i.t injection. In the Ad re-dosing (repeated viral administration) experiment, 7 days after the 1st i.t. viral administration (or 14 days after the 1st i.v. viral administration), the mice were injected with 2nd dose of indicated Ad (1x10⁹ pfu AdRSVp16 or AdRSVlacZ) either by i.v. or i.t. route. In some experiments, the
2nd dose of Ad also contained 1x10^9 pfu AdRSVTGFb1. At indicated days prior or after Ad administration, the blood was collected from mice and the serum was purified as described below.

D. Mouse blood collection and serum purification

The blood samples were collected from mice by retro-orbital bleeding puncture at indicated days either prior to or after Ad administration. A sufficient large number of mice were used, so no single mouse would be subjected to draw blood more than once in every two-week period. The serum was purified from blood by placing blood samples at room temperature for 3 hr and followed by centrifuging at 5,000 rpm for 4 min. The serum was carefully transferred to a clean tube from the blood clot and kept frozen at −70°C until use.

E. ELISA assay for measuring anti-Ad antibody

The anti-Ad antibody in mouse serum was measured by standard enzyme-linked immunosorbent assay (ELISA). AdRSVLacZ (1x10^9 pfu per well in 50 µl of 0.05 M sodium carbonate buffer, pH 9.6) was coated onto 96-well plates overnight at 4°C. After washing, the wells were blocked in 1%BSA in PBS overnight at 4°C. The following day a serial of diluted serum samples (100 µl/well) were added to the wells and incubated for 4 hr at 37°C. After washing, goat anti-mouse IgG conjugated with horseradish peroxidase (Promega, Madison, WI) was added and incubated for 2 hr at 37°C. Substrate, 3, 3′, 5, 5′-tetramethylbenzidine (Promega) was added and incubated for 10 min at room temperature. The reaction was stopped by 1M H₂SO₄ and absorbance at 450 nm (OD₄₅₀) was measured.

F. Assay for measuring effect of neutralizing anti-Ad antibody on block of the secondary Ad infection

To detect neutralizing anti-Ad antibody in the serum and its blocking effect on the secondary Ad infection, PPC-1 cells (2x10⁶/well) were plated in 96-well plate and incubated overnight. The mouse serum purified as described above was diluted in a series of concentration from 1/4 to 1/2048 in culture medium. The dilutions were incubated with AdRSVLacZ at 37°C for 1 hr. The mixture was used to infect PPC-1 cells in 96-well for 90 min at 37°C at multiplicity of infection ( moi) of 5. After replacing with the fresh medium, the cells were incubated at 37°C for 48 hr. The cells were fixed for 5 min at 4°C in 2% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde in PBS and rinsed three times with PBS. The staining reaction was performed on cells by incubating overnight at 37°C in 1mg/ml X-gal (Gibco BRL), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl₂ in PBS. The concentrations of serum dilution were recorded that gave 50% blue cells as compared to control cells that used pure AdRSVLacZ.

III. Results

A. Ad-induced immune response varies with route of viral administration

To examine and compare the immune response induced by Ad of different delivery routes and the immunological tolerance by tumor itself, the kinetics (i.e., the process of the production) of anti-Ad antibody produced in mouse serum was analyzed. The immune-competent mice were inoculated subcutaneously (s.c.) with prostate cancer TRAMP-C2 cells first to establish allograft tumors. When tumors developed to about 50 mm³ size, the mice were injected (day 0) by either i.v. or i.t. with 1x10⁹ plaque forming units (pfu) AdRSVP16, respectively. At day 3, 7, 10, 14, and 17, the blood was collected and the purified serum was used for the anti-Ad antibody ELISA assay for measuring the anti-Ad antibody titer. As shown in Figure 1, there was a minimal immune response observed within 72 hours after the first viral infection regardless of i.v. or i.t. injection. The difference between the two delivery route started to appear after one week, when i.v. injection induced a stronger immune response as reflected by the increased anti-Ad antibody in the serum (the higher OD₄₅₀ value) than i.t. injection. The Ad-induced immune response by i.v. injection kept increasing over time; in contrast, the immune response induced by i.t. injection was lagging and reached the plateau at around day 10 before starting to wane at two week after viral injection (Figure 1). These results indicated that Ad induced a lasting and significantly higher immune response by i.v. injection than by i.t. injection; the latter only caused a mild, flat and transient immune response.

B. The Ad-induced immune response is mainly caused by adenoviral proteins rather than by therapeutic genes

To analyze whether the recombinant AdRSVP16-induced immune response was mainly caused by Ad genomic proteins or by the transgene p16, we replaced AdRSVP16 with another Ad, AdRSVLacZ, for the same i.t. experiment. AdRSVLacZ shares the exactly same Ad genome framework as AdRSVP16 except that it carries a different transgene. With the same dose of either AdRSVP16 or AdRSVLacZ by i.t. injection, a similar level and time-course pattern of immune response were observed between these two Ads: As representative shown in Figure 2, at both day 3 and 7 after the viral administration, AdRSVP16 and AdRSVLacZ caused similar levels of anti-Ad antibody in sera. This result indicates that the Ad-induced immune response is mainly caused by the native Ad proteins.

C. Blocking effect of neutralizing anti-Ad antibody on 2nd Ad infection

To correlate the anti-Ad antibody titer (Ad-induced immune response) with the actual inhibition of the repeated Ad administration, we performed neutralizing anti-Ad antibody assay that measured the blocking effect of anti-Ad antibody in serum on the secondary Ad infection. Immune competent mice were first inoculated with TRAMP-C2 cells to establish s.c. allograft prostate tumor. When the tumors reached the volume of about 50 mm³, the first two groups of mice were i.t. injected with 1x10⁹ pfu AdRSVLacZ and AdRSVP16 per tumor, respectively; the third group of mice were i.v. injected into the tail vein with 1x10⁹ pfu AdRSVP16. At the day prior to Ad administration and at day 3, 7, 14, 28, and 56 after Ad administration, respectively, the blood samples were collected from mice and the sera were purified as described above. This assay (Table 1) showed that i.v.
injection caused significantly stronger immune response compared with that of i.t. injection. For example, the levels of neutralizing anti-Ad antibody that was able to inhibit the function of the 2nd Ad (as presented by 2nd AdRSVlacZ infection on PPC-1 cells --- the intensity of blue cells after X-gal staining) from serum on day 14 after i.v. injection is more than 16-fold higher (1/64 dilution vs 1/4 dilution, the row of 2 weeks, Table 1) than its counterpart of i.t. injection, demonstrating the presence of a much higher titer of neutralizing anti-Ad antibody in the serum of mice administrated by i.v. viral injection.

Also as shown in Table 1, the titer of neutralizing anti-Ad antibody by i.v. injection that gave 50% inhibition on the 2nd Ad infection was about 1:32-1:64 dilution at one week after administration. Then, it kept increasing over time up to 8 weeks after viral administration, reaching 1:516 dilution (the more diluted serum, the higher potency of its anti-Ad neutralizing ability). In contrast, for all the groups administrated by i.t. route, all the counterpart titers were low, even the most concentrated dilution (1:4 dilution) gave no or much less inhibition on 2nd AdRSVlacZ infection on PPC-1 cells. The only detectable “peak” that was able to inhibit 50% of the 2nd Ad infection was between 1:4-1:8 and 1:8-1:16 dilution (3rd row, 1 week, Table 1). This inhibitory ability waned in two weeks after viral administration and thereafter. These results indicated, by comparing to those from i.v. injection, that i.t. injection gave much lower titer of neutralizing anti-Ad antibody and mild immune response to interfere with the function of the 2nd (repeated) administrated Ad.

Also noticeably, the titer of neutralizing anti-Ad antibody is not associated with which kind of transgene (i.e., p16 or LacZ) it is, as both AdRSVp16 and AdRSVlacZ gave similar range of titer (Table 1); therefore, these results again demonstrated that Ad-induced immune response is mainly caused by Ad proteins rather than by transgenes. Together with data from Figure 1 and Table 1 which also suggests that tumor tissue itself may develop immunological tolerance, these combined results suggest that directly intratumoral injection (i.e., intraprostatic injection in a clinical setting) of Ad at a reasonable interval (i.e. after every 10-14 days) should be considered as a rational approach of local delivery of viral vectors for cancer gene therapy.

Table 1: Fold of dilutions of Ad neutralized antibody from the first adenoviral infection that gave 50% inhibition of the secondary AdRSVlacZ infection

<table>
<thead>
<tr>
<th>Time of serum collection before and after the 1st viral infection</th>
<th>Route of first adenoviral administration</th>
<th>Route of second adenoviral administration</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>intravenous (i.v.) injection of AdRSVp16</td>
<td>intratumoral (i.t.) injection of AdRSVlacZ</td>
</tr>
<tr>
<td>Pre-immune</td>
<td>none*</td>
<td>none</td>
</tr>
<tr>
<td>72 hours</td>
<td>&gt;1/4***</td>
<td>1/4 – 1/8</td>
</tr>
<tr>
<td>1 weeks</td>
<td>1/32 – 1/64**</td>
<td>&gt;1/4</td>
</tr>
<tr>
<td>2 weeks</td>
<td>1/64</td>
<td>&gt;1/4</td>
</tr>
<tr>
<td>4 weeks</td>
<td>1/64 – 1/128</td>
<td>&gt;1/4</td>
</tr>
<tr>
<td>8 weeks</td>
<td>1/516</td>
<td>&gt;1/4</td>
</tr>
</tbody>
</table>

Immuno-competent BL57/c mice were inoculated with mouse prostate cancer cell line Tramp C2 (5×10⁶ cells/per mouse) to establish the subcutaneous tumors. When tumor became 50 mm³ in size, 1×10⁹ pfu adenovirus (AdRSVp16 or AdRSVlacZ, the first viral infection) was injected either intravenously (i.v.) or intratumorally (i.t.) to each mouse. The blood samples were collected before (pre-immune) and after (at 72 h, 1, 2, 4, 8 weeks) adenovirus administration. Serial dilutions of the serum purified from blood were preincubated with AdRSVlacZ at 37°C for 1 h, then the treated adenoviral vectors (AdRSVlacZ) were used to transduce human prostate cancer cell line PPC-1 (at moi=5) in 96-well plate. The PPC-1 cells were fixed and stained with X-gal 48 h post AdRSVlacZ transduction (i.e., secondary infection) and the blue cells were counted under light microscope. (The lower dilution (i.e., 1/516), the higher potency of the neutralized antibody to inhibit the 2nd Ad infection).

*All pre-immune sera had no inhibition for the secondary AdRSVlacZ infection (shown as none), that is, serum without any dilution still gave no inhibition of the secondary AdRSVlacZ’s infection on PPC-1 cells. **The fold dilution of mouse serum (Ad neutralized antibody) that gave 50% inhibition to the secondary Ad infection (AdRSVlacZ on PPC-1 cells) is indicated. For example, 1/32 means a serum dilution of 1:32. The 50% inhibition was determined by comparing each well for the positively stained blue cells to the control PPC-1 cell wells that were transduced with untreated AdRSVlacZ (i.e., the AdRSVlacZ without serum). ***Any dilution more concentrated than 1:4 fold dilution (i.e., >1/4) is considered to contain no or minimal anti-Ad neutralized antibody.
D. Anti-Ad antibody titer and TGFβ1 effect on anti-Ad antibody production in the repeated Ad administration

Because it was reported that 2nd administrated Ad is eliminated by the host mainly because of the existing neutralizing anti-Ad antibody (47), it was important for us to examine the existence and extent of anti-Ad antibodies after viral re-dosing, which directly correlates to the immune response induced by the 2nd Ad administration. We were also interested in examining whether TGFβ1 has a suppression effect on host immune response towards the 2nd administrated Ad. To accomplish these goals, immune competent mice were administrated with the 1st Ad and then re-dosed with 2nd Ad (AdRSVlacZ) with and without AdRSVTGFβ1, a recombinant Ad expressing an active form of TGFβ1 protein.

To determine the kinetic immunological responses after repeated Ad administration, we first examined the time-course immune response from mice that had been injected with Ad by i.t. route on both 1st and 2nd viral administration. C57BL/6 mice were immunized by i.t. injection of 1x10⁹ pfu AdRSVP16 first, one week later, a 2nd dose of (repeated administration or re-dosing) i.t. Ad administration was performed (day 0) by either using 1x10⁹ pfu AdRSVlacZ alone, or 1x10⁹ pfu each of AdRSVlacZ and AdTGFβ1, or 1x10⁹ pfu each of AdRSVlacZ and AdRSV2C9 (a control Ad carrying an unrelated cDNA transgene). On day 3, 7, 10, 17 and 24 after the 2nd viral administration, sera were purified from blood and the levels of anti-Ad antibody were evaluated by ELISA assay. As shown in Figure 3, even with AdRSVlacZ alone as a 2nd viral administration by i.t. route, the levels of anti-Ad antibody after the 2nd Ad administration were significantly higher and more persistent than that induced by the 1st Ad administration by i.t. route (compare the OD₄₅₀ values shown here) in Figure 3 with i.t. route in Figure 1, demonstrating that the repeated Ad administration, even by i.t. route, caused a high level of anti-Ad antibody; and consequently, this resulted in a higher immunologic response. Moreover, although all three groups showed elevated levels of anti-Ad antibody in serum over the time after the 2nd Ad administration, the group containing AdRSVTGFβ1 had a significantly lower level of anti-Ad antibody compared to the other two groups without AdRSVTGFβ1. For example, on day 17 after Ad re-dosing, the level of anti-Ad antibody (OD₄₅₀ value) in the group with co-administration of AdRSVlacZ and AdTGFβ1 is only 50% of the group with co-administration of AdRSVlacZ and AdRSV2C9, and about 56% of the group with AdRSVlacZ alone (Figure 3), suggesting that TGFβ1 is able to suppress the production of anti-Ad antibody. To demonstrate that the TGFβ1-mediated reduction of anti-Ad antibody production is a general phenomenon rather than specific to certain dilution of sera for the ELISA assay (such as 1:32 dilution we used in the assay of Figure 3), we randomly selected a time point (day 10), made a series of sera dilution on day 10’s sample, and then performed anti-Ad antibody ELISA assay for each dilution. As shown in Figure 4, all sera dilutions consistently demonstrated that TGFβ1 expression

![Figure 1: Kinetics of anti-Ad antibodies in serum. Immune-competent mice C57BL/6 were s.c. inoculated with prostate cancer cell line TRAMP-C2 (5x10⁶ cells/per mouse) to establish the allograft tumors. When tumors became 50 mm² in size, 1x10⁹ pfu AdRSVP16 were injected by either i.v. or i.t. route into each mouse (n=5 in each group), respectively. At the indicated times after viral administration, blood was collected and the serum was purified. The levels of anti-Ad antibody in serum were measured using anti-Ad antibody ELISA assay as described in M&M section. The OD₄₅₀ values shown here are from serum dilution 1:32.](image1.png)

![Figure 2: Comparison of anti-adenovirus antibodies in serum induced by different transgenes. Immune-competent mice C57BL/6 were inoculated with TRAMP-C2 (5x10⁶ cells/per mouse) to establish the subcutaneous tumors. When tumors became 50 mm² in size, 1x10⁹ pfu AdRSVlacZ or AdRSVP16 was i.t. injected to each mouse (n=5 in each group), respectively. At the indicated times after viral administration, blood was collected and the serum was purified. The levels of anti-adenovirus antibodies in serum were measured using anti-Ad ELISA assay. The OD₄₅₀ values shown here are from serum dilution 1:32. The differences between AdRSVlacZ and AdRSVP16 groups at both day 3 and 7, respectively, are statistically insignificant (P>0.05).](image2.png)
reduced levels of neutralizing anti-Ad antibody after the 2nd i.t. Ad administration. Therefore, it is appropriate and representative to show ELISA assay results at 1:32 sera dilution. In addition, the level of lacZ transgene expression, as examined by X-gal staining of the tumor sections on day 7 after the 2nd viral administration, was stronger in mice with co-administration of AdRSVlacZ and AdTGFβ1 than that with co-administration of AdRSVlacZ and AdRSV2C9 (not shown). Thus, expression of TGFβ1 in repeated Ad administration is useful for attenuating neutralizing anti-Ad antibody and enhancing the effect of the 2nd therapeutic Ad for repeated viral administration.

Figure 3: Immune response of mice to repeated i.t. viral administration in the presence and absence of TGFβ1. Immune-competent mice C57BL/6 were inoculated with TRAMP-C2 cells (5×10⁶ cells/per mouse) to establish the subcutaneous tumors. When tumors became 50 mm² in size, all the mice were i.t. injected with 1×10⁹ pfu AdRSVp16. Seven days after the 1st viral administration, the mice were divided into three groups (n=5 for each group): group 1 mice were i.t. injected with 1×10⁹ pfu AdRSVlacZ alone to each mouse; group 2 mice were i.t. injected with 1×10⁹ pfu each of AdRSVlacZ and AdRSVp16; group 3 mice were i.t. injected with 1×10⁹ pfu each of AdRSVlacZ and AdRSV2C9, an adenovirus expressing an unrelated gene product cytochrome P450 (as a control in the absence of AdRSVp16). At the indicated times after the 2nd viral administration, serum were collected. The levels of anti-Ad antibody in serum of these mouse groups were measured using anti-Ad antibody ELISA assay. The OD₄₉₀ values shown here are from serum dilution 1:32 from each day. *The differences between the group with co-administration of AdRSVlacZ plus AdRSVp16, and the other two groups, respectively, are statistically significant (P<0.05).

E. Suppression of Ad-induced immune response by TGFβ1 in repeated Ad administration

To determine the immunological responses after repeated Ad administration by both i.v. route, C57BL/6 mice were immunized by i.v. injection of 1×10⁹ pfu AdRSVp16 first, two weeks later, a 2nd dose i.v. administration was performed (day 0) by either using 1×10⁹ pfu each of AdRSVlacZ and AdTGFβ1, or 1×10⁹ pfu each of AdRSVlacZ and AdRSV2C9, respectively. All immunized mice in the latter group died 3 days after 2nd i.v. Ad administration, indicating a stronger immune response that caused the death of the mice. In contrast, all mice i.v. injected with 2nd dose of Ad in the presence of AdRSVTGFβ1 (i.e., AdRSVlacZ plus AdRSVTGFβ1) survived for the entire experimental process. On day 3, 7, 10, 17 and 24 after the 2nd viral administration, blood samples were collected from this survived mouse group and sera were purified, and the levels of anti-Ad antibody were evaluated by ELISA assay. As expected, the level of neutralizing anti-Ad antibody was high after the 2nd i.v. viral administration (Figure 5). These results demonstrate that TGFβ1 can partially suppress the production of neutralizing anti-Ad antibody primed by i.v. Ad re-dosing, while preventing the severe immunological-response consequences. While it is difficulty to tell whether TGFβ1 has more suppressive effect on immune response by i.t. route versus i.v. route after repeated Ad administration (as all mice in two consecutive i.v. Ad administration in the absence of TGFβ1 died so comparison cannot be made between groups with and without TGFβ1 presence), it appears practical to apply repeated Ad administration by i.t. route, as even both in the presence of TGFβ1, the immune response elicited by i.t. route was significantly lower that that by i.v. route (Figure 5). These results suggest that TGFβ1 may be useful as an immune suppressive agent in repeated administration of Ad by i.t. route.

Figure 4: Effect of TGFβ1 on suppression of anti-Ad antibody production in i.t. Ad delivery route. Blood samples were collected and sera were purified from the three mouse groups as described above in Figure 3 legend. The levels of anti-Ad antibody in serum collected were measured using anti-Ad antibody ELISA assay. The representative anti-Ad antibody titer (serum dilution) is shown for the day 10 after the 2nd i.t. viral administration. In brief, the sera were diluted in D-MEM medium in two-fold steps starting from 1:8 dilutions, these dilutions were then applied to AdRSVlacZ-coated 96-well plates, and the plates were incubated at 4°C overnight. The OD₄₉₀ values for each serum dilution were measured following standard anti-Ad antibody ELISA assay.
An important and yet not solved issue in gene therapy is the host immune response against the viral-mediated gene transfer, especially when an Ad vector is used (Yang et al., 1994). Ad does not integrate into the host cell chromosome and therefore the therapeutic transgene expression is transient (Dong et al., 1996). Even in an immune-competent nude mice model, Ad-mediated expression of lacZ reporter gene in a human prostate xenograft tumor waned significantly within two weeks after i.t. viral injection (Lu, 2001). Together with the fact that not 100% of prostate cancer cells comprising the tumor are transduced at one time and a high-dose associated toxicity of Ad, the repetitive administration of Ad is therefore required to achieve a sustained and effective gene therapy of cancer. However, repeated exposure to viral antigen could provoke immune reaction of host that may prevent subsequent transductions by Ad vectors. In order to overcome these problems and reduce host immune response that rejects the repeated administration of therapeutic Ad, three main strategies are used. First, to modify the vectors in a way to sustain the transgene expression so that there is no need for repeated administration. Second, to modify the vectors in order to decrease their immunogenicity. Third, to tame the host immune response to viral antigens. In an attempt for the first strategy, aspects of different vectors are combined to achieve stable genetic transduction and efficient delivery in vivo. An adenoviral-retroviral chimeric vector system has been developed that takes the advantages of both adenoviral and retroviral vectors, that is, the high in vivo transduction efficiency rendered by adenovirus and stable integration of transgene into the host genome rendered by retrovirus (Feng et al., 1997). In an attempt for the second strategy, antigenic viral proteins are eliminated by deleting viral genes. For example, a helper-virus independent adenoviral vector with multiply deletion of E1, polymerase, and preterminal protein, has been developed and it exhibited a capability of long-term gene transfer in vivo together with a significantly reduced hepatic toxicity (Hodges et al., 2000). The third strategy includes suppression of host immune system by co-administration of an immune-suppressing agent or viral cytokines to induce tolerance to viral antigens, or abrogation of the T cell function with antibodies. Ad-mediated expression of CTLA4IgG, which blocks the CD28-B7-mediated costimulatory signal and consequently inhibits T cells activation and prevents T cells-mediated immune responses (Nakagawa et al., 1998), or direct CTLA4IgG administration (Kay et al., 1995; Kay et al., 1997) effectively suppressed anti-Ad immunogenicity and prolonged Ad-mediated gene expression.

Although it is well known that Ad causes immune response to secondary Ad administration and thus hampers the repeated Ad-mediated gene transfer, the systemic comparison among different viral delivery routes and the optimal elapsed time between repeated viral deliveries have not been extensively studied before. This study has compared immune responses against secondary Ad challenge from i.t. or i.v. administration of primary Ad. The best delivery route and optimal elapsed time between viral injections, which sustain the transgene expression and elicit the minimal immune response was provided. In addition, the effect of TGFß1 on suppression of anti-Ad antibody and sustaining of transgene in secondary Ad challenge was also explored.

The previous studies by other groups indicated that development of the humoral response to Ad injection is dependent on the route and dose of administration (Gahery-Segard et al., 1997; Chen et al., 2000). Chen et al. (Chen et al., 2000) have determined the minimal dose of Ad vector required to elicit the detectable production of neutralizing antibodies when delivering the Ad expressing luciferase (AdRSV.L) by the intramuscular route. By using Ad doses ranged from $10^2$ to $10^{10}$ pfu, neutralizing antibodies were not detectable in mice immunized with less than $1 \times 10^7$ pfu Ad. The first evidence of neutralizing antibody production was at a dose of $1 \times 10^7$ pfu of AdRSV.L; this was also the minimum dose where detectable luciferase expression was observed in muscle tissue. An immunizing dose of $10^7$ pfu AdRSV.L resulted in an increase in both luciferase expression and production of neutralizing anti-Ad antibody (Chen et al., 2000). Based on their results, we have selected $1 \times 10^7$ pfu as dose of Ad administration in our study.
Our anti-Ad antibody ELISA assay (Figure 1) and anti-Ad neutralized antibody assay (Table 1) consistently showed that i.v. route of viral administration caused a much higher host immune response than i.t. route, although this difference is marginal within 72 h after the first viral injection. However, the immune potency caused by i.v. injection kept increasing over time, whereas the level of neutralizing anti-Ad antibody elicited from i.t. injection reached plateau at around at day 10 and maintained an overall much weaker immune response. This observation was consistently reflected in the ability of anti-Ad neutralized antibody in the sera to inhibit second Ad infection (Table 1): Transgene (LacZ) expression by AdRSVlacZ in the presence of anti-Ad neutralizing antibody decreased remarkably with the increase of neutralizing antibody (Table 1), demonstrating that the immune response (level of neutralizing anti-Ad antibody) caused by i.t. Ad administration was weaker than that by i.v. route (Table 1). Several studies suggest that tumor tissue itself may develop immunological tolerance (Hay N, 2005; Ermolaeva et al., 2008; Essers et al., 2009). Taken together, these results suggest that directly intratumoral (or intraprostatic situation in the clinical setting) injection of Ad at a reasonable interval (i.e., after every 10-14 days) should be considered as a rational approach for solid tumor (such as prostate cancer) gene therapy.

The importance of TGFβ1 in immune regulation and tolerance has been increasingly recognized (Li et al., 2006). TGFβ1 is a potent regulatory cytokine with diverse effects (Rubtsov and Rudensky, 2007). The pivotal function of TGFβ1 in the immune system is to maintain tolerance via the regulation of lymphocyte proliferation, differentiation, and survival (Li et al., 2006). Although TGFβ1 is known to have multiple suppressive actions on T cells, B cells, dendritic cells, and macrophages (Greulich and Flavell, 2002; Ahmadzadeh and Rosenberg, 2005; Marie et al., 2005; Thomas and Massague, 2005; Li et al., 2006; Marie et al., 2006; Otani et al., 2009), the exact mechanism of TGFβ1’s suppression of Ad-induced immune responses remains to be defined. Several previous studies showed that exogenous expression of TGFβ1, either by naked DNA or in Ad-mediated gene transfer, results in tolerance of host immune system by decreasing production of immunological cytokines and increasing production of endogenous regulatory cytokines (D’Ovidio et al., 1999; Chan et al., 2000). It is likely that TGFβ1 suppresses Ad-induced immune response by blocking B cell activation and regulating other immune response components such as T cells, macrophages, Dendritic cells and natural killer cells (Li et al., 2006; Marie et al., 2006; Otani et al., 2009).

Based on the reports that TGFβ1 can suppress the immune response induced by virus (Lu et al., 1999a; Reinhold et al., 1999), we have analyzed whether TGFβ1 can suppress the immune response specifically to the repeated administrated Ad. Our results have shown that administration of AdRSVTGFβ1, which expresses an active form of TGFβ1, can lower the level of anti-Ad neutralizing antibody after second Ad administration and prolongs the expression of transgene carried by Ad. Remarkably, the prevention of death by co-delivery of AdRSVTGFβ1 in mouse group challenged with two consecutive i.v. injection of Ad demonstrates that TGFβ1 has a suppressive effect on the Ad-induced immunological response: The immune response by two consecutive i.v. injection of Ad was so strong that all mice died in that group; however, in the presence of TGFβ1 the mice survived the two consecutive i.v. injection of Ad. Moreover, the effect of TGFβ1-mediated suppression on the production of neutralizing anti-Ad antibody in our study was probably underestimated—due to the fact that we used AdRSVTGFβ1 to express TGFβ1 protein. AdRSVTGFβ1, as an Ad itself, would also elicit immune response. While the ideal delivery of TGFβ1 should be in a less immune-prone manner such as co-delivery of TGFβ1 protein form (rather than as AdRSVTGFβ1) to the mice, this study has served as a “proof of concept” to demonstrate that immune response suppressor, such as TGFβ1, can be a great adjuvant for gene therapy involving repeated Ad administration in order to attenuate the viral-induced host immune response and thus boost the therapeutic effects.

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References
Lu et al: Study of immune response against, and TGFβ1 effect on secondary adenoviral vector administration


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