The role of coagulation factors VII, VIII, and IX on the tropism and inflammatory effect of Adenovector on liver and breast cell lines

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Abstract

Introduction: Adenovectors are promising vectors for gene delivery to the target cells. During gene therapy, AdV interact with plasma components particularly vitamin K-dependent coagulation factors. In this study, we analyzed the comparison between cell entrance, inflammatory patterns, and innate immune induction which induced by the Adenovector and Adenovector coated by FVII, FVIII, and FIX on two cell-lines; HepG2, MCF7.

Methods: The Adenovector expressing GFP (AdV_{GFP}) was prepared and then loading of AdV by coagulation factors were analyzed by zeta potential measurement. The non-toxic MOI of vector employed alone or in complex with coagulation factors VII, FVIII, and FIX applied on HepG2 and MCF7 cell lines. The transduction rates of complexes were analyzed by fluorescent microscopy and flow cytometry. The expression levels of innate immune genes (PKR, STING, IRF-3 and MX-1) were measured by Real-time PCR. Also the level of IL-6 and IL-1 β were evaluated by using ELISA assay.

Results: The loading of Adenovector by FVII and FIX decreased the zeta charge of the complex particles and enhanced the entry rate in both HepG2 (FVII/AdV: 38.3% and FIX/AdV: 61.9%) and MCF7 (FVII/AdV: 31.2% and FIX/AdV: 36.6%). The expression of IL-6 cytokine enhanced when AdV exposed to FVII (P value: 0.005) in MCF7 and also FVII (P value: 0.01) and FIX (P value: 0.009) in HepG2. Adenovector coated by FVII and FIX could significantly alter the expression pattern of innate immune genes.

Conclusion: The findings are highlighted the role of FVII and in particular FIX in facilitating the entry of vector into the cells; also they are enhanced the inflammation and innate immune responses. Interestingly, FVIII had no effect or even adverse effect on entry, inflammation, and innate immune induction.

Introduction

For centuries viruses have evolved naturally to deliver their genome to cells with high efficiency. Adenovirus vector (AdV) is one of the most used gene-therapy platforms because it has remarkable efficiency of transduction, high amount of gene expression, scaling-up potency in industry and large capacity for transgenes (1). By administrating AdV intravascularly, > 99% of AdVs are accumulated in the liver due to the high affinity to hepatocytes (2, 3). More importantly, the tropism of the AdV could be affected by plasma components such as antibodies, complement, and especially coagulation factors. When Adenovector is injected into the blood, vitamin K-dependent coagulation factors such as FVII and FIX and FX shield the vector and alter the tropism to target cells (4). Historically, in 2005, the old accepted model of adenovirus entrance which was a CAR-dependent pathway was challenged. In this pathway, adenovirus could not infect hepatocytes. The new pathway, preliminarily, has been called "coagulation-factor dependent" and indicates the role of FIX and complement factor C4BP in the tropism of Adenovirus toward hepatocytes (5, 6). Then, other studies suggest the role of other coagulation factors such as FX and FVII on AdV tropism (7, 8). It is interestingly demonstrated that FX has more responsibility like

protection of AdV from blood natural antibodies. Thus, the vector may properly achieve the hepatocytes and be transduced (9). Recently, Ghahestani et al. demonstrated that FVII and particularly FX can leave impacts on the rate of virus entry and the amount of induced innate immunity (10). Therefore, molecular studies about the exact interactions of vectors give us a better perspective on vector biodistribution.

As the prevalence of Adenovirus is wide among the human population, anti-Adenoviral neutralizing antibodies act as a barrier to achieve efficient transduction. Moreover, intravenous injection of AdV might result in lethal consequences such as complement activity, cytokine storm, and induction of inflammatory cascade. Innate immune response to AdV might be an important reason for the failures among usage of AdV in clinical trials (11–13). The antibody-opsonized AdV could be taken up by innate immune cells via FC-receptor mediated pathway. This way, innate immune responses would be enhanced (14). The elevation of innate immune gene expressions such as TNF- α , MIP-2, and IP-10 follows from administering AdV intravascularly (15). One of the most studied innate immune pathways induced by AdV is TLR/NF κ B. Both TLR-dependent and TLR-independent pathways are leading to type 1 interferon production (16). Sayaka Tsuzuki et al. demonstrated that the enhancement of type 1 interferon after adenovirus injection results in activating TLR9/MyD88-dependent and independent pathways (17). Although the E1 and E2A mutated AdV induce less inflammation, there is still a long way to improve vectors that cause no inflammation (12).

Although several studies have been done to put the spotlight on immune induction of AdV, yet there are still information gaps available to fill out. It is still unclear how exactly AdV influences the inflammation and what is the responsibility of the coagulation factors in this process. In this study, the correlation between AdV-coagulation factors VII, VIII, IX complexes and innate immunity in hepatocyte cell-line (HepG2) and also breast cancer cell-line (MCF-7) were evaluated for the first time in order to give a better perspective of AdV targeting in gene therapy.

Materials And Methods

Cell culture

Both HepG2 and MCF-7 cells (obtained from Iranian Biological Resource Center, Tehran, Iran) were cultured in Dulbecco's Minimal Eagle Medium (DMEM) supplemented with 12% Fetal Bovine Serum, 2 mM L-glutamine, 100 units penicillin/mL and 100 mg/mL streptomycin which known as complete MDem. Both cell-lines were adherent cells which detached by trypsin/EDTA and they were grown at 37°C in a humidified atmosphere of 5% CO₂.

Adenovector preparation and titting

Adenovirus type 5 with the GFP reporter gene (AdV_{GFP}) which was used in this study has been propagated and purified as described before (18). In order to determine the AdV_{5GFP} concentration TCID₅₀ was employed on Ad293 cells. In addition, to get an optimal concentration for experiments different MOI of the vector was evaluated in MCF-7 and HepG2 cell lines. Therefore, these two cell lines

were separately seeded in 96 well plates and then dilutions of vector were added and cell death (trypan blue exclusion test) and typical cytopathic effect was thoroughly monitored for 48hrs.

ZETA potential measurement

So as to determine whether the mentioned coagulation factors shield the AdV properly we measured the electrostatic potential of each complex. The Zeta potential of vector particles alone or in combination with coagulation factors were determined by ZETA-check (Microtrac, Meerbusch, Germany) as described before (18). The test repeated four times; however, the first assessment omitted due to the possibility of inhomogeneity. Thus, the mean of the three round tests considered as the final data.

In vitro transduction of coagulation factor loaded Adenovectors

Three clotting factors VII, VIII, and IX were kindly provided by Dastgheib hospital Children Unit. Coagulation factors VII (FVII, AryoSeven™) and VIII (FVIII, Saman daroo™) were recombinant human factors whilst coagulation factor IX (FIX, Biotest™) was human purified factor. The Physiological concentration of FVII, FVIII, and FIX are 0.5µg/mL, 100ng/mL, and 5µg/mL, respectively (7, 19). All three factors were solved in injection water and stored at -70°C. In order to prepare the vector-coagulation factor complexes, coagulation factors at physiological concentration were added to the vector in 50µl PBS and incubated in 37°C for 1 hour. Two mentioned cell-lines seeded the day before to reach 90% confluency for transduction. Serum-free DMEM replaced to the complete media and complexes added smoothly to the wells. Totally, there were 5 groups including AdV solely, AdV/FVII, AdV/FVIII, AdV/FIX, and control negative which were cells without receiving anything more. At the end of the transduction, plate was incubating at 37°C in 5% CO₂ incubator. In order to perform ELISA and real-time PCR assays, plates were incubated for 48 hours and 6 hours, respectively.

Transduction rate analysis by fluorescent microscopy and Flowcytometry

We exposed seeded cell-lines to AdV_{GFP} and AdV/coagulation factors VII, VIII, and IX for 48h prior to GFP analysis. It is obvious that for negative control non-infected cell in the plate was needed. Firstly, we used fluorescent microscopy in order to subpopulation of bright transduced cells. Afterwards, flow cytometry assay employed to characterize these cells. The exposure time of the camera was 10.587 ms and 38.17 ms for the HepG2 and MCF-7 cell lines, respectively. For capturing the pictures, sensitivity of the CellSense software was set up to ISO 100. Flow cytometric analysis was used for the better evaluation of the transduction using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Each sample washed three times in PBS and collected by centrifuge (950RPM, 1min). 100µl of each sample was diluted in 200µl PBS then 10,000 cells were calculated and analyzed by flow cytometry. GFP expression seen just at one gated cell population. The data analyzed using FlowJo V10 (Tree Star, Ashland, OR, USA).

Real-time Polymerase chain reaction

Total RNA was extracted by RNX-Plus solution (Sinaclon Inc, Iran) and quality of the RNAs was observed by gel electrophoresis. Afterwards, quality and quantity of each sample measured by using a Nanodrop spectrophotometer (Nanodrop; Thermo Fisher Scientific, Wilmington, DE, USA). Proper RNAs stored at -70°C freezer. To assess validity of viral transduction, extracted RNAs were subjected to RT-qPCR. Reverse transcription was performed by EasyTM cDNA synthesis kit (Parstous Inc, Iran) according to the manufacturer's instructions. The input RNAs for cDNA synthesis were 1 mg. The synthesized cDNAs were used as templates for RT-qPCR assay. Real-time polymerase chain reaction was performed using the Step one system and the RealQ Plus 2× Master Mix Green (Ampliqon A/S, Odense, Denmark). 4 specific innate immunity mediator genes which assessed were *PKR*, *MX-1*, *IRF-3*, *STING1* and internal control was *PGK* for Real-time PCR normalization. All PCRs were performed like the following procedure: 95°C for 15 min followed by 40 cycles of 95°C for 15 s, proper annealing temperature (showed in table 1) for 1 min, and an extension at 72°C for 30 s. Melting curves verify the quality of each sample. Finally, fold changes were calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1 The primers used in the Real-time PCR assay. The table shows 4 genes which evaluated in our study and the reference gene which was PGK

genes	Sequences (5'-3')	Annealing temperature
PKR	F:ACGCTTTGGGGCTAATTCTTG R:CCCGTAGGTCTGTGAAAACTT	58°C
MX-1	F:TAATAAAGCCCAGAATGCCATC R: ATGAGTGTCTTGATCTTATACC	57°C
IRF-3	F: GTGATGGTCAAGGTTGTG R: GTGGCTGTTGGAAATGTG	57°C
STING-1	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	62°C
PGK (ref)	F : TAAAGCCGAGCCAGCCAAAA R : CTCCTACCATGGAGCTGTGG	60°C

Cytokine analysis

The secretion of inflammatory cytokines by the HepG2 and MCF7 cell lines were quantified using a multiplex Enzyme linked absorbance assay. Briefly, supernatants were collected for ELISA assay after transduction and incubation of either AdV or AdV/coagulation factors complexes at 48h. Proinflammatory cytokines (IL-6, IL-1β) were measured by commercial ELISA kits (Karmania pars gene) using the manufacturer's recommendations. The lower detection limit of the IL-1β and IL-6 were 2 pg/ml, and 3 pg/ml, respectively. Absorption was measured via an ELISA reader at 450 nm.

Statistical analytic

ELISA test and Real-time PCR test were performed at least 3 times. Data were expressed as the mean standard error of the mean and analyzed using one-way ANOVA or the Kruskal-Wallis test using SPSS software and for graph designing we used GraphPad Prism 5. To determine significant differences in differential gene or protein expression control and experimental groups $p = <0.5$ was considered as statistically significant difference between the group means.

Results

The cytotoxic effect of AdV_{GFP} on HepG2 and MCF7 cell-lines

After different dilutions of AdV_{GFP} exposed to cell lines, the MOI of 1-2 was determined as the optimal concentration without significant cytopathic effect on both cells. The trypan blue exclusion test and morphological view shows that in this concentration less than 10% of cell exhibited sign of death and necrosis however, by the increasing the titer, the CPE appeared to be significantly enhanced especially at MOI > 10.

The measurement of particle charge

The dispersive behavior of the AdV solely and the AdV/F VII and IX in the liquid were remarkably different. The charge of the AdV was -39.6 and the zeta potential of the FVII and FIX complexes were -41.6 and -41.9, respectively. Therefore, the zeta charge of AdV became more negative when coated with these blood clotting factors. On the other hand, the differentiation between zeta potential charge of the AdV/F VIII and AdV was negligible.

The transduction efficiency

One question in this study was to determine whether both vitamin-K blood clotting factors such as 7 and 9 and ordinary coagulation factors like FVIII can affect tropism and entry of Adenovector or not. To address this, fluorescent microscopy and flow cytometry assays were employed. Fluorescent microscopy showed that in both cell-types coagulation factors VII and IX exhibited enhancing transduction efficiency and increasing the GFP signal density on adenovector, as shown in Figure 1 and 2. Although coagulation factor VIII could not highly affect the tropism of AdV on both cell-lines, the transduction result showed nearly similar pattern of virus entry to cells as AdV alone.

More precisely, Flow cytometry analysis also showed that AdV_{GFP} loaded with FVII and FIX contribute to escalating the entry rate of Adenovector to both cell lines (Figure 2). In fact, FVII and FIX could increase the entry rate in HepG2 up to 38.3% and 61.9%, respectively, in comparison with AdV_{GFP} which was 40.3%. Furthermore, in MCF7 cell-line coagulation factors VII and IX can increase the entry of Adenovector to 31.2% and 36.6% while the entry rate of simple AdV_{GFP} was 23.1%. Turning next to FVIII, data showed no significant effect of FVIII in adenovector transduction rate on HepG2. Even more interesting, though, is

the decreasing effect of FVIII on Adenovector entry into MCF7 indicating an inhibitory impact for that factor.

Cytokine analysis

Secretion of inflammatory cytokines, IL-6 and IL-1 β , were analyzed on both cell lines by ELISA assay (Figure 5). All treated cell groups could not be affected in terms of the secretion level of IL-1 β cytokine. On the other side of the scale, secretion level of IL-6 in HepG2 cells raised remarkably when it is treated with AdV_{GFP} shielded by factors including AdV/FVII and IX (P = 0.01 and 0.009), however, this escalation is more significant with FIX. In the case of the production of IL-6 in MCF-6, cells which are exposed to AdV/FVII demonstrated a significant increase in cytokine level comparing to the Adenovector alone (P = 0.046).

The expression pattern of innate immune mediators (PKR, STING-1, MX-1, IRF3)

Gene expression analysis of liver cell (HepG2) and mammalian gland cell (MCF-7) were evaluated by Real-Time PCR after 6 hrs incubation with different Adenovectors. RT-qPCR results revealed that some innate immune mediator genes induced by AdV and coagulation factors shielded Adenovectors (Figure 4).

With regards to PKR gene, which is an indicator of innate immunity, on both cell-lines expression of this gene affected by AdV/FVII and AdV/FIX. Expression of PKR gene upregulated more than 10 fold (P = 0.035) by AdV/FVII and less than 10 fold (P = 0.05) by AdV/FIX on HepG2. On MCF7, this gene could be increased by both AdV/FVII (P value < 0.001) and AdV/FIX (P value < 0.01) in comparison with FVIII complex. In contrast, AdV/FVIII didn't left any significant change over expression of PKR when evaluated thereby.

In terms of innate immune induction by STING-1, an important alteration was the upregulation of this gene on MCF7 cells when exposed to AdV/FIX. Compared to control negative (P: 0.019) and AdV/FVIII complex (P value: 0.011) this gene risen up to more than 6 fold. However, expression pattern of STING-1 on all treated groups on HepG2 cells remained unchanged.

Expression pattern of MX-1 up-regulated by all type of Adenovectors including AdV (P value: 0.037), AdV/FVII (P value: 0.001), and AdV/FIX (P value: 0.006). As it is clear in figure 6. AdV coated with FVII and FIX could increase the MX-1 expression level near 6 folds. On the other side, on MCF7 cells this escalation of MX-1 expression is more obvious on AdV/FIX group (P value: 0.01).

Expression pattern of IRF-3 gene remarkably induced by AdV/FIX on HepG2 cell-line and the level of IRF-3 grow round about 8 fold. This growth has significant meaning by cell as control negative (P value < 0.001), AdV/FVIII (P value: 0.001), AdV/FVII (P value: 0.004), and Adenovector alone (P value: 0.001). On the other hand, expression pattern of IRF-3 on MCF7 cells mostly induced by Adenovector (P value: 0.014) and AdV/FVII (P value: 0.032) and increased by these two factors.

Discussion

The loading of coagulation factors on administrated Adenovector is critically important during gene therapy. Therefore, understanding the effect of vitamin K-dependent coagulation factors on tropism, the transduction rate, innate immune responses, and inflammation is very crucial issue to be investigated.

In this study, we once again reaffirm that AdV utilized a coagulation factor-dependent pathway to enter the cells as Shayakhmetov et al. described before (6). Coagulation factors VII and IX which were K-dependent vitamins could shield AdV and have a role to enter the cells. Coagulation factor VIII not only could not effect on zeta charge of AdV; it also could not influence the transduction efficiency. Interestingly, FVIII even downregulates the transduction rate of AdV in MCF7 cells. Although studies showed that soon after the injection of AdV they accumulate in hepatocytes, we propose the tendency of AdV to breast cells by the same pathway which it enters the hepatocytes (20). In fact, coagulation factors VII, IX, and X which are K-dependent blood clotting factors share Gla-EGF1-EGF2-SP common domain structure. They provide a bridge between cells and AdV by HSPG mediation which is abundant in epithelial cells (21, 22). However, not all K-dependent coagulation factors which bind to AdV could efficiently bind HSPG. Indeed two adjacent FVII molecules make stable dimer via their SP domains so this dimerization reduces the ability of binding AdV/FVII to HSPG (23). This fact could explain why transduction efficiency in our results via AdV/FVII was slightly less than AdV/FIX. The high affinity of AdV shielded with FIX and VII to hepatocytes was not surprising but we showed that these coagulation factors could even affect the affinity of AdV to MCF-7. However, the rate of this tendency was irrefutably lower than hepatocytes.

Despite the fact that employing AdV in gene delivery is efficient, Vigorous inflammatory and immunogenic effect soon after AdV administration limits the time of expression (24). Even more worrying, though, is that as AdV is too inflammatory and immunogenic, and this immune response may be responsible for the mortality in mouse and non-human primate models (25). Doronin et al. suggested that Adenovirus type C in blood binds to the coagulation factor X with high affinity and this attachment could trigger antiviral inflammatory responses via the TLR/NF- κ B pathway since coagulation factor activation can induce inflammatory responses (26). In contrast, another study determined that FX could protect AdV in blood from natural antibodies and complement until the vector could achieve the liver (9). In our study, we showed that coating AdV with coagulation factors VII and IX could have an effect on the rate of AdV entrance, eminently. IL-6 analysis determined the inflammatory effect of FIX on hepatocytes and FVII in both cell-lines. However, the secretion pattern of IL-1 β on MCF7 and HepG2 cells remained unchanged. This could be due to the limit of our study about not employing time-course study. It should be underlined that FVIII not only could not impact viral entry; also it does not have a significant effect on inflammation and innate immune induction. The previous study in our research group revealed that the inflammatory influence of adenovirus complexed with FVII by analyzing TNF- α and IL-1 β on LX-2 cell type (18). These results are consistent with the studies which demonstrate the inflammatory responses to AdV. To be more precise, the expression level of several inflammatory cytokines in a wide range of cell types from macrophages to epithelial and endothelial cells are escalating by AdV and these escalation is

affected by specific coagulation factors (10, 27). Apart from cytokines, we determined the innate immune induction of AdV and particularly when it is coated with coagulation factors VII and IX. Except for the STING-1 gene which could not be affected by all test groups on HepG2, other experimented genes were raised up to a significant level when they were exposed to AdV or AdV/FVII and FIX. To be exact, the FIX complex upregulates the expression pattern of PKR, MX-1 and IRF-3 and FVII complex increases the MX-1 and PKR genes on HepG2 cell line. Also, the FIX complex could have an increasing effect on PKR, STING-1, and MX-1 and the FVII complex could raise the expression of IRF-3 and PKR on MCF7 cell line. These genes distinctively indicate innate immunity. In the same way, studies suggest that when FX binds to AdV, innate immunity detects the complex as a pathogen-associated molecular pattern via the TLR-NF κ B pathway (18, 26). There is a contrary study that found no significant correlation between innate immune responses and AdV/FX; however, they experiment the human mononuclear phagocytes (28).

By way of conclusion, we once again showed the importance of vitamin K-dependent coagulation factors on the behavior of Adenovector. The findings suggested that AdV binding to FVII and FIX could increase the transduction rate of the vector and this enhancement may influence the cellular responses to the detection of the vector. Therefore, inflammation and innate immune responses would be remarkably higher than when AdV is unable to attach to FVII and in particular FIX.

Declarations

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Conflict of interest

There are no conflicts of interest.

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Figures

A.HepG2

B.MCF7

Figure 1

Fluorescent microscopy of live HepG2 and MCF7 cells transduced by AdVGFP and AdV/coagulation factors. Fluorescent microscopy of AdVGFP solely and Adenovector shielded with coagulation factor VII, FVII, and IX which after 48h incubation. A(1-4) Fluorescent microscopy image of HepG2. B(5-8) Fluorescent microscopy image of MCF7 (1, 5) AdVGFP. (2, 6) AdVGFP/coagulation factor VII. (3, 7) AdVGFP/coagulation factor VIII (4, 8) AdVGFP/coagulation factor IX

Figure 2

Histogram flow cytometry data of (A) HepG2 and (B) MCF7. 10,000 HepG2 and MCF7 cells were detached after 48hrs and analyzed. (1, 5) AdVGFP. (2, 6) AdVGFP/FVII. (3, 7) AdVGFP/FVIII (4, 8) AdVGFP/FIX

Figure 3

The inflammatory pattern of AdVGFP and complexed with coagulation factors in HepG2 and MCF7. (A, B) IL-6 and IL-1 β secretion of HepG2 cells with or without coagulation factors VII, VIII, and IX. (C, D) IL-6 and IL-1 β secretion of MCF7 cell line untreated or treated with AdVGFP and AdV/coagulation factors. Data from all experiments are averages (n=6) and bars represents standard deviations. All units are based on pictograms per milliliter

Figure 4

The expression pattern of PKR, STING-1, MX-1, IRF-3. (A, B) A refers to HepG2 innate immune gene expression pattern and B refers to MCF7 cell line. (1-8) Four innate immune gene mediators which evaluated by Real-time PCR assay. AdVGFP with or without coagulation factors incubated for 6hrs after transduction and then alteration of gene pattern assessed