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ScienceDirect
International Journal of Pharmaceutics 354 (2008) 9–15
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Mini review

Innate immune response induced by gene delivery vectors

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Received 6 June 2007; received in revised form 6 June 2007; accepted 11 June 2007
Available online 16 June 2007

Abstract

Gene therapy is a clinical strategy that has the potential to treat an array of genetic and nongenetic diseases. Vectors for gene transfer are the essential tools of gene therapy. For gene therapy to be successful, an appropriate amount of the therapeutic gene must be delivered into the target cells without substantial toxicity. A major limitation of the use of gene therapy vectors is the innate immune responses triggered by systemic administration of such vectors. It is essential to overcome vector-mediated innate immune responses, such as production of inflammatory cytokines, the maturation of antigen-presenting cells and tissue damage, because the induction of these responses not only shortens the period of gene expression but also leads to serious side effects. Viral vectors (for example, adenovirus (Ad) vectors) have been assumed to be more potent in inducing innate immune responses in spite of their high transduction efficiency since they contain pathogenic proteins. However, recent studies have demonstrated that not only viral vectors but also nonviral vectors, such as lipoplex (liposome/plasmid DNA complex), can induce innate immune responses. Indeed, nonviral vectors including lipoplex induce comparable or larger levels of innate immune response than viral vectors. In this review, we present an overview of the innate immune responses induced by Ad vector and lipoplex, which are used primarily for *in vivo* gene transfer.

Keywords: Adenovirus vector; Lipoplex; Innate immunity

1. Introduction

Gene therapy provides new hope as a therapeutic method for both genetic and nongenetic diseases. Various types of gene delivery vectors have been developed to improve the efficiency of *in vivo* gene expression, and have been employed in many clinical trials. The vectors for gene delivery are usually classified as viral or nonviral vectors. Viral vectors, at present, dominate in clinical trials because they are highly efficient in transducing cells; however, viral vectors are immunogenic and potentially mutagenic. In 1999, there was a fatal accident in Pennsylvania caused by the systemic administration of adenovirus (Ad) vector (Marshall, 1999; Raper et al., 2003). This accident was due to the over-activation of innate immunity triggered by the injection of heavy doses of Ad vectors. Thus, the immune response induced by gene therapy vectors is a significant problem, which must be overcome (Marshall, 1999).

The systemic administration of Ad vectors induces both innate and adaptive immune responses with its humoral and cell-mediated components. In the case of adaptive immune response, capsid antigens are largely responsible for specific immunity toward Ad vectors. In the first generation Ad vector lacking the E1 gene, leaky expression of viral genes from the vector stimulates an immune response against Ad vector-transduced cells (Yang et al., 1994; Yang et al., 1995). The cytotoxic T lymphocyte (CTL) response can be elicited against viral gene product and/or transgene products expressed in the transduced cells. To reduce cell-mediated immune response against viral gene products expressed in the transduced cells, "helper-dependent (HD)" or "gutted" Ad vectors, in which all viral genes are deleted except the inverted terminal repeat (ITR) sequences at both ends and the packaging signal, have been developed. The deletion of all viral protein-coding regions from the Ad genome improves the prospects of Ad vectors for long-term gene expression,

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doi:10.1016/j.ijpharm.2007.06.012

suggesting that immunogenic toxicities induced by HD Ad vectors are greatly reduced (Palmer and Ng, 2005). Humoral virus-neutralizing antibody responses against the Ad capsid itself are another limitation, preventing transduction upon the subsequent administration of vectors of the same serotype. Because hexons are primarily targeted by neutralizing antibodies, hexon modification has been reported to allow for escape from neutralizing antibodies (Roberts et al., 2006). As other strategies, Ad vectors belonging to subgroups other than Ad type 5, such as Ad type 11 or 35, or to species other than human, have also been developed (Farina et al., 2001; Sakurai et al., 2003a; Seshidhar Reddy et al., 2003; Vogels et al., 2003; Holterman et al., 2004). Compared with adaptive immunity to Ad vectors, the mechanism of innate immune response is less understood. It is essential to elucidate the complete mechanism of Ad vector-mediated innate immune response in order to develop safe Ad vectors.

Nonviral vectors have recently gained increasing attention since they do not contain any pathogenic proteins and are therefore more likely to be safe (Niidome and Huang, 2002). However, Li et al. demonstrated that the systemic administration of lipoplex induces high levels of inflammatory cytokines (Li et al., 1999). The production of inflammatory cytokines mediates the suppression of gene expression and tissue damage (Qin et al., 1997; Loisel et al., 2001; Sellins et al., 2005). Thus, innate immune response is the most important problem to be overcome for both viral and nonviral vectors. In this paper, we review innate immune response induced by Ad vector and lipoplex, which are used for *in vivo* gene transfer in contrast with other vector systems.

2. Innate immune response to Ad vectors

Ad is a nonenveloped virus containing an icosahedral protein capsid with a diameter of approximately 80 nm (Shenk, 2001). At least 51 serotypes of human Ad have been identified, and Ad serotype 5 (Ad5) and Ad2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad vectors are the most efficient class of vector in terms of delivering genes into both dividing and non-dividing cells. They have a large packaging ability for the incorporation of foreign genes and can easily be grown to high titers (Wilson, 1996). Additionally, they can transduce foreign genes efficiently into both cultured cells *in vitro* and many target organs *in vivo*. These advantageous features lead to increasing numbers of clinical applications for Ad vectors. By July of 2006, Ad vectors had been used in 26% of all gene therapy protocols (out of a total of 305 protocols) in gene therapy (Journal of Gene Medicine, Website http://www.wiley.co.uk/genmed/clinical/) worldwide. Systemic Ad vector application, however, is limited due to its activation of cellular, humoral and innate immune responses (Schnell et al., 2001; Zhang et al., 2001; Liu et al., 2003; Muruve, 2004; Xu et al., 2005). Among these, innate immune response against Ad vectors is the most poorly understood. It is essential to clarify the mechanism of innate immune response triggered by the systemic administration of Ad vectors in order to achieve a safe method of gene therapy using Ad vectors.

2.1. Origin of cytokine/chemokine production induced by Ad vectors

The intravenous injection of Ad vectors results in the immediate production (1–6 h post-injection) of cytokines/chemokines (De Geest et al., 2005; Shayakhmetov et al., 2005; Hartman et al., 2007b; Kiang et al., 2006; Koizumi et al., 2006, 2007; Manickan et al., 2006; Sakurai et al., 2007; Yamaguchi et al., 2007). As shown in Table 1, various kinds of cytokines/chemokines are released by the systemic administration of Ad vector.

Intravenously injected Ad vectors are delivered primarily to the liver and spleen. In the liver, Ad vector is likely to be equally distributed to the parenchymal (hepatocytes) and non-parenchymal (Kupffer and endothelial) cells, depending on the dose injected (Koizumi et al., 2003, 2006). Since these tissues contain many immune cells including liver Kupffer cells, splenic dendritic cells (DCs) and macrophages, these cells are assumed to be responsible for the production of inflammatory cytokines.

The depletion of Kupffer cells in mice by intravenous injection of gadolinium chloride ($GdCl_3$) eliminates the Ad vector-induced release of tumor necrosis factor (TNF)-α, but does not suppress the production of interleukin (IL)-6, suggesting that there might be other sites of inflammatory cytokine production (Lieber et al., 1997). The depletion of DCs and tissue macrophages in mice by intravenous injection of liposomes encapsulating dichloromethylene-bisphosphonate (Cl_2MDP) results in a marked inhibition of IL-6 and IL-12 production (Zhang et al., 2001). Splenic DCs and macrophages isolated from Ad vector-injected mice secrete high levels of inflammatory cytokines (Zhang et al., 2001). When the mice are splenectomized, IL-6 production is decreased upon Ad vector injection (De Geest et al., 2005). Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of the liver and spleen after systemic Ad injection suggests that the spleen, but not the liver, is a major site of inflammatory cytokine production (Koizumi et al., 2007). These results indicate that immune cells in the spleen should be a major source of inflammatory cytokine production.

Excessive complement activation has also been reported to be involved in Ad vector-mediated innate immune responses (Kiang et al., 2006). Ad vectors bind to blood factors, such as factor IX, factor X and lactoferrin, leading to liver transduction and hepatotoxicity (Shayakhmetov et al., 2005; Johansson et al., 2007; Parker et al., 2006). The interaction of Ad vector with blood factors might also play a role in the induction of innate immune responses.

2.2. Intracellular mechanism of cytokine/chemokine production

Toll-like receptors (TLRs), which sense specific molecular patterns present in microbial components, are major receptors involved in the activation of innate immune response. Following the recognition of microbial components by TLRs, they, except for TLR3, transduce intracellular signaling through the adaptor protein, myeloid differentiation primary response gene 88 (MyD88), leading to the production of inflammatory cytokines

Table 1
Level of cytokine production *in vivo* by systemic administration of Ad vector or lipoplex

Vector	Strain of mice used	Injected dose of vectors (/mouse)	Peak level of cytokine productions (pg/ml)			Other cytokines/chemokines determined	Reference
			IL-6	IL-12	TNF-α		
Ad-hAAT	C3H/HeJ	10^{10} VP	1300	–	1250	–	Lieber et al. (1997)
Ad-LacZ	DBA/2	$10^{8–9}$ PFU	–	–	–	MIP-2, IL-10, RANTES, MCP-1	Muruve et al. (1999)
Ad-LacZ	C57BL/6	3×10^{11} VP	1200	1000	–	–	Zhang et al. (2001)
Ad-LacZ	DBA/2	10^{11} VP	–	–	–	IP-10, MIP-2	Tibbles et al. (2002)
Ad-AT_4	C57BL/6	10^{11} VP	2500	–	–	–	De Geest et al. (2005)
Ad-GFP	C57BL/6	10^{11} VP	1700	–	–	IFN-γ	Shayakhmetov et al. (2005)
Ad-LacZ	C57/BL6J	1.5×10^{11} VP	1000	1200	–	CXCL1, MIP-1α, MCP-1, RANTES, IL-5	Hartman et al. (2007b)
Ad-Luc	C57BL/6	10^{11} VP	600	–	–	–	Koizumi et al. (2006)
Ad-LacZ	C57BL/6	1.5×10^{11} VP	1200	1000	–	CXCL1, IFN-γ, RANTES, IL-1β	Kiang et al. (2006)
Ad-GFP	C57BL/6Ncr	10^{11} VP	5000	1000	–	–	Manickan et al. (2006)
Ad-Luc	C57BL/6	10^{11} VP	800	800	ND	MIP-2, IFN-α, IFN-β, IFN-γ	Koizumi et al. (2007)
Ad-Luc	C57BL/6	5×10^{10} VP	500	1000	ND	–	Sakurai et al. (2007)
Ad-Luc	C57BL/6	3×10^{10} VP	200	–	–	–	Yamaguchi et al. (2007)
Liposome–protamine–DNA complex	CD-1	50 μg DNA	–	–	20000	IFN-γ	Li et al. (1999)
Liposome–protamine–DNA complex	C57BL/6	25 μg DNA	–	1200	5000	IFN-γ	Whitmore et al. (1999)
Lipoplex	Swiss mouse	50 μg DNA	4500	–	350	IFN-γ	Loisel et al. (2001)
Lipoplex	CDF1	25 μg DNA	–	3000	7000	IFN-γ	Sakurai et al. (2002)
Lipoplex	CDF1	25 μg DNA	–	–	900	IFN-γ	Sakurai et al. (2003a,b)
Lipoplex	CD-1	25 μg DNA	–	2000	1500	IFN-γ	Liu et al. (2004)
Lipoplex	C57BL/6	33 μg DNA	10000	250	300	MIP-1α, RANTES, IL-10, IFN-γ	Zhao et al. (2004)
Lipoplex	BALB/c	25 μg DNA	–	–	500	–	Kuramoto et al. (2006)
Lipoplex	C57BL/6	25 μg DNA	800	3000	600	–	Sakurai et al. (2007)

VP: viral particle; PFU: plaque forming unit; ND: not detectable.

and interferons (IFNs) by activating nuclear factor kappa B (NF-κB) and interferon regulatory factors (IRFs), respectively (Hemmi et al., 2000; Zhao et al., 2004; Kawai and Akira, 2006) (Fig. 1).

Recently, it has become clear that DCs are divided into two major subsets, conventional DCs (cDCs) and plasmacytoid DCs (pDCs); the former play a role as professional antigen-presenting cells, while the latter act as major type I IFN producers in viral infection (Colonna et al., 2004; Yoneyama et al., 2004, 2005). The stimulation of bone marrow precursors *in vitro* with Flt3-ligand leads to differentiation into both cDCs (Flt3L-cDCs) and pDCs (Flt3L-pDCs). IL-6 production in Flt3L-cDCs is TLR9/MyD88-dependent, while type I IFN production is TLR9-indepedent (Basner-Tschakarjan et al., 2006; Yamaguchi et al., 2007). On the other hand, IL-6 production in Flt3L-pDCs and peripheral macrophages by Ad vectors occurs in a TLR9/MyD88-independent manner (Yamaguchi et al., 2007). These results suggest that the recognition of Ad vector by immune cells occurs not only in a TLR-dependent manner, but also in a TLR-independent manner (Fig. 1). The specific sensor receptor and/or signaling pathway used for the activation of innate immune responses to Ad vector might depend on the type of cell. In contrast to the MyD88-dependent IL-6 production in cDCs, TLR9- or MyD88-deficient mice show no decrease in serum IL-6 levels after Ad vector administration (Yamaguchi et al., 2007), suggesting that not only DCs, but also other kinds of cells such as macrophages and endothelial cells might also produce cytokines *in vivo*.

The activation of intracellular signaling for cytokine production by Ad vector has also been studied in nonimmune cells such as HeLa cells and A549 cells (Bruder and Kovesdi, 1997; Bowen et al., 2002; Hartman et al., 2007a). The activation of the Raf/mitogen-activated protein kinase (MAPK) pathway by Ad vectors results in the production of IL-8 or IP-10 in non-immune cells (Bruder and Kovesdi, 1997; Tibbles et al., 2002). The activation of Akt/protein kinase B, protein kinase A (PKA) and the p38/MAPK pathway are also involved in the production of inflammatory cytokines (Suomalainen et al., 2001; Liu et al., 2005). The Ad vector-mediated production of inflammatory cytokines/chemokines in nonimmune cells is associated with NF-κB activation, as in the case of immune cells (Borgland et al., 2000; Morelli et al., 2000; Bowen et al., 2002; Liu et al., 2005; Hartman et al., 2007a). Taken together, these findings suggest that nonimmune cells might also be involved in the innate immune response induced by the systemic administration of Ad vector.

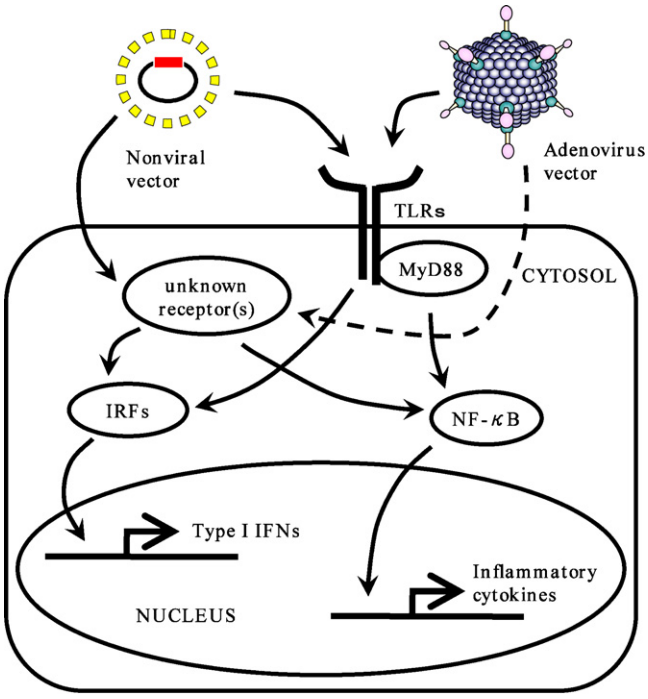

Fig. 1. General scheme of Ad vector- or nonviral vector-induced inflammatory gene expression in immune cells. TLR: toll-like receptor; MyD88: myeloid differentiation primary response gene 88; IRF: interferon regulatory factor; NF-κB: nuclear factor kappa B; IFN: interferon.

2.3. Development of an improved Ad vector which induces less innate immune response

An understanding of the biology of host response to Ad vectors will impact the design of future generations of these agents by allowing researchers to focus on reducing their immunogenicity and improving their safety. To reduce the production of inflammatory cytokines by the systemic administration of Ad vectors, we and others have developed new types of Ad vectors (De Geest et al., 2005; Koizumi et al., 2006, 2007). The non-specific distribution of Ad vectors in tissue after *in vivo* gene transfer is due to the relatively broad expression of coxsackievirus and adenovirus receptor (CAR) (the primary receptor), α_v integrin (the secondary receptor) and heparan sulfate (the tertiary receptor). The modified Ad vector ablated for CAR, α_v integrin and heparan sulfate binding, which we have named "triple mutant Ad vector", reduces cytokine production *in vivo*, suggesting that the binding of Ad vector with these receptors might be involved in the induction of innate immune response, although the mechanism behind this remains unknown (Koizumi et al., 2006). The fiber-modified Ad vector containing a stretch of lysine residues (K7 (KKKKKKK) peptide), AdK7, decreases the production of inflammatory cytokines (IL-6, macrophage inflammatory protein (MIP)-2 and IFN-γ, but not TNF-α, regulated on activation, normal T cell expressed and secreted (RANTES), IFN-α and IFN-β) due to the reduced spleen distribution of AdK7 compared with that with conventional Ad (Koizumi et al., 2007). Since the interaction of fiber with CAR is reported to be responsible for cytokine production in nonimmune cells (Liu et al., 2003; Tamanini et al., 2006), the substitution of Ad5 fiber for the fiber of other types of Ad vectors which do not bind to CAR, such as Ad7, Ad35 and Ad41, is another strategy for reducing innate immune responses (Shayakhmetov et al., 2004; Schoggins et al., 2005; Ni et al., in press). The intravenous injection of modified Ad vector containing Ad type 35 fiber shows lower IL-6 and TNF-α levels compared to the injection of conventional Ad vector (Shayakhmetov et al., 2004; Ni et al., in press), suggesting that CAR-binding activity might participate in the activation of innate immune responses *in vivo*. Another approach is to modify Ad vector with monomethoxypolyethylene glycol (MPEG). PEGylation reduces vector uptake in the spleen, resulting in the suppression of cytokine production (De Geest et al., 2005). The development of improved Ad vectors targeting a specific tissue or cell type with reduced distribution to immune cells is an important approach to avoid the induction of innate immune responses (Mizuguchi and Hayakawa, 2004).

3. Innate immune response to lipoplex

Although viral vectors have high transduction efficiency, safety concerns regarding their use in humans make nonviral vectors an attractive alternative. Cationic liposome has proven to be a useful tool for the delivery of genes into cells in nonviral forms (Felgner et al., 1987, 1995; Zhu et al., 1993; Liu et al., 1995). Nonviral vectors have advantages with respect to simplicity of use, ease of large-scale production, and lack of specific immune response (Liu and Huang, 2002). Genetic immunization with plasmid DNA vaccines has proven to be a promising tool in conferring protective immunity in various experimental animal models of infectious diseases or tumors, however this indicates that plasmid DNA has the ability to induce immune responses (Sakurai et al., 2003b; Prud'homme, 2005; Bolesta et al., 2006). As in the case of viral vectors, innate immune responses and tissue damages are induced by the systemic injection of lipoplex even though it contains no viral components (Li et al., 1999; Whitmore et al., 1999; Loisel et al., 2001; Sakurai et al., 2002, 2003b, 2007; Liu et al., 2004; Zhao et al., 2004; Kuramoto et al., 2006). The systemic administration of cationic polymer/plasmid DNA complex (polyplex) is also known induce innate immune response (Gautam et al., 2001).

The deletion of macrophages in tissue by intravenous injection of $GdCl_3$ decreases the production of TNF-α and IL-12 by lipoplex, suggesting that tissue macrophages containing liver Kupffer cells and spleen macrophages are closely involved in inflammatory cytokine production following the systemic administration of lipoplex (Sakurai et al., 2002). The trigger of the innate immune response is likely to be the bacterial origin of the plasmid DNA, which is incorporated in the lipoplex. Hemmi et al. report that bacterial DNA, such as plasmid DNA, is recognized by TLR 9 (Hemmi et al., 2000). Plasmid DNA and bacterial DNA contains a much higher frequency of unmethylated CpG motifs (also known as immunostimulatory CpG motifs) than does mammalian DNA (Scheule, 2000; Zhao et al., 2004; Yasuda et al., 2005). The production of inflammatory cytokines induced by the systemic administration of lipoplex is greatly, but not completely, suppressed in $TLR9^{-/-}$ mice (Zhao

et al., 2004), indicating that the recognition of CpG motifs in plasmid DNA by TLR9 is crucial for the induction of innate immune responses induced by lipoplex. In contrast, the absence of CpG motif in plasmid DNA greatly reduces cytokine production, although it also does not completely eliminate it (Sakurai et al., 2007). Another study has shown that the methylation of CpG motifs in plasmid DNA partly suppresses the production of inflammatory cytokines (Whitmore et al., 1999). These findings indicate that the interaction of the CpG motifs in the plasmid DNA with TLR9 plays a role in the innate immune responses, but that there is another as yet unknown mechanism underlying the induction of innate immune responses, independently of the CpG motifs. Recently, Ishii et al. reported that double-stranded B-form DNA triggers the production of type I IFNs and chemokines through a TLR-independent mechanism (Ishii et al., 2006). They suggest that there is (are) unknown receptor(s) in cytoplasm for the recognition of DNA, leading to the activation of innate immune responses (Fig. 1). The identification of these unknown receptors which sense foreign DNAs would provide a strategy for reducing the innate immune response induced by lipoplex.

As a strategy to reduce the innate immune response induced by lipoplex, Liu et al. developed a new type of lipoplex, safeplex, which efficiently delivers genes with less induction of innate immune response by co-delivering DNA and the inflammatory suppressor dexamethasone (Liu et al., 2004). Recent reports demonstrate that lipopolysaccharide-induced TNF-α production is suppressed with the pre-injection of NF-κB decoy, whose double-stranded oligonucleotides contain an NF-κB binding sequence (Higuchi et al., 2005, 2006), suggesting that NF-κB decoy might be another suppressor of lipoplex-induced cytokine production.

As described above, both Ad vector and lipoplex have the ability to activate innate immune responses by systemic administration of the vectors. Table 1 shows the levels of cytokine production induced by Ad vector or lipoplex. These results clearly indicate that the induction of inflammatory cytokines, such as IL-6, IL-12 and TNF-α, is greater when lipoplex is injected, than with Ad vector, even though it is commonly believed that nonviral vectors are safer to use in gene therapy than viral vectors. Thus, it is essential to pay close attention to the innate immune responses induced by nonviral vectors as well as to those induced by viral vectors.

4. Conclusion

To achieve the desired therapeutic effects, gene therapy vectors must be able to safely deliver genes of interest to the designated target and to ensure their safe expression for an appropriate amount of time. Recently, improved Ad vectors and lipoplex have been developed to decrease inflammatory toxicity. However, those new vectors do not completely suppress the induction of the innate immune responses that may occur with the systemic administration of the vectors. A greater understanding of the mechanism of induction of innate immune responses by gene therapy vectors is essential for the development of next-generation safe gene therapy vectors.

Acknowledgement

This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

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