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THE INDUCTION OF INFLAMMATION BY ADENOVIRUS VECTORS USED FOR GENE THERAPY

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1. ABSTRACT

The ability to repair or enhance an individual's genetic make-up provides the sublime opportunity to ameliorate or eliminate many clinical disorders that affect mankind. Gene therapy is thus a revolutionary clinical strategy that may potentially treat an array of genetic and non-genetic diseases, as well as a novel method for drug delivery and vaccination. To these ends, adenovirus vectors are currently the most promising means to deliver specific genes of interest into target cells of the patient. A major limitation of the use of adenovirus vectors in clinical trials, however, is the rapidly induced inflammatory response against these infectious particles. This review aims to describe the cellular and molecular mechanisms involved in the adenovirus-mediated inflammatory response.

2. INTRODUCTION

Viruses are obligate intracellular parasites of living but non-cellular nature. They are ultra-microscopic (20-300 nm diameter), metabolically inert agents composed of DNA or RNA and a protein coat. As they are only capable of replication within living host cells, infection is an essential stage of their life cycle. Throughout the course of evolution, therefore, viruses have become highly developed agents specialized in infection and transfer of genetic material to cells of other organisms. Nature may thus be considered to have provided us with vectors finely tuned for efficient gene transfer. On the other hand, as viruses have evolved into professional infectious gene-transferring agents, so our immune system has evolved to counter-attack the efforts of would-be pathogens. The immune response against invading pathogens is comprised of an immediate inflammatory reaction (innate immunity), followed by a more slowly developed antigen-specific defense (acquired/adaptive immunity).

Adenoviridae are non-enveloped viruses with a 30-40 kb linear double-stranded DNA genome that are extensively studied and developed for gene therapy applications. Replication-deficient adenovirus (Ad) vectors have several advantages, including the ability to package large quantities of DNA, ease to produce and broad cell tropism (1). First-generation adenovirus vectors are derived from E1/E3 deleted wild-type adenoviridae. The experimental and clinical experience with these vectors has revealed significant host immune responses that limit their safety and efficacy *in vivo*. First generation Ad vectors efficiently induce adaptive immunity, responses that have been well characterized by in various models of gene transfer with these agents 1. Newer generations of Ad vectors have been deleted of the entire coding region (helper-dependent Ad vectors) to increase DNA carrying capacity and to alleviate antiviral host adaptive immunity (2). The development of helper-dependent Ad vectors has minimized the host adaptive response and improved the efficacy and duration of gene transfer *in vivo* (3, 4).

Increasingly, Ad vectors are recognized to activate innate immune mechanisms following transduction. The elicitation of inflammation has been a particularly difficult obstacle when employing Ad vectors for the purposes of somatic gene therapy. The viral titer required to achieve efficient gene transfer and therapeutic benefit is many logs greater than that of a normal wild-type infection. This serves to exacerbate the acute inflammatory reaction and introduces the additional hazard of profound damage to healthy tissue and significant morbidity in transduced hosts (5, 6). Furthermore, the inflammatory reaction to Ad vectors significantly reduces gene transfer efficiency and vector persistence (7, 8). In contrast, for the purposes of vaccine development or cancer immune

therapy, adenovirus vectors are ideal in that its potent adjuvant effect is essential to effectively immunize hosts or cause a bystander effect. A further understanding of viral-mediated inflammation is therefore required in order to improve the efficacy and safety of gene therapy for all applications. In this review, the mechanisms of adenovirus-induced inflammation *in vivo* and *in vitro* will be discussed. Consequently, learned manipulation of the acute inflammatory response may provide us the means to progress and realize the aims of clinical gene therapy.

3. THE BIOLOGY OF ADENOVIRUS VECTOR-INDUCED INFLAMMATION *IN VIVO*

Ad vectors are recognized to induce inflammation *in vivo*. In immunocompetent hosts, Ad vectors induce inflammation of transduced tissues several days (>5 days) following transduction. This delayed inflammatory response corresponds to antigen specific adaptive immunity that has been well characterized by numerous groups (9-13). However, Ad vectors are also noted to induce acute dose-dependent inflammation of transduced tissues prior to the development of adaptive immune responses in pre-clinical and clinical trials utilizing these agents (5, 14-17). In the early clinical trials using Ad vectors to deliver the cystic fibrosis transmembrane conductance regulator (CFTR) gene to patients with cystic fibrosis, early local inflammation was noted in patients receiving the highest titers of AdCFTR intranasally (17). Similar findings were seen in patients receiving increased titers of AdCFTR administered intrabronchially (16). In a human trial of ornithine transcarbamylase (OTC) deficiency, increasing titers of a first generation Ad vector expressing human OTC resulted in early, dose-dependent liver inflammation, thrombocytopenia and a systemic flu-like illness that included fever and myalgias (5). Trapnell and co-workers characterized the early inflammatory response to Ad vectors in a study of AdCFTR gene transfer to the lung of cotton rats (14). Ad vectors administered intranasally induced dose-dependent lung inflammation within 3 days. Inflammatory infiltrates consisted predominantly of neutrophils, although mononuclear cells were also present. McCoy *et al* confirmed similar findings in the lung, wherein incomplete and transcription-defective adenovirus particles induced quantitatively similar pulmonary inflammation compared to intact, competent viral vectors, thus suggesting that the early inflammatory response induced by Ad vectors was transcription independent (15). These early findings suggested that Ad vectors induced an early, dose dependent inflammatory response that occurred independent of viral gene transcription or the adaptive immune system.

3.1. Adenovirus Vectors Induce Early Cytokine Gene Expression *In vivo*

The observation of early Ad vector-induced inflammation resulted in numerous studies examining the role of cytokines and chemokines in this process. Ad vectors induce numerous inflammatory genes *in vivo*. These include the cytokines TNFalpha, IL-6, IL-1beta, interferon-gamma, IL-12 and the chemokines MIP-2, IP-10, RANTES, MIP-1alpha, MIP-1beta and MCP-1. In addition, genes that are involved in leukocyte trafficking such as adhesion molecules are also expressed confirming the complexity of the early inflammatory response to Ad vectors (6, 7, 18-21). The time course of cytokine and chemokine induction by adenovirus vectors has been well characterized. In the lung, Otake *et al* have demonstrated the induction of MIP-2, MIP-1alpha and RANTES within hours of adenovirus transduction (7). In similar studies, we demonstrated the induction of multiple chemokine mRNAs in the liver within 60 minutes of Ad vector transduction in mice. Chemokine genes upregulated included RANTES, MIP-1alpha, MIP-1beta, MCP-1, IP-10 and MIP-2 (18). The induction of chemokines by Ad vectors is significant and correlates with the inflammatory response within the transduced organs. In the lung, the modulation of inflammation with corticosteroids was associated with reduced serum levels of TNFalpha, IL-6, MCP-1 and MIP-1alpha and improved transgene expression (7). In the liver, blocking MIP-2 (a neutrophil chemoattractant) with neutralizing antibodies reduced Ad vector induced liver injury confirming the role of this chemokine in mediating neutrophil recruitment and liver injury (18).

The expression of pro-inflammatory mediators following transduction with Ad vectors occurs prior to significant viral gene transcription suggesting that the adenovirus particle or capsid triggers this response. UV/psoralen inactivation of Ad vectors renders the virions transcription defective, but does not affect the structure of the particle or its ability to transduce cells (22). Administration of UV/psoralen-inactivated Ad vectors to mice induced identical chemokine mRNA expression patterns as mice receiving transcription-competent Ad vectors confirming that viral gene transcription is not required to activate this host response (18). Similarly, Wilson and co-workers demonstrated in mouse and non-human primate models the activation of innate responses by transcription-defective adenovirus particles (6, 21). Consistent with previous studies, serum IL-6, TNFalpha, IL-12 levels and liver toxicity occurred within hours in a dose dependent manner and were induced equally in animals receiving transcription competent or defective Ad vectors.

Recently, we examined the temporal course of cytokine and chemokine mRNA expression in the liver following transduction with first generation Ad vectors (23). Following intravenous administration, Ad vectors induced a biphasic course of cytokine and chemokine gene expression (Figure 1). The first phase corresponds to the innate immune system and occurred independent of Ad vector transcription. The inflammatory response was transient and did not extend beyond 24 hours following a sub-lethal dose of Ad vector. Following this early phase, a relatively quiescent period of inflammatory gene expression occurred in the liver with only basal levels of TNFalpha, IP-10 and MIP-2 mRNA expressed at 72 hours. At 4-5 days following Ad vector administration, a second peak of inflammatory gene expression developed with increasing levels of TNFalpha, IP-10, MCP-1, MIP-1alpha and MIP-1beta mRNA in the liver. The second peak of inflammatory gene expression however was not seen

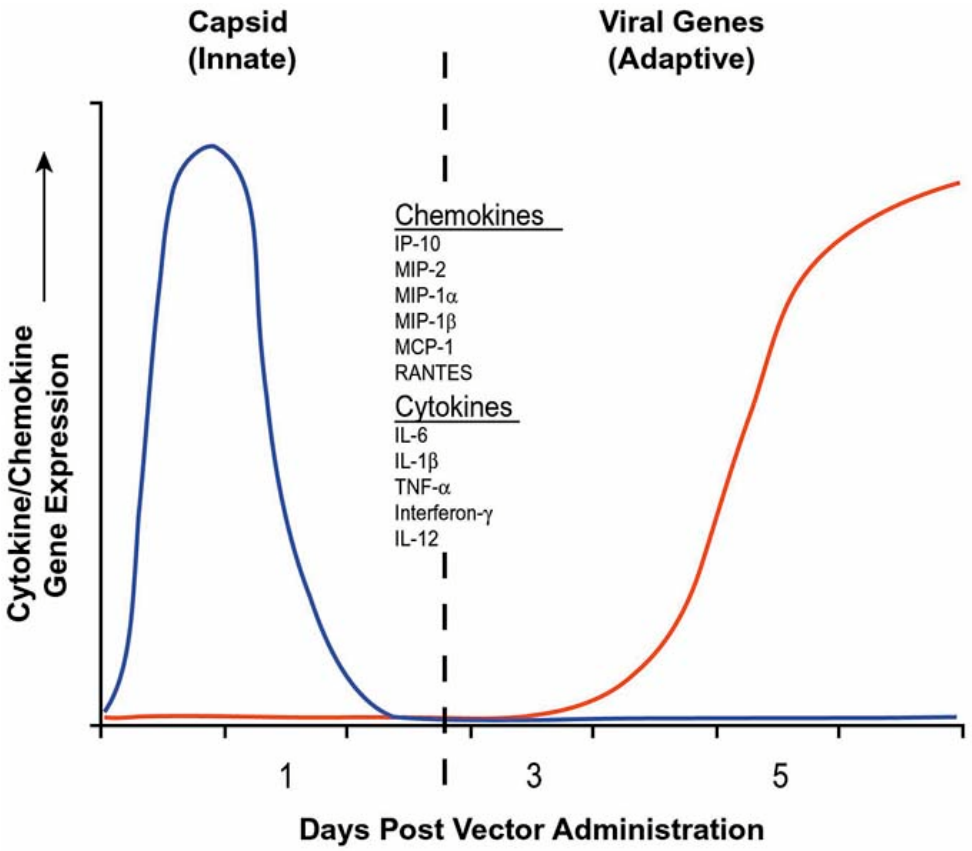

Figure 1. Biphasic time course of cytokine and chemokine expression in immunologically naïve mice receiving first generation adenovirus vectors.

following the administration of UV/psoralen inactivated Ad vectors, confirming that this response was dependent on viral gene transcription and thus corresponds to the adaptive immune response. Other groups have also noted the biphasic nature of the host response to first generation Ad vectors (24). In summary, these studies show that Ad vectors activate the innate immune system and cause the expression of multiple chemokines and cytokines that result in the inflammation observed in tissues transduced with these agents.

3.2. Macrophages and Target Cells Mediate Adenovirus Induction of Chemokines/Cytokines

It is well accepted that the adenovirus particle is capable of inducing the expression of inflammatory cytokines and chemokines. The cells that express these genes are also being recognized using cell depleting strategies and targeted gene therapy vectors. Liver resident macrophages (Kupffer cells), act as a first line of defense for many pathogens invading the liver. Studies have suggested that Kupffer cell blockade in the liver reduces adenovirus-induced inflammation and improves gene transduction (25, 26). Gadolinium chloride ($GdCl_3$), when administered systemically, is taken up by Kupffer cells and results in apoptosis and reduced phagocytic ability (26, 27). Similarly, liposome encapsulated biphosphonates (liposome-Cl_2-MDP) can also deplete liver Kupffer cells as well as dendritic cells in the spleen (28, 29). Studies employing gadolinium chloride or liposome-Cl_2-MDP prior to Ad vector administration in mice have shown improved hepatocyte transduction and reduced serum TNFalpha, IL-6 and IL-12 expression, suggesting that these cells contribute to the inflammatory response induced by these agents (24-26). However, in the study by Lieber *et al*, while a reduction of TNFalpha in the serum was observed following Kupffer cell blockade, NFκB activation in the liver remained unchanged. These results suggested that liver cells other than macrophages were activated following Ad vector transduction (26). In studies performed in our laboratory, direct liver activation by Ad vectors was assessed by measuring liver mRNA levels following Kupffer cell blockade with $GdCl_3$. Regardless of Kupffer cell status, Ad vectors very efficiently induced inflammatory gene mRNA expression in the liver (23). This observation confirms that Ad vectors are capable of inducing inflammatory gene expression in non-hematopoietic target cells. In the absence of resident macrophages, Ad vector interactions with endothelium likely played a significant role in the early inflammatory response (20, 23). Taken together, these results show that adenovirus vectors activate resident macrophages and dendritic cells in the early phase following administration. The induction of these cells is responsible for cytokine gene expression, particularly TNFalpha and IL-6. However,

adenovirus vectors can also activate non-hematopoietic target cells directly, including endothelium.

3.3. The Early Activation of Innate Immunity is Required for Optimal Adaptive Immunity Against Adenovirus Vectors

The early induction of chemokines and cytokines by adenovirus vectors causes acute inflammation, and in severe cases, significant morbidity in transduced hosts. In addition to the acute consequences of inflammation, numerous studies have demonstrated the importance of the early innate response in reducing gene transfer efficiency and vector persistence (7, 8). The innate immune response induced by Ad vectors is also a key event in the development of adaptive antiviral immunity. Benihoud *et al* demonstrated reduced humoral responses in TNFalpha/lymphotoxin-alpha deficient mice, findings that were echoed by Elkon *et al* (19, 30). In the latter study, the lack of TNFalpha also markedly prolonged transgene expression highlighting the importance of this cytokine in the host response to Ad vectors. In contrast, similar studies in IL-6 deficient mice did not significantly impact the host response to Ad vectors (31). Wilson and co-workers demonstrated that profound depletion of dendritic cell and macrophage populations with liposome-Cl_2-MDP reduced TNFalpha and IL-6 expression and also reduced adenovirus specific cytotoxic T-lymphocyte responses (24).

4. THE MOLECULAR BIOLOGY OF ADENOVIRUS VECTOR-INDUCED INFLAMMATION

A thorough understanding of the mechanism by which the adenovirus particle activates the innate arm of the immune system is required to improve the efficacy and safety of adenovirus-mediated gene therapy in humans. The studies performed *in vivo* have demonstrated early, capsid-mediated activation of innate immunity by Ad vectors. The induction of chemokines and cytokines results in inflammation, which reduces gene transfer efficiency and damages transduced tissues. In addition, the induction of pro-inflammatory genes is also required for optimal adaptive immunity. The implied role of the adenovirus capsid or particle suggests that events triggered during Ad vector-cell interactions and entry ultimately result in the cascade of inflammation and innate immunity observed *in vivo*. Numerous studies have examined the molecular biology of the adenovirus capsid-induced inflammation and will be reviewed in the following section.

4.1. Adenovirus Vectors and Inflammatory Gene Expression

Ad vectors induce the expression of various cytokines and chemokines in innate inflammatory cells such as macrophages in addition to non-hematopoietic targets such as epithelial and endothelial cells. The dose dependent inflammatory response observed *in vivo* also exists *in vitro*. Studies by Hidaka *et al* examining Ad vector transduction *in vitro* demonstrated a saturation effect, where increasing vector titers was not associated with increased transduction (32). Cell type and high affinity receptor density are likely factors that determine the titer at which vector saturation occurs. Consistent with this, a saturation effect is also observed in regards to Ad vector induction of inflammatory genes. In human peripheral blood mononuclear cells (PBMC), Higginbotham and co-workers demonstrated a 2-3 fold induction of IL-8 and IL-6 by Ad vectors despite a 100 fold increase in input titer (33). Similarly, in murine epithelial-derived cells, Ad vector titers increased beyond 30,000 particles/cell was associated with no further increase in expression of the chemokine IP-10 (34). These observations are relevant for *in vivo* gene therapy. First, escalating titers of adenovirus vectors may not translate into improved gene transfer efficiency and may worsen the inflammatory response to these agents. Second, many of the adverse inflammatory effects related to the adenovirus particle may be avoided by simply using lower titers (35).

Moncytes and resident macrophages are essential effector cells of the innate response to viral infection. In the lung, Zsengeller *et al* showed the rapid accumulation of Ad5LacZ vectors in alveolar macrophages 10 minutes after vector administration and the upregulation of inflammatory cytokines and chemokines IL-6, TNFalpha, MIP-2, and MIP-1alpha by these cells (36). *In vitro*, transduction of the macrophage cell line RAW264.7 with Ad vectors increased TNFalpha expression as early as 2 hours post transduction (36). Vector internalization and endosomal escape was required for Ad vector-induced TNFalpha expression since chemical inhibition of endosome acidification and/or lysis attenuated TNFalpha expression (36). Combining this observation with the lack of transgene expression in macrophages would suggest that the triggering event might reside after endosomal escape but prior to nuclear localization of adenovirus vectors. Higginbotham *et al* demonstrated in human PBMC the expression of cytokines and chemokines induced by Ad vectors *in vitro* (33). At a dose of 1000 pfu per cell, serotype 5 adenovirus (Ad5) vectors caused a minimal release of TNFalpha and IL-1beta, a significant upregulation of IL-6 and RANTES, and a steady increase of GM-CSF, MIP-1alpha, Gro-alpha, and IL-8 over a 96-hour window. The use of UV-psoralen inactivated vector particles or empty capsids did not diminish the inflammatory response confirming the importance of the viral particle or capsid in this process (33).

Adenovirus vectors also induce the expression of inflammatory genes in cells outside the blood. Ad vectors and UV-psoralen inactivated adenovirus particles induced the expression of the chemokines RANTES, IP-10 and MIP-2 within 6 hours of transduction in epithelial-derived cells lines (18). Numerous groups have shown the Ad vector induction of chemokines including RANTES, IP-10 and IL-8 in HeLa cells, the human respiratory epithelial cell line A549, mouse renal epithelial cells (REC) and the mouse insulinoma cell line TGP61 (18, 37-40). The activation of pro-inflammatory genes appears to be a direct effect that does not require a second messenger or cytokine such as TNFalpha, IL-1beta, or interferon-gamma. RANTES and IP-10 induction by Ad vectors in HeLa cells and the mouse epithelial derived cell line, REC, occurred in the absence of these cytokines suggesting that the adenovirus particle is capable of directly activating non-

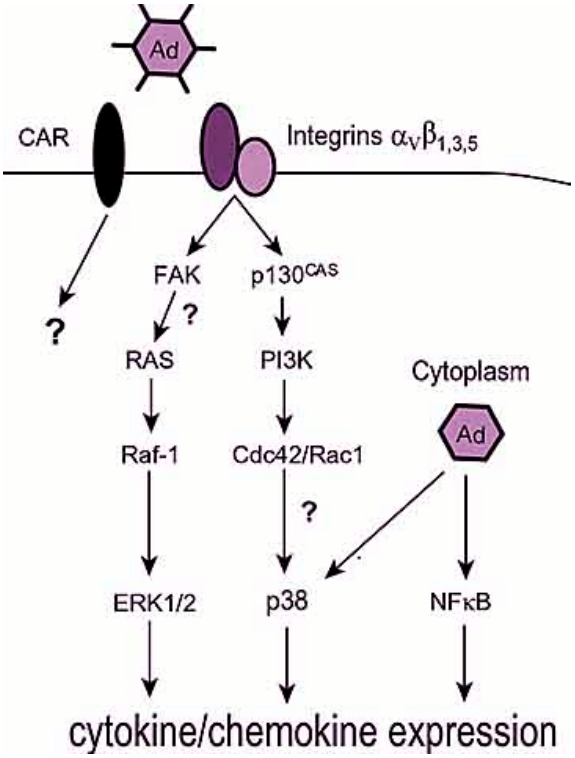

Figure 2. General scheme of adenovirus induced signaling. Unknown or speculative pathways are marked by "?".

acrophage target cells to express inflammatory mediators (39, 40). In support of this view, studies by Reich *et al* demonstrated that adenoviral transduction of HeLa cells induced transcription of interferon-stimulated genes in the absence of any protein synthesis, indicating that cellular activation by adenovirus was not due to viral replication or interferon production (41). Interestingly, the adenovirus E1A gene product suppressed transcriptional induction of interferon-stimulated promoters, possibly as a mechanism of immune evasion. Thus, adenovirus vectors induce a broad array of inflammatory genes in innate effector cells and vector target cells that underlie the inflammatory response to these agents *in vivo*.

4.2. Adenovirus Vector-Induced Signaling and Host Inflammatory Responses

The *in vivo* and *in vitro* studies demonstrating early activation of host innate immune responses by the adenovirus capsid or particle point to a significant role for adenovirus-cell interactions or cell entry in triggering inflammation and subsequent anti-viral immunity. In epithelial cells, adenovirus vectors attach to and enter cells through interactions with CAR receptor and $alpha_V$ integrins. While signal transduction related to CAR binding has yet to be demonstrated, integrins are involved in a wide variety of signaling events regulating protein kinases, growth factor receptors, and organization of actin cytoskeleton (42, 43). Numerous signaling pathways are utilized by group C adenoviridae to internalize and infect cells, and thus it is not surprising that the process of viral cell entry may trigger inflammatory gene expression (Figure 2). Bruder and Kovesdi have shown Ad vector induction of the chemokine IL-8 in HeLa cells is dependent on signaling via ERK. Raf-1, the downstream effector of the Ras GTP binding protein, was activated as early as 5 minutes after Ad5LacZ transduction in HeLa cells. This was followed by p42/MAPK phosphorylation 10 minutes after Ad5LacZ transduction. Inhibiting signaling via this pathway effectively reduced IL-8 expression suggesting for the first time that Ad vectors induced early signaling events that activated host defense mechanisms (38).

Studies from our laboratory have demonstrated the activation of the MAP kinases p38 and ERK within 10 minutes of Ad vector cell entry in a mouse kidney-derived epithelial cell line (REC cells) (34). Interestingly, signaling via JNK was not activated in these cells following Ad vector transduction. Chemical inhibition of p38 and ERK signaling blocked Ad vector induction the chemokine IP-10 proving a link between early vector-induced signaling and pro-inflammatory gene expression (34). We have also shown a significant role for NFkappaB in adenovirus vector induction of chemokine genes (39, 40). In HeLa and REC cells, Ad vectors induced the nuclear translocation of NFkappaB within 2 hours resulting in the transcriptional activation of IP-10 and RANTES genes. The inhibition of NFkappaB activity by overexpressing the natural inhibitor, IkappaBalpha effectively blocked adenovirus vector induction of both these chemokines (39, 40).

The early activation of signaling and subsequent chemokine gene expression confirms that the viral cell entry process is an early event that triggers the host inflammatory response to Ad vectors. Several studies have evaluated the role of adenovirus cell surface receptors in the induction of inflammatory gene expression. The high affinity receptor CAR does not appear to be specifically required for the adenovirus vector signal activation (34, 39, 44). In REC cells, the CAR-ablated fiber knob mutant, AdL.F 45 induced similar levels of IP-10 gene expression compared to the wild type capsid vector AdLuc when corrected for differences in transduction efficiency (34). Furthermore, group B adenovirus particles that do not use CAR as a high affinity receptor (46), induced both IP-10 and RANTES equally in epithelial cell lines suggesting that adenovirus vector induced inflammatory gene expression can occur independent of CAR (34, 39). Ad vectors activate cell signaling via RGD-dependent interactions with $alpha_V$ integrins (43). Studies by others and us suggest, however, that these interactions may not be essential for activation of inflammatory pathways in epithelial cells (34, 39, 47). RGD peptides did not affect p38 activation following Ad2 infection in HeLa cells (47). Similarly, we demonstrated a reduction in Ad vector induction of RANTES following competition with RGD peptides, though a parallel reduction in transduction, however, was also seen. Studies in CAR deficient cells confirmed that vector interaction with $alpha_V$-integrins in the absence of internalization was insufficient to induce the expression of RANTES (39). These observations suggest that vector internalization rather than RGD-dependent integrin interaction is the critical step in the activation of a transduced cell. Studies with the RGD-deleted vector, AdL.PB (48), confirmed that the induction of pro-

inflammatory signals was mainly RGD-independent. At equal levels of transduction, AdL.PB activated similar levels of p38 and IP-10 gene expression as the wild-type vector AdLuc (34) supporting the notion that efficient activation of inflammatory signals and gene expression requires vector internalization.

The importance of internalized particles to induce an inflammatory response has been suggested by several studies. Trapnell and coworkers demonstrated that inhibition of adenovirus internalization and endosomal escape attenuated TNFalpha gene expression in alveolar macrophages (36). In a similar vein, studies in our laboratory have shown that impairing endosomal escape with bafilomycin A1 or ammonium chloride significantly reduced REC cell p38 and ERK activation by adenovirus vectors. A significant reduction in IP-10 gene expression was also seen (34). Furthermore a temperature sensitive adenovirus mutant that has a defect in endosomal penetration was unable to activate p38 in HeLa cells (47). These data suggest that the activation of pro-inflammatory signals by adenovirus vectors occurs mainly in a post-internalization step, likely following endosomal penetration.

5. FUTURE PERSPECTIVES

Adenovirus vectors are promising agents for human gene therapy. They have numerous advantages, particularly their ability to package large quantities of DNA and their episomal gene expression that removes the risk of insertional mutagenesis. Ad vectors induce significant inflammation that can be an advantage or not depending on the clinical application. A global understanding of Ad vector biology is required to fully realize the potential of these agents for human gene therapy. While our understanding of Ad vector induced immunity has grown considerably over the past decade, more research is required. Newer generations of gutted adenovirus vectors have greatly diminished the adaptive immune response to these vectors and improved the efficiency and duration of gene transfer. However, the viral capsid remains, and it is now clear that the adenovirus particle can trigger a wide variety of events in host cells and tissues. A greater understanding of the innate immune response to Ad vectors *in vivo* is needed in addition to understanding the molecular basis of Ad vector-induced signaling. For example, the trigger point and exact upstream mediators of ERK and p38 signaling are not known. The viral determinants that trigger inflammatory signaling also need to be determined. It is possible that Ad vectors could be re-engineered to lack these properties. *In vivo*, the role of innate effector cells and their interactions with Ad vectors is also poorly understood. It is therefore essential to understand the innate response to adenovirus vectors to improve the safety profile of these agents when an inflammatory response is not desired or to enhance the response when an adjuvant effect is desired. Finally the involvement of other innate signaling pathways has yet to be explored. The demonstration that respiratory syncitial virus envelope proteins recognize and activate Toll-like receptor-4 raises the possibility that a different spectrum of innate signaling may be involved in the inflammatory response to adenovirus vectors (49). Ultimately research in this area will result in strategies to modify the vector capsid or the host and improve the application of adenovirus vectors in humans.

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