Soluble components in body fluids as mediators of adenovirus infections.

Mari Nygren
To my family
Abstract

Adenoviruses (Ads) are highly prevalent in the human population, especially in children where about every 10th acute respiratory infection is caused by Ads. Adenoviruses cause mild infections in otherwise healthy persons, whereas in immunocompromised patients, an Ad-infection can be lethal. Around 60 different types of Ads have been isolated, and these types are divided into seven species: A to G. Adenoviruses commonly infect airways, eyes, and intestines, but some Ads also infect urinary tract, tonsils, and liver.

Before viral infection of a cell, the virus needs to attach to a cell surface receptor. The first cellular receptor identified and demonstrated to be used by Ads in vitro was the coxsackie and adenovirus receptor (CAR). Ads from most species bind to CAR via a capsid protein called fibre. However, CAR is not always accessible for Ads to bind in vivo, which makes it plausible that there exists other alternative receptors that Ads use in their infection of cells.

Except for binding directly to cellular receptors, Ads have been reported to bind and attach to cells via soluble components in body fluids. In 2005, Shayakhmetov et al. found that the soluble components coagulation factor IX (FIX) and complement component C4 binding protein (C4BP) could function as bridges between Ad type 5 (Ad5) and hepatocytes (Shayakhmetov et al., 2005). The cellular receptors for Ads complexed with FIX and C4BP were found to be heparan sulphate proteoglycans (HSPG) and low density lipoprotein receptor-related protein (LRP). Not long after this discovery, the coagulation factors X, VII and protein C were also found to bridge Ad5 to hepatocytes (Parker et al., 2006). Importantly, these findings explained why Ad-based vectors mainly targeted the liver after administration in the blood.

In this project, I have been working with Ad5 (species C), Ad31, Ad18, and Ad12 (all from species A) and cell lines that represent the natural tropism for these viruses. We have found that Ad5, Ad31, and Ad18 can use coagulation factors (Ad5 uses FIX and FX, whereas Ad31 and Ad18 only use FIX) as bridges during attachment and infection of epithelial cells from the respiratory tract, eyes, and the intestines which corresponds to the natural tropism of these Ads (Jonsson et al., 2009, Lenman et al., 2011). Moreover, we suggest that FIX and FX bind to the hexon protein of Ad5, Ad18, and Ad31, but not to the Ad12 hexon protein, thus explaining why these factors did not promote Ad12 infection. Finally, in all cases, the Ad:coagulation factors complexes attached to target cells via heparan sulphate, which is also present on non-hepatic epithelial cells.

From these results, we suggest that Ads have evolved to use coagulation factors for binding to and infection of cells outside the liver, i.e. cells that represent the Ad tropism, and that this may be an important mechanism used by Ads to infect cells that do not have or present CAR accurately.
Adenovirusinfektioner är vanligt förekommande hos människor, särskilt hos barn där ca var tionde akut luftvägsinfektion orsakas av adenovirus (Ad). Adenovirus ger upphov till milda infektioner hos friska personer, medan en Ad infektion kan vara dödlig för en immunsupprimerad patient. Närmare 60 olika typer av Ads har isolerats, och dessa typer är indelade i sju undergrupper (species); A till G. Olika Ad orsakar olika sjukdomar, beroende på vilken typ av cell/vävnad/organ som de infekterar, dvs beroende på deras tropism.

Innan ett virus infekterar en cell, måste viruset binda till densamma. Den första cellulära receptor som identifierades kallas för coxsackie- och adenovirus receptorn (CAR). De flesta, men inte alla, Ad binder CAR. Eftersom CAR inte alltid är tillgänglig för Ad, är det troligt det finns andra alternativa receptorer som Ad kan binda till innan de infekterar mänskliga celler.

Förutom att binda direkt till cellulära receptorer, så har Ads rapporterats binda till celler via lösliga faktorer i kroppsvätskor. Shayakhmetov och hans kollegor fann bl.a. att koagulationsfaktorn FIX bildar komplex med Ad och fungerar som brygga mellan Ad typ 5 (Ad5) och celler i levern (Shayakhmetov et al., 2005). De visade också att detta kompleks binder till kolhydrater på cellytan som kallas för heparan sulfat. Kort efter denna upptäckt, visade en annan grupp att koagulations faktorerna FX, FVII & protein C, också fungerar som bryggor mellan Ad5 och leverceller (Parker et al., 2006).


I detta projekt har jag arbetat med Ad5, Ad31, Ad18 och Ad12 och med de typer av celler som dessa Ad typer brukar infektera; celler från andningsvägar, ögon och tunntarm. Våra resultat tyder på att Ad5, Ad31 och Ad18 kan använda koagulationsfaktorer, (Ad5 använder FIX och FX, medan Ad31 och Ad18 endast använder FIX), som bryggor vid binding till och infektion av dessa celler (Jonsson et al., 2009, Lenman et al., 2011). Vi fann också att dessa komplex mellan Ad och koagulationsfaktorer band till heparan sulfat cell på cellytan.

Från dessa upptäckter kan man förmoda att Ad har utvecklats att använda sig av koagulationsfaktorer för att lättare binda och infektera celler utanför levern, dvs celler som motsvarar Ad tropism, även när CAR inte finns tillgängligt.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>Ads</td>
<td>Adenoviruses</td>
</tr>
<tr>
<td>AD</td>
<td>Adenoid-degenerating agent</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenovirus death protein</td>
</tr>
<tr>
<td>AHC</td>
<td>Acute haemorrhagic disease</td>
</tr>
<tr>
<td>APC</td>
<td>Adenoidal-pharyngeal-conjunctival</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AP</td>
<td>Activation peptide</td>
</tr>
<tr>
<td>ARD</td>
<td>Acute respiratory disease</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxackie- and adenovirus receptor</td>
</tr>
<tr>
<td>C4BP</td>
<td>Complement component C4 binding protein</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
</tr>
<tr>
<td>DSGL-2</td>
<td>Desmoglein-2</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl phosphatidylcholine</td>
</tr>
<tr>
<td>E genes</td>
<td>Early genes</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EKC</td>
<td>Epidemic keratoconjunctivitis</td>
</tr>
<tr>
<td>FIX, FX, FVII etc.</td>
<td>Coagulation factor IX, X, VII etc.</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>G/I</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>Gla</td>
<td>γ-carboxy glutamate</td>
</tr>
<tr>
<td>Hep I and III</td>
<td>Heparinase I and III</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>HVR</td>
<td>Hyper variable region</td>
</tr>
<tr>
<td>ICTV</td>
<td>International committee on taxonomy of viruses</td>
</tr>
<tr>
<td>Lf</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>L genes</td>
<td>Late genes</td>
</tr>
<tr>
<td>LPLD</td>
<td>Lipoprotein lipase deficiency</td>
</tr>
<tr>
<td>LRP</td>
<td>Low-density lipoprotein related protein</td>
</tr>
<tr>
<td>MHC I</td>
<td>Major histocompatibility complex I</td>
</tr>
<tr>
<td>MLP</td>
<td>Major late promoter</td>
</tr>
<tr>
<td>NIID</td>
<td>National institute for infectious diseases (Japan)</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>PC</td>
<td>Protein C</td>
</tr>
<tr>
<td>RGD</td>
<td>(arg-gly-asp)-motif</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SR</td>
<td>Scavenger receptor</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TP</td>
<td>Terminal protein</td>
</tr>
<tr>
<td>VA RNA</td>
<td>Virus associated RNA</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>wt</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
Publication list

Paper I
Coagulation factors IX and X enhance binding and infection of adenovirus types 5 and 31 in human epithelial cells.
Mari I. Jonsson, Annasara E. Lenman, Lars Frängsmyr, Cecilia Nyberg, Muhamed Abdullahi and Niklas Arnberg.

Paper II
Coagulation factor IX mediates serotype-specific binding of species A adenoviruses to host cells.
Annasara Lenman, Steffen Müller, Mari I. Nygren, Lars Frängsmyr, Thilo Stehle and Niklas Arnberg.

Not included in this thesis:
Adenoviruses use lactoferrin as a bridge for CAR-independent binding to and infection of epithelial cells.

Aim of thesis

This thesis characterizes the interactions between wt Ads and components in body fluids during Ad binding to cells and infecting their natural target cells in vitro. In addition, this thesis identifies Ad capsid proteins and cellular receptors that are involved in these interactions.
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1 Introduction

1.1 History

In 1953, Rowe and his colleagues, while searching for the agent responsible for respiratory infections, first identified adenoviruses (Ads). Using human adenoids, they isolated an agent: adenoid-degenerating (AD) agent (Rowe et al., 1953). In 1954, Hilleman and Werner isolated an unknown agent from human tracheal cells in a study of influenza among military recruits (Hilleman & Werner, 1954). These new agents- Rowe´s AD and Hilleman and Werner´s unidentified agent- and another new agent causing acute respiratory disease (ARD) among military recruits were found to be related (Riley-Vargas et al., 2004). These agents comprised a group that shared several clinical syndromes: sore throat, fever, cough, etc. Huebner and his associates proposed the term adenoidal-pharyngeal-conjunctival (APC) agents as the group name (Huebner et al., 1954). By this time, many names were used for infectious agents that were probably the same. In 1956, this problem of terminology was solved when these new respiratory-tract agents were named adenoviruses (Enders et al., 1956). Since then, several types of avian, reptilian, amphibian, and other mammalian Ads, including nearly 60 types of human Ads, have been isolated and characterized. Adenoviruses were initially found to cause the common cold and later also for other respiratory, intestinal, and ocular diseases. In 1962, Trentin et al. discovered that human Ad type 12 can cause cancer in hamsters (Trentin et al., 1962). However, Ads have not yet been found to be a causative agent of cancer in humans (Green et al., 1980). This ability of Ads to induce tumours made Ads interesting as a model in the study of oncogenesis. Adenoviruses have also been used as models for studies of a large number of other cell biology functions such as splicing of mRNA and DNA replication. Today, Ads are extensively explored as gene therapy vectors.

1.2 Classification

According to the International Committee on Taxonomy of Viruses (ICTV), the hierarchy for adenoviral taxa is family, genus, and species. Ads are further divided into types, genotypes, and isolates. Here, I will only explain how Ads are classified according to family, genus, species, and types.

The family of Ads is called Adenoviridae, which is further divided into five genera (according to ICTV): Mastadenovirus (isolated from mammals); Aviadenovirus (isolated from birds); Atadenovirus (isolated from reptiles and birds); Siadenovirus (isolated from reptiles and birds); and Ichtadenoviruses (isolated from fish). Species were formerly called subgenera or subgroups. Human Ads are divided into seven species: A, B, D, E, F, and G. Different species can be distinguished from one another by studying hemagglutination properties, phylogenetic distance, homology, tropism/symptoms, and many other methods (Benko, 2012). The conserved regions in the core of the hexons appear to be appropriate for distinguishing among different genera and species (Wadell, 2000, Ebner et al., 2005). To date, nearly 60 types of Ads have been isolated. Methods used to distinguish different Ad-types include haemagglutination-inhibition test (HI), neutralization test and homology studies (Wadell, 1999, Benko, 2012).
The hypervariable regions (HVRs) localized at the top of the hexons can be used to characterize the types within particular species (Crawford-Miksza & Schnurr, 1996, Ebner et al., 2005, Norrby & Wadell, 1969). In addition, fibres carry type specificities (Wadell et al., 1980, Norrby & Wadell, 1969). According to the methods mentioned above, Ads have been divided into species and types (Table 1).

Currently, the adenovirus research community has not fully agreed on how to classify Ads although two classifications have been proposed: the system should primarily be based on genomic sequences (Seto et al., 2011) or both genomic sequences and on established serological typing-methods (Aoki et al., 2011). However, the following table is still commonly accepted.

Table 1. Ad taxonomy and tropism.

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotype/ Type</th>
<th>Tropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
<td>Intestine, Airways</td>
</tr>
<tr>
<td>B</td>
<td>3, 7, 11, 14, 16, 21, 34, 35, 50, 55</td>
<td>Eyes, Airways, Urinary tract</td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6, 57</td>
<td>Eyes, Airways, Tonsils, Liver</td>
</tr>
<tr>
<td>D</td>
<td>8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54, 56</td>
<td>Eyes, Intestine</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>Eyes, Airways</td>
</tr>
<tr>
<td>F</td>
<td>40, 41</td>
<td>Intestine</td>
</tr>
<tr>
<td>G</td>
<td>52</td>
<td>Intestine</td>
</tr>
</tbody>
</table>

Note: Ads have only been investigated and classified as serotypes up to Ad51. Adenovirus 52 and above are only named as types.

1.3 Structure

The Ad is a non-enveloped DNA virus about 90 nm in diameter. Structural proteins build up the protein shell (the capsid) of the Ad particle containing the double stranded (ds) DNA and some non-structural proteins. These proteins are designated with Roman numerals in order of decreasing molecular mass. No. I is obliterated, as this “protein” has been found to be an aggregate of molecules. The proteins are divided into major, minor and core proteins (Table 2, Figure 1.). The major and the minor proteins are structural proteins, whereas the core proteins are often referred to as non-structural proteins. In 1966, the major proteins were named hexon (protein II), fibre (IV), and penton base (III) (Ginsberg et al., 1966)
1.3.1 Major capsid proteins: hexon, fibre and penton base

The major proteins of Ads constitute the icosahedral capsid of the virus particle, which contains a 35-kb long ds DNA. At each of the 12 corners of the virus particle, a penton resides that consists of the penton base with a protruding fibre. A fibre knob is situated at the end of each fibre. The icosahedral structure of Ad and the major capsid proteins were discovered in 1959 (Horne et al., 1959). Each hexon is surrounded by six neighbouring hexons, and each penton base is surrounded by five hexons. The hexon is a major brick that builds the capsid, whereas the fibre is responsible for attachment to cells and the penton base is responsible for internalization of the Ads into cells.

1.3.1.1 The hexon

The most abundant of the capsid proteins in the Ad particle are the hexons; each Ad particle consists of 240 hexon trimers. The hexon consists of both variable and conserved regions (Pring-Akerblom & Adrian, 1993). At the top of each hexon-monomer, there are seven flexible type-specific loops: the hypervariable regions (HVRs) (Crawford-Miksza & Schnurr, 1996). The HVRs render the Ad types their specific antigenicity, as different HVRs bind to different receptors, antibodies, or cells.

1.3.1.2 The fibre

The fibre, which mediates attachment of the virus to the primary cellular receptor, consists of three monomers: a trimer. One of these trimers is situated at each corner of the Ad particle. Most Ad-types have only one fibre, but Ad40 and Ad41 have two different fibres (one long and one short). Each fibre monomer is composed of three domains:

1. An N-terminal domain that binds to the penton base.
2. A central shaft with slight flexibility. The shaft is composed of repeats of an approximately 15-residue long motif. Different types of Ads have different no of repeats, from six (Ad3) to 22 (Ad2 & Ad5).
3. A C-terminal domain; fibre knob, that binds to the primary cellular receptor.

1.3.1.3 The penton base

The penton base is a pentameric protein where each monomer contains conserved RGD (arg-gly-asp)-motifs. These motifs bind to secondary cellular receptors during internalization of the Ad particle. The pentameric penton base and the trimeric fibre form a complex - a penton - at each corner of the virion (Berk, 2007). The RGD-motif is conserved in all Ads except in species F Ads - Ad40 and Ad41 (Albinsson & Kidd, 1999).

1.3.2 Minor capsid proteins cement proteins IIIa, VI, VIII, and IX

The minor capsid protein IIIa is involved in assembly, protein VI participates in disruption of the endosomal membrane during infection and proteins VIII and IX stabilize interactions between hexons (Berk, 2007). These are the known functions of minor capsid proteins, but these proteins may also have other, so far unidentified, functions.
### 1.3.3 Non-structural proteins core proteins

The terminal protein (TP), and the polypeptides V, VII, and X (the latter also known as µ: Mu) bind the ds linear Ad DNA, forming the core of the particle. Other examples of non-structural proteins include DNA-binding protein (DBP), which binds viral DNA and initiates replication, viral protease p23, which cleaves and subsequently completes the assembly of certain precursor proteins into infectious virions and Ad death protein (ADP), which promotes virus release from cells (Berk, 2007).

#### Table 2. Adenovirus proteins

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Type of protein</th>
<th>No of proteins in Ad2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexon; pII</td>
<td>Major structural</td>
<td>240 trimers</td>
</tr>
<tr>
<td>Penton base; pIII</td>
<td>Major structural</td>
<td>12 pentamers</td>
</tr>
<tr>
<td>pIIIa</td>
<td>Minor structural</td>
<td>60 monomers</td>
</tr>
<tr>
<td>Fibre; pIV</td>
<td>Major structural</td>
<td>12 trimers</td>
</tr>
<tr>
<td>pV</td>
<td>Core protein</td>
<td>-</td>
</tr>
<tr>
<td>pVI</td>
<td>Minor structural</td>
<td>60 hexamers</td>
</tr>
<tr>
<td>pVII</td>
<td>Core protein</td>
<td>-</td>
</tr>
<tr>
<td>pVIII</td>
<td>Minor structural</td>
<td>-</td>
</tr>
<tr>
<td>pIX</td>
<td>Minor structural</td>
<td>80 trimers</td>
</tr>
<tr>
<td>μ; pX</td>
<td>Core protein</td>
<td>-</td>
</tr>
<tr>
<td>TP</td>
<td>Core protein</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 1. A schematic picture of the Ad particle with the structural proteins: major, minor and, core proteins. Reprinted from (Nemerow et al., 2009) with permission from the corresponding author and the publisher.
1.4 Adenovirus life cycle

The Ad life cycle can be divided into six steps:

1. Adsorption;
2. Penetration/Internalization;
3. Uncoating;
4. Replication, transcription, and translation;
5. Assembly of virus particles; and

These steps are divided into the early phase (step 1-4) and the late phase (step 4-6). The early and late phases are separated by the onset of viral replication. The early phase ends after the expression of early genes (step 4), and the late phase begins with the expression of late genes (step 4). During the early phase, which lasts for about five to six hours, so-called early proteins are produced. These proteins modulate expression of viral late genes and host cell genes. Late genes, which are expressed during the late phase, encode structural proteins, which build up the Ad capsid (Berk, 2007).

Figure 2. Adenovirus life cycle. CAR, coxsackie- and adenovirus receptor; DBP, DNA-binding protein; E, early; L, late; MLP, major late promoter; pTP, precursor terminal protein; TP, terminal protein. Reprinted from (Lenaerts et al., 2008) with permission from the corresponding author and the publisher.
Description of the main steps of the Ad life cycle in vitro:

1. **Adsorption**
   The fibre on the Ad particle bind to the cellular receptor.

2. **Penetration/Internalization**
   The RGD motif on the penton base on the Ad particle then binds to the cellular integrins αvβ3 or αvβ5 (Wickham et al., 1994, Wickham et al., 1993). The Ad ends up in an endosome in the cell through a clathrin coated membrane invagination (Varga et al., 1991).

3. **Uncoating**
   The low pH in the endosome dissociates the fibres and the protein VI from the capsid (Greber et al., 1993). The pVI disrupts the endosomal membrane and the Ad is released from the endosome (Greber et al., 1993, Wiethoff et al., 2005). The partially dissociated Ad particle is then transported to the nucleus along microtubules (Mabit et al., 2002, Kelkar et al., 2006, Leopold et al., 2000). Protein VI has also been shown to be involved in the transport of Ads to the nucleus along microtubules (Wodrich et al., 2010). At the nuclear pore complex (NPC), Ad DNA is imported into the nucleus (Greber et al., 1997).

4. **Replication, transcription, and translation**
   In the nucleus, transcription (by cellular RNA-polymerase II (Peterson, 1986)) and replication (by viral DNA-polymerase) of Ad DNA occurs. The mRNA is transported into the cytosol where it is translated to proteins. The human Ad genome contains five early transcription units (E1A, E1B, E2, E3, and E4), three delayed early transcription units (IX, IVa2 and E2 late), and one late transcription unit (major late), which is processed to give five families of late mRNA (L1 to L5). The replication and transcription of Ad genes take place in the following order:
   A. **Transcription of early genes**
      Early genes encode viral DNA polymerase, proteins that stimulate further expression of viral genes or down regulate the expression of cellular genes, proteins that modulate the cellular immune response to viral infections, etc.
   B. **Replication of viral DNA**
      During replication, large amounts of viral DNA are generated for further gene expression and packaging into new virions.
   C. **Transcription of late genes.**
      The replication of viral DNA results in the activation of the delayed early genes and the late genes. Delayed early genes encode proteins needed for viral replication and packaging of capsids. Activation of the Ad major late promoter (MLP) leads to transcription of late genes, which encode structural proteins that build up the virus particle (i.e., hexon, fibre, and penton) and proteins that mediate virus assembly, etc.

   In addition, Ads also transcribe a set of RNAs that are not translated, termed the virus associated RNAs (VA RNAs) which take part in combating cellular defence mechanisms.

5. **Assembly of virus particles**
   Late proteins are transported from the cytosol to the nucleus where the assembly of virus particles occurs; structural proteins build up empty capsids, which are filled with viral DNA and core-proteins. Up to $10^5$ virus particles can be assembled in each cell.

6. **Exit**
   About three days following infection, Ad progeny are released by cell lysis, triggered by adenovirus death protein (ADP) (Tollefson et al., 1996).
1.5 Receptors

When Ads first encounter a cell, it has to bind to a primary cellular receptor to attach to the cell. This attachment takes place either directly to a cell-bound receptor (e.g., CAR), or indirectly via soluble components in body fluids (e.g., coagulation factors). After attachment to the primary receptor, internalization occurs as the result of Ads binding its cell-bound secondary receptor (integrins).

1.5.1 Cell-bound receptors

The first cell-bound receptor used by Ads was the coxsackie and adenovirus receptor (CAR), which was identified in 1997 (Bergelson et al., 1997, Tomko et al., 1997). After this discovery, several other cell membrane bound receptors for Ads have been found.

1.5.1.1 Coxsackie and adenovirus receptor (CAR)

CAR is a protein that belongs to the immunoglobulin super family. About 40 years ago, certain coxsackie viruses and Ads were found to infect cells by binding to the same cellular receptor (Lonberg-Holm et al., 1976). In 1997, CAR was identified as the cellular receptor for Ad2 and Ad5 and some coxsackie B viruses (Tomko et al., 1997, Bergelson et al., 1997). Since then, Ads from all species (except species B) have been found to bind CAR (Roelvink et al., 1998). CAR is expressed in tight junctions on epithelial cells in the respiratory tract, small intestine, brain, heart, pancreas, and liver (Tomko et al., 2000), on endothelial cells lining blood vessels (Carson et al., 1999, Vincent et al., 2004, Zanone et al., 2007) and on lymphatic endothelial cells (Vigl et al., 2009). CAR promotes cell-to-cell adhesion between epithelial cells. This adhesion is achieved at the outermost part of CAR; that is, D1 binds to D1 of another CAR-molecule on neighbouring cells (Cohen et al., 2001).

However, several findings suggest that CAR may not be the only, or not the main, cellular receptor used by Ads for binding to and infecting cells:

1. In vitro, where respiratory epithelial cells are non-polarized and CAR is expressed all over the cell surface, Ads easily infect the cells. In vivo, however, respiratory epithelial cells are polarized and express CAR only basolaterally and laterally. In vivo, when Ads first encounter respiratory epithelial cells via the apical side, the infection-ability of Ads via CAR is poor (Walters et al., 1999, Excoffon et al., 2010, Zabner et al., 1997).
2. In vivo, the expression of CAR is low or absent on primary T-cells (Chen et al., 2002), which is a natural target cell type for species C Ads.
3. When Ads produce capsid proteins in the cells, an excess of fibres is made. These free fibres are secreted basolaterally and bind CAR molecules, which are localized on the basolateral and lateral membranes. Fibre disrupts CAR-CAR dimers resulting in disruption of junctional integrity. This allows Ads to filter between the cells and escape from the apical side. In this way, CAR functions to release Ads from cells rather than to promote Ads binding to cells (Walters et al., 2002). This is a totally different function of CAR than the discovery of CAR as a receptor for Ads.
4. Removal of the CAR-binding ability of adenoviral vectors does not change their capacity to transduce cells in vivo (Alemany & Curiel, 2001).
1.5.1.2 Integrins
Integrins have many functions: they make cells adhere to each other and to the extracellular matrix, they function in intracellular signalling, they function as cellular receptors for pathogens, etc. Integrins are composed of one α-subunit and one β-subunit, which can dimerize into about 24 combinations. Ads initially bind cells via receptors other than integrins (e.g., CAR, coagulation factors). After this initial binding, Ads bind to integrins for internalization into the cells. The first integrins shown to be used by Ads in vitro were the dimers αvβ3 and αvβ5 (Wickham et al., 1993). It has been shown that Ads bind to these integrins via the RGD motif on the penton base (Wickham et al., 1993, Bai et al., 1993) In addition, several other integrin-dimers on cells that Ads bind have been identified.

Ads have also been shown to bind other membrane bound primary receptors, sialic acids (Arnberg et al., 2000a, Arnberg et al., 2000b), CD46 (Defer et al., 1990, Gaggar et al., 2003, Marttila et al., 2005, Sirena et al., 2004, Persson et al., 2009), desmoglein-2 (DSGL-2) (Wang et al., 2011), scavenger receptors (SR) (Xu et al., 2008), CD80 and CD86 (Short et al., 2006), vascular cell adhesion molecule-1 (VCAM-1) (Chu et al., 2001), MHC I (Hong et al., 1997), and HSPG (Smith et al., 2003, Dechecchi et al., 2001). The functions of CD80, CD86, VCAM-1, MHC I, SRs, and HSPG have not been confirmed, so more research is needed to uncover their importance as receptors for Ads.

1.5.2 Soluble components
In contrast to directly binding to cellular receptors, Ads have also been reported to use soluble components in body fluids as adaptors that can bridge the virus to cellular receptors. Examples of soluble components used by Ads include coagulation factors, lactoferrin (Lf), and dipalmitoyl phosphatidylcholine (DPPC).

1.5.2.1 Coagulation factors
Coagulation factors, also called clotting factors, are proteins in the blood plasma that are involved in the forming of blood clots at sites of damaged blood vessels. The coagulation factors consist of about 13 proteins, indicated by Roman numerals (I-XIII), and their regulators. The coagulation factors are generally serine proteases, and activate other coagulation factors by cleaving them at specific serine residues. The coagulation factors are synthesized mainly in the liver, and circulate in the blood system as inactive enzyme precursors, so-called zymogens. Coagulation factors can be found in body fluids other than blood. They can be exudated from the plasma to the respiratory system as a response to inflammation in the respiratory epithelium (Battista et al., 1980, Persson et al., 1991). As a response to inflammation, various coagulation factors can also be produced directly by bronchial epithelial cells (Perrio et al., 2007) or secreted from macrophages (Fox, 2010). Moreover, FIX has been found in saliva (Denny et al., 2008, Nour-Eldin & Wilkinson, 1957).

The coagulation cascade is divided into three phases: the initiating phase, the amplification phase, and the propagation phase. The initiating phase is also called the extrinsic or tissue factor (TF) pathway. The amplification phase and the beginning of
the propagation phase are also called the intrinsic or the contact pathway. The rest of the coagulation cascade is common for the intrinsic and the extrinsic pathways. The initiation phase is started upon vascular injury when TF on the negatively charged surfaces of TF-presenting cells or macrophages is exposed to coagulation factors in the blood. Tissue factor is expressed by cells that are normally not exposed to flowing blood, such as sub-endothelial cells and cells surrounding blood vessels. When a blood vessel is injured, TF is exposed to coagulation factors in the blood. This exposure starts a cascade of activation of coagulation factors, which ends with the final propagation phase, in which fibrin forms a clot at the site of the wound. Because most of these reactions require Ca\(^{2+}\)-ions, citrate and EDTA work as anti-coagulants for blood samples. When a clot clogs a wound, the coagulation cascade has fulfilled its purpose and is terminated by a control system. Protein C (PC), which takes part in the control system of coagulation, deactivates certain coagulation factors, terminating the formation of the fibrin clot.

The vitamin K dependent zymogens FVII, FIX, FX, and PC share a common domain architecture; they all consist of five protein domains (Figure 3). These are the γ-carboxy glutamate (Gla) domain, two copies of the epidermal growth factor (EGF) domain, an activation peptide (AP), and a C-terminal catalytic domain, which carries out the catalytic cleavage. When the zymogen is cleaved at serine residues in the AP, the zymogen is activated and can cleave other precursors. The N-terminal EGF domain in FIX and FX has been shown to be partly responsible for binding TF (Zhong et al., 2002). The Ca\(^{2+}\)-ion dependent Gla-domain binds phospholipids in the cell surface membrane (Freedman et al., 1996). This Gla domain of FX has also been shown to bind the hexon of Ad5, and the catalytic domain of FX has been shown to bind HSPG on hepatocytes (Waddington et al., 2008, Alba et al., 2012).

![Figure 3. A schematic picture of the structure of the vitamin K dependent zymogens. GLA: γ-carboxy glutamate; EGF: epidermal growth factor; AP: activation peptide; and the catalytic domain.](image)

In 2005, Shayakhmetov et al. found that the soluble component FIX could function as a bridge between Ad5 and hepatocytes (Shayakhmetov et al., 2005). The cellular receptor for FIX was found to be HSPG. Not long after this discovery, the coagulation factors FX, protein C, FIX, and FVII were also found to bridge Ad5 to hepatocytes (Parker et al., 2006). Heparan sulphate proteoglycans consist of core proteins linked to polysaccharides. Heparan sulphate (HS), a sulphated polysaccharide produced by most eukaryotic cells, are expressed on the cell surface (Bernfield et al., 1999). By interacting with a large number of ligands, HSPGs influence a number of biological processes, such as cell-cell and cell-matrix adhesion, motility, growth, and signalling (Varki, 1993, Bernfield et al., 1999). Heparan sulphate proteoglycans have been shown to serve as cellular receptors for a number of bacteria, parasites, and viruses (Rostand & Esko, 1997).
Other soluble components in body fluids used by Ads for binding to cells include Lf (Johansson et al., 2007) and DPPC (Balakireva et al., 2003). The importance of Lf and DPPC as soluble components that bridge Ads to cellular receptors has not been validated; more research is needed.

1.6 Pathology and epidemiology

Adenovirus infections occur worldwide in humans and in animals, although human Ad types are generally not pathogenic to animals and vice versa (Wold, 2007). Animals that can become infected by Ads include birds, cows, monkeys, dogs, and cats. Adenoviruses enter the host via the mouth, the nasopharynx, or the ocular conjunctiva. The virus then multiplies initially in the pharynx, conjunctiva, or the small intestine, which means that Ad-infections mainly occur in these regions. Epithelial cells are the primary cell type to be infected by Ads (Boyer et al., 1959). When species C Ads reach and infect the tonsils (which are aggregates of lymphoid tissues located in the pharynx), these can persist for months or years without viral production and thus giving no symptoms (Neumann et al., 1987). After stimulation, these cells can cause viral reactivation resulting in RNA transcription, DNA replication, and infectious virus production (Garnett et al., 2009).

Respiratory infections commonly lead to pharyngitis (Ad1-7), acute respiratory disease (ARD) (Ad4, 7), and pneumonia (Ad3, 7). These diseases are particularly common in young children and military recruits. Acute respiratory disease is an acute febrile respiratory infection of short duration characterized mainly by cough and sore throat. Some other types, including Ad31, have also been reported to cause respiratory infections in otherwise healthy immune competent individuals (Wang & Feldman, 1976, Johansson et al., 1989, Adrian & Wigand, 1989, Johansson et al., 1991, Harsi et al., 1995).

Infections of the conjunctiva tend to develop into pharyngo-conjunctival fever (Ad3, 4, 7), which may be spread via swimming pools, or epidemic keratoconjunctivitis (EKC) (Ad8, 19, 37). Pharyngo-conjunctival fever can also be caused by Ad5 (National Institute for Infectious Diseases; NIID, in Japan, http://www.nih.go.jp/niid/en/iasr-e.html). All species C viruses can infect the eye. In Japan, for example, they accounted for 25-50% of all pharyngo-conjunctival fever cases between 2008 and 2012. In the United States, Ad5 (and Ad8, 19, and 37) are most commonly associated with ocular diseases (Gordon et al., 1996).

Enteric infections may be caused by species F Ads (Wold, 2007), but also by other species, such as species A Ads (Schmitz et al., 1983, Adrian & Wigand, 1989). Most of the enteric infections are subclinical, but some enteric infections cause diarrhoea. Infections by species A Ads are relatively common. Among immune competent individuals, the prevalence of neutralizing antibodies to Ad31 and Ad18 has been shown to be only second to members of species C and Ad3 (Vogels et al., 2003). In immunocompromised patients, Ad31 may also cause pneumonia and hepatitis (Hierholzer, 1992). Infections caused by Ad31 in immunocompromised patients tend
to be more severe than those caused by other species A or other Ads (Hierholzer, 1992, Johansson et al., 1991).

Adenoviruses may also reach the bloodstream, and cause viremia (Wold, 2007). In this way, other organs (e.g., the kidney) may become infected by Ads (Wold, 2007). Adenoviruses can cause genitourinary infections, that may develop into cervicitis and urethritis (both caused by Ad37) or cystitis (Ad11). In young boys, Ad11 and Ad21 may cause acute haemorrhagic disease (AHC) (Wold, 2007). Occasionally, the liver is infected by Ads, but the route of infection is unknown, perhaps by latent Ads that have infected transplanted organs (e.g., lymphocytes) (Wold, 2007) or via viremia. However, hepatitis in immune competent humans is probably not caused by Ads.

The immunocompromised host is a patient with an immunodeficiency disease (e.g., AIDS) or a patient being treated with cytotoxic and immunosuppressive drugs (e.g., cancer patients or patients undergoing organ or tissue transplantation). In immunocompromised patients, Ad infections may spread throughout the whole body, becoming generalized, and localized to the respiratory tract, liver, urinary tract, abdominal organs, etc. (Hierholzer, 1992). Patients who are immunocompromised are not more often infected with Ads than immune competent hosts, but the outcome of the infection may be more serious and even fatal (Wold, 2007). The symptoms caused by Ads resemble those seen in immune competent individuals with a few additions listed in Table 4. Most common diseases caused by Ads in immunocompetent individuals and immunocompromised patients are listed in Table 3 and 4, respectively.

The seroprevalence (prevalence of neutralizing antibodies in serum) towards Ads has been determined in several studies: the seroprevalence towards species A-E among 100 individuals from Belgium was examined by Vogels et al. (2003), and the seroprevalence towards species F among 300 individuals in Japan and among 300 individuals in Bangladesh have been studied by Shinozaki et al. (1987) and Jarecki-Khan and Unicomb (1992), respectively (Table 5). These studies showed that antibodies to species C Ads were the most common, followed by antibodies to species A > species B (Ad3 and Ad7) > species E > species D > species F (Vogels et al., 2003, Shinozaki et al., 1987, Jarecki-Khan & Unicomb, 1992). In the case of Ad5, 85-90% of the neutralizing antibodies are directed towards the hexon, whereas the rest of the antibodies are probably directed towards the fibre and the penton (Roberts et al., 2006).
Table 3. Common diseases caused by Ads in immunocompetent individuals.

<table>
<thead>
<tr>
<th>Species</th>
<th>Site of infection</th>
<th>Disease</th>
<th>Common Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-C, E</td>
<td>Respiratory tract</td>
<td>Pharyngitis</td>
<td>Ad1-7 (B, C &amp; E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ARD</td>
<td>Ad4 &amp; 7, 14 (E &amp; B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pneumonia</td>
<td>Ad3 &amp; 7 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(in some cases Ad31, 14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tonsillitis</td>
<td>Ad5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenoiditis</td>
<td>Ad5</td>
</tr>
<tr>
<td>B-E</td>
<td>Ocular tract</td>
<td>Pharyngoconjunctival fever</td>
<td>Ad3, 4 &amp; 7 (B &amp; E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EKC</td>
<td>(in Japan also Ad5)</td>
</tr>
<tr>
<td>A, F</td>
<td>Gastrointestinal tract</td>
<td>Enteritis</td>
<td>Ad40, 41 (F) &amp; 31 (A)</td>
</tr>
<tr>
<td>B, D</td>
<td>Genitourinary tract</td>
<td>Cervicitis</td>
<td>Ad37 (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urethritis</td>
<td>Ad37 (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cystitis</td>
<td>Ad11 (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AHC</td>
<td>Ad11, 21 (B)</td>
</tr>
</tbody>
</table>

ARD: Acute Respiratory Disease; EKC: Epidemic Keratoconjunctivitis; AHC: Acute Haemorrhagic Cystitis. References to the data presented in this table can be found in the text above and in (Wold, 2007).

Table 4. Common diseases caused by Ads in immunocompromised patients. These correspond to infections in immunocompetent individuals with the additions shown in this table.

<table>
<thead>
<tr>
<th>Species</th>
<th>Site of infection</th>
<th>Disease</th>
<th>Common Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-F</td>
<td>Respiratory tract</td>
<td>Pneumonia</td>
<td>Ad2-5, 7, 31, 34, 35,</td>
</tr>
<tr>
<td>A, C</td>
<td>Liver</td>
<td>Hepatitis</td>
<td>Ad31, 1, 2, 5</td>
</tr>
<tr>
<td>A, C</td>
<td>Gastrointestinal tract</td>
<td>Enteritis</td>
<td>Ad31, Ad5</td>
</tr>
<tr>
<td>B</td>
<td>Genitourinary tract</td>
<td>AHC</td>
<td>Ad 7, 11, 34, 35</td>
</tr>
</tbody>
</table>

AHC: Acute Haemorrhagic Cystitis. References to the data presented in this table can be found in the text above and in (Hierholzer, 1992).
Table 5. Seroprevalence towards Ads species A-F.

<table>
<thead>
<tr>
<th>Species</th>
<th>Seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40-70%</td>
</tr>
<tr>
<td>B</td>
<td>40-70% (Ad3 &amp; Ad7), &lt;20% (the rest of species B)</td>
</tr>
<tr>
<td>C</td>
<td>50-80%</td>
</tr>
<tr>
<td>D</td>
<td>5-35%</td>
</tr>
<tr>
<td>E</td>
<td>45%</td>
</tr>
<tr>
<td>F</td>
<td>10-50%</td>
</tr>
</tbody>
</table>

References to the data presented in this table can be found in (Vogels et al., 2003, Shinozaki et al., 1987, Jarecki-Khan & Unicomb, 1992).

1.7 Viral vectors.

Adenoviruses have several features that make them suitable as vectors: Ads can be prepared in high quantities and Ads do not integrate their genome into the host genome, so they pose minimal risk for insertion mutagenesis and cancer. Moreover, as Ads are non-enveloped, they are stable for packaging as lyophilized preparations. This stability makes it possible to store and transport the Ads so patients can receive them without a need for a cold chain (Khare et al., 2011). Until 2013, Ads were, with 23.2%, the main viral vector used in gene therapy clinical trials worldwide (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Most of the recombinant adenoviral vectors used in gene therapy applications are based on serotypes 2 and 5 (Schmitz et al., 1983), but other serotypes are continuously being explored as vectors.

Despite huge efforts and resources put into gene therapy, only very few viral-based vectors have been approved. In 2003, China was the first country in the world to license a gene therapy vector to treat cancer (Pearson et al., 2004). This viral vector, called Gendicine, consists of an Ad5-backbone with the p53-gene, a naturally occurring tumour suppressor gene. A gene-therapy vector called Glybera, made by a Dutch firm, was given commercial approval in Europe for the first time in 2012 (http://www.uniqure.com/news/163/182/First-gene-therapy-in-Western-world-receives-positive-opinion-in-Europe-from-CHMP.html). Glybera is an aden-associated viral (AAV) vector that contains the gene encoding an enzyme called lipoprotein lipase, which breaks down certain kinds of fat. The therapy is for a rare genetic condition called lipoprotein lipase deficiency (LPLD). People with the disorder cannot break down certain types of fat in the bloodstream.
2 Results and discussion

In this project, I have examined the mechanisms used by wt Ads for binding to and infection of natural target cells. This includes coagulation factors found in different body fluids and their interactions with virions and host cells.

2.1 Components in body fluids that promote Ad infection

We found earlier that human Lf, which is present in various body fluids, can bridge Ad5 to epithelial cells from the ocular and the respiratory tract and to T-cells (Johansson et al., 2007). Other groups have found that Ad5 can be bridged to lung epithelial cells via DPPC, a pulmonary surfactant present in the lungs (Balakireva et al., 2003), or to hepatocytes via C4BP, a component from the complement system, and FIX, a component from the coagulation system (Shayakhmetov et al., 2005). In 2006, it was found that FVII, FX and protein C, components from the coagulation system can bridge Ad5 to hepatocytes (Parker et al., 2006). Components from the complement system, which are involved in the host’s innate immune response to pathogens, and components from the coagulation system, which are involved in the clotting of blood, are mainly but not only present in the blood. Coagulation factors can be exudated from the plasma to the respiratory system as a response to inflammation in the respiratory epithelium (Battista et al., 1980, Persson et al., 1991). Various coagulation factors can also be produced directly by respiratory epithelial cells as a response to inflammation (Perrio et al., 2007). Coagulation factor IX has also been found in saliva (Denny et al., 2008, Nour-Eldin & Wilkinson, 1957), suggesting that Ads in the mouth can use coagulation factors for binding to and infection of cells. A plausible scenario is that coagulation factors exist in many body fluids after exudation from plasma, but no evidence exists for this. We hypothesized that they may also be present in other body fluids, such as those that surround the cells and tissues that are infected by Ads. As a result of these findings, we investigated whether different body fluids had any impact on Ad infection of their natural target cells. We performed infection experiments with plasma, tear fluid, saliva, breast milk, human epithelial cells from the cornea (HCE) and the lung (A549), and one Ad type from each species (Table 6, corresponding to Table 1 in paper I). We found that infection by Ad5 (species C) was enhanced by plasma, tear fluid, and saliva, whereas infection by Ad31 (species A) was enhanced only by plasma. We saw a slight inhibiting effect by all body fluids on Ad belonging to species B and D to F. The reason for this inhibiting effect could be the presence of protective antibodies towards Ads, which neutralize the virus.
Table 6. Effects of body fluids on Ad infection of HCE and A549 cells. (This table corresponds to Table 1 in paper I).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plasma</th>
<th>Tear fluid</th>
<th>Saliva</th>
<th>Breast milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCE</td>
<td>A549</td>
<td>HCE</td>
<td>A549</td>
</tr>
<tr>
<td>Ad31 (A)</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ad3 (B1)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ad35 (B2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ad5 (C)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Ad37 (D)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ad4 (E)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ad41 (F)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

“+” and “++” denote a 0 to 300% and a >300% increase in the number of infected cells, respectively, whereas a dash denotes no increase or a small decrease (<50%).

HCE: human corneal epithelial cells; A549: human lung epithelial cells.

2.2 FIX and FX promote Ad5-, Ad31-, and Ad18-infection, but not Ad12-infection

As mentioned above, other groups have found that certain complement components and coagulation factors can promote Ad5 infection of hepatocytes (Shayakhmetov et al., 2005, Parker et al., 2007). As these components are present mainly in blood and as we found that blood promoted both species C and A infection (Table 5), we screened a set of complement components and coagulation factors to elucidate whether these components could be responsible for our results. We performed infection and binding experiments with Ad5 and all species A Ads: Ad31, Ad18 and Ad12. We used the complement components C3b, iC3b, C3dg, C4b, and C4dg, the coagulation factors FIX, FX, FVII, and protein C, and epithelial cells that correspond to the natural target cells of species C and species A Ads: HCE-, A549-, and epithelial cells originating from the intestine: FHs74Int-cells.

We found that FIX and FX enhanced Ad5-binding to and infection of cells, whereas only FIX could promote Ad31- and Ad18-binding to and infection of cells. None of the tested coagulation factors could promote Ad12-infection of cells. Results from infection experiments with Ad5 and species A Ads in A549 cells are shown in Figure 4 (Figure 4a corresponds to Figure 1a in paper I, and Figure 4b corresponds to Figure 4a in paper I and 1b in paper II). Similar results were obtained in experiments in HCE- and FHs74Int-cells (data not shown).

Coagulation factor X has been shown to bind the HVRs 5 and 7 of the Ad5 hexon (Alba et al., 2009, Waddington et al., 2008). Because FIX and FX are highly homologous (Leytus et al., 1986), we assumed that the Ad31:FIX interaction is also mediated by the hexon protein. Therefore, we investigated the different complexes...
between FIX, FX, and hexons from various Ad types in detail using surface plasmon resonance (SPR) (Figure 2 and Figure 3 in paper II). SPR-experiments were performed as follows: ligands (Ad5, Ad31, or Ad12 hexons) were immobilised on a chip and the analyte (FIX or FX) was injected to flow over the chip. In this way, we obtained affinity data for Ad31:FIX, Ad5:FX, and Ad12:FIX complexes. To obtain data from the Ad5:FIX interaction, we changed places of the ligand and analyte. That is, we immobilized FIX and injected Ad5 hexon as analyte. From these SPR experiments, we concluded that the Ad31 hexon binds to FIX with similar affinity (nM) as the Ad5 hexon binds to FX or FIX, whereas FX does not bind to the Ad31 hexon at all (data not shown). Moreover, no binding was detected between FIX or FX and the Ad12 hexon (data not shown). The Ad31 hexon:FIX complex was found to be the most stable complex, as the average $t_{1/2}$ of the Ad31 hexon:FIX complex has a 10-fold higher value than the Ad5:FIX/FX complexes. This implies that Ad31 has evolved to use FIX to a larger extent than Ad5 has evolved to use FX.

We also evaluated the homology of the HVR-5 and HVR-7 of species A Ads by comparing their amino acid sequences. We found a closer homology between Ad18 and Ad31 compared to Ad12. This homology explain why FIX only promotes Ad31 and Ad18, but not Ad12 binding to and infection of cells.

Figure 4a. FIX and FX promote Ad5 infection of A549-cells.  
(This figure corresponds to Figure 1a in paper I)
2.3 Low concentrations of FIX and FX promote efficient Ad-infection

Coagulation factors probably exist in several body fluids, but the concentrations of coagulation factors in body fluids other than blood is not known. Coagulation factors have been found in saliva (Denny et al., 2008, Nour-Eldin & Wilkinson, 1957) and may also be found in the respiratory system as a response to inflammation (Battista et al., 1980, Persson et al., 1991, Perrio et al., 2007). Depending on in which body fluid Ads are located, they may contact different concentrations of FIX and FX.

To test whether FIX and FX could exert their promoting effect on Ad binding to and infection of cells at other concentrations than at their physiological concentrations in plasma (Figure 4), we performed binding experiments with different FIX and FX concentrations. We found that FIX promoted Ad5-binding to and infection of target cells at physiological concentration (5 µg/ml in blood), whereas FX exerted similar effects at 0.1 µg/ml, corresponding to only 1% of its physiological concentration (10 µg/ml in blood). Also, only 1% of the physiological concentration of FIX in plasma was needed for efficient promoting effect of Ad31-binding to cells (Figure 5a and 5b). These figures correspond to: Figure 2a and Figure 4c, respectively, in Paper I. Our results from *in vitro* experiments, indicating that Ad5 and Ad31 can use FIX and FX at concentrations lower than plasma concentrations, suggest that FIX and FX in other
body fluids than blood may be of importance for Ad infections in vivo and at other locations than the liver, such as the respiratory tract, the eyes, or the intestines.

This hypothesis was further supported by the results from the SPR experiments: the affinity between Ad31 and FIX was strong (3nM) and this K_D value was much lower than the physiological concentration of FIX in plasma (85nM; 5µg/ml), indicating that very low concentrations of FIX may promote Ad31-binding.

![Figure 5a. FIX and FX promote binding of Ad5 to A549- and HCE-cells in a dose dependent manner. (This figure corresponds to Figure 2a in paper I)](image)

![Figure 5b. FIX promotes binding of Ad31 to A549- and HCE-cells in a dose dependent manner. (This figure corresponds to Figure 4c in paper I)](image)
2.4 Cellular receptors for Ad:FIX/FX complexes

2.4.1 FIX- and FX-mediated Ad5-binding to cells are dependent on HS

Heparan sulphate (HS) is a glycosaminoglycan (GAG) that occurs as a proteoglycan, such as HSPG, on cell surfaces. In this form, HS functions as a cell surface receptor for signalling peptides, microbes etc. and by promoting cell-cell adhesion (Bernfield et al., 1999). HSPG may function as cellular receptors on hepatocytes for Ads complexed with FIX or FX (Shayakhmetov et al., 2005, Parker et al., 2006). This interaction makes it easier for Ad5-based vectors, when complexed to FIX or FX, to bind and transduce this cell type. Adenoviruses that have reached the blood stream (mostly Ad-based vectors) are transported to the liver and then bridged to hepatocytes via FIX or FX. This bridging may explain why viral vectors based on Ad5 are entrapped in the liver instead of reaching their targets (such as tumours). Here, we wanted to investigate whether HS is a cellular receptor when FIX and FX promote Ad5- and Ad31-binding to and infection of HCE, A549, and FHs74Int cells. To test this, we performed binding and infection experiments using heparin (Figure 6) and heparinase I (Hep I) (Figure 7). Heparin is a highly sulphated, extracellular GAG and resembles the less sulphated, membrane-bound HS. Incubation of Ad5 and Ad31 with heparin decreased the promoting effect of FIX and FX, indicating that HS is needed for FIX- and FX-mediated Ad5 and Ad31 infection of cells (Figure 6a corresponding to Figure 3a in paper I and Figure 6b corresponding to Figure 5a in paper I). Heparinase I cleaves the membrane-bound HS and consequently inhibits binding of FIX/FX to HS on cells. Incubation of HCE, A549, or FHs74Int cells with Hep I diminished the promoting effect of FIX and FX on Ad5-binding to cells. This finding indicates that HS is a cellular receptor for FIX- and FX-mediated Ad5-binding to these cells. On the other hand, incubation of Ad31 with HCE, A549, and FHs74Int cells pre-treated with Hep I resulted in little or no difference in binding or infection, a finding that makes the importance of HS for Ad31 infections unclear (Figure 7a corresponding to Figure 3e in paper I and Figure 7b corresponding to Figure 6a in paper I). Thus, these experiments indicated that FIX-mediated Ad31-binding to epithelial cells may not include HS or may involve GAGs other than HS that are not sensitive to Hep I.

![Graph](image)

Figure 6a. Cell surface heparan sulphate is required for FIX- and FX-mediated Ad5-binding to A549-cells. (This figure corresponds to Figure 3a in paper I)
Figure 6b. Cell surface heparan sulphate is required for FIX-mediated Ad31-binding to A549-cells. (This figure corresponds to Figure 5a in paper I)

Figure 7a. Cell surface heparan sulphate is required for FIX- and FX-mediated Ad5-binding to A549-cells. (This figure corresponds to Figure 3e in paper I)

Figure 7b. Cell surface heparan sulphate does not seem to be required for FIX-mediated Ad31-binding to A549-cells. (This figure corresponds to Figure 6a in paper I)
2.4.2 **FIX-mediated Ad31-binding to cells is dependent on HS and/or LRP**

When Shayakhmetov et al. discovered that HSPG functions as a cellular receptor on hepatocytes for the Ad5:FIX complex, they also found that the cellular receptor LRP could have this function (Shayakhmetov et al., 2005). The LRP receptor is a transmembrane protein that functions in signal transduction and endocytosis of nutrients and vitamins. To evaluate whether FIX-mediated binding of Ad31 depends on HS and/or LRP, we first performed binding experiments using Lf, which can bind to both HS and LRP (Ji & Mahley, 1994). Lactoferrin is a positively charged iron-binding antimicrobial glycoprotein that binds a variety of GAGs (e.g., HS, chondroitin sulphate and heparin) and other sulphated polysaccharides (Mann et al., 1994). We found that Lf inhibited the promoting effect of FIX on Ad31-binding to FHs74Int-cell in a dose-dependent way (Figure 4 in paper II). These results indicated that FIX-mediated Ad31-binding depends on HS and/or LRP.

2.4.3 **FIX-mediated Ad31-binding to cells is dependent on GAGs, but not LRP**

To further evaluate whether FIX binds HS and/or LRP during enhancement of Ad31-binding to cells, we performed binding experiments with Chinese hamster ovary (CHO)-cells with different expression levels of GAGs (including HS) and LRP. We found that FIX promoted Ad31-binding efficiently to CHO-cells that express all GAGs, but also to cells that lack LRP. This finding implies that FIX-promoted Ad31-binding does not depend on LRP. We also found that FIX mediated a reduced Ad31-binding to cells that do not express HS and an even further reduced binding to GAG-deficient cells (Figure 5 in paper II). These findings indicate that GAGs other than HS may contribute to FIX-mediated Ad31-binding. In summary, these results suggested that FIX-mediated Ad31-binding to cells might depend on several GAGs (including HS), but not LRP. In similar binding experiments with Ad5, we found that both FIX and FX promoted Ad5-binding to CHO-cells expressing GAGs.

2.4.4 **HS is the most important GAG used for FIX-mediated Ad31-binding**

After discovering that the Ad31:FIX complex may bind several different GAGs (including HS), we wanted to identify which these GAGs are. We performed binding experiments using the following soluble GAGs: heparin, heparan sulphate, chondroitin sulphate A, chondroitin sulphate B (also called dermatan sulphate), and chondroitin sulphate C (Figure 6 in paper II). These GAGs differ in disaccharide construction and degree of sulphation, for example, heparin is highly sulphated and has the highest negative charge.

We found that the promoting effect of FIX or FX on Ad31-resp. Ad5-binding to FHs74Int-cells was strongly inhibited by heparin and heparan sulphate but only slightly inhibited by dermatan sulphate and chondroitin sulphate A. Similarly, the
promoting effect of FIX on Ad5-binding to FHs74Int-cells was strongly inhibited by heparin and slightly inhibited by heparan sulphate, dermatan sulphate, and chondroitin sulphate A. Chondroitin sulphate C did not influence the results in any of the experiments. These results imply that the most important cellular receptor for FIX-mediated Ad31-binding to cells is HS.

From these experiments performed with non-polarized cells in vitro, we hypothesized that FIX and FX may also promote Ad binding to HS in vivo, as HS is expressed apically on polarized cells in vivo (Mertens et al., 1996), unlike CAR (Zabner et al., 1997, Walters et al., 1999).

In paper I, we concluded that Hep I inhibited the promoting effect of FX on Ad5 infectivity, but Hep I only weakly inhibited the promoting effect of FIX on Ad31 infectivity. We then learned that there are different types of heparinases and that these cleave different domains of HS/heparin. Therefore, using other heparinases than Hep I, we set out to examine whether HS is a cellular receptor for the Ad31:FIX complex. We found that Hep III inhibited both FIX:Ad31 and FX:Ad5, but to different degrees: Hep III inhibited the promoting effect of FX on Ad5-binding to a larger extent (about 90%) than the promoting effect of FIX on Ad31-binding (about 70%) (Figure 7 in paper II). The effect of Hep I on Ad31:FIX binding was less apparent; Hep I inhibited the promoting effect of FIX on Ad31-binding only 20%, whereas the promoting effect of FX on Ad5-binding was inhibited by Hep I to the same degree as by Hep III (90%). These results indicate that both the Ad31:FIX and Ad5:FX complexes bind to HS, but probably to different regions. The Ad31:FIX complex preferably binds to less sulphated regions (cleaved by Hep III), whereas the Ad5:FX complex binds to both less sulphated (cleaved by Hep III) and highly sulphated regions (cleaved by Hep I) of HS on cell surfaces equally well. At this point, we cannot exclude, however, that other molecules may also contribute to FIX-mediated Ad31-binding to cells.
3 General discussion and concluding remarks

In this study, we have characterized the interactions between wt Ads belonging to species C (Ad5) and A (Ad31, Ad18, and Ad12) and coagulation factors FIX and FX during Ad-binding to and infection of epithelial cells corresponding to the natural tropism of these Ads. We found that FIX and FX mediated wt Ads attachment and subsequent infection of their natural target cells (Figure 8a) (Jonsson et al., 2009, Lenman et al., 2011). The natural target cells of species C and A Ads are mainly epithelial cells from respiratory tract and the gastrointestinal (G/I) tract. Thus, a main conclusion from these results is that FIX and/or FX may contribute to wt Ad infections in these tissues. Based on our results, one may assume that Ads have evolved to use components in body fluids for more efficient binding to and infection of cells outside the liver (e.g., airways and the intestines). Although these results were obtained from in vitro experiments, they may also be of importance in vivo. Our results thus contribute to further knowledge of wt Ad infections of their natural target cells.

Other groups have found that FIX and FX mediate binding of and transduction of Ad5-based vectors to hepatocytes (Figure 8b) (Shayakhmetov et al., 2005, Parker et al., 2006). The effect of this Ad:FIX/FX interaction is that Ad-based vectors are trapped in the liver. This is fine if the liver is the target for Ad-based vectors, but if we want the vector to reach other organs, cells or tissues, this effect is not wanted. To circumvent the problem with Ad-based vectors being trapped in the liver, trials have been made with higher doses of Ad-based vector administered to the patient so that at least some of the vector would reach the target. But this resulted in toxic immune response and death of a patient (Raper et al., 2003). To avoid FIX or FX from binding to Ads, several methods can be used, e.g., mutation of the hexon to which FIX and FX bind (Vigne et al., 1999); use other Ad types as Ad-based vectors that do not bind FIX/FX (Gao et al., 2003, Reddy et al., 2003); or PEGylation of the vector (Alemany et al., 2000). The Ad:FIX/FX interactions with hepatocytes found by Shayakhmetov and Parker are mainly useful for understanding the hepatic tropism of the Ad-based vector and further development of Ad-based vectors.

However, the interaction between Ad5 and FX was recently found to have an additional effect: FX shields Ad5 from attack by natural antibodies and complement components, thus protecting Ads from opsonization by innate immunity (Figure 8c) (Xu et al., 2013). In this way, the host cannot protect itself from Ad infections, and wt Ads may cause diseases. The results from this group, add to our knowledge of infections by wt Ads and may contribute to the understanding of why some Ad infections are persistent. On the other hand, this process protects Ad-based vectors from being destroyed by innate immunity, although it does not stop the Ad-based vectors from being trapped in the liver. Thus, these results are also useful when designing Ad-based vectors for gene therapy.

The relevance of FIX and FX on Ad binding depends on the type of Ad:FIX/FX interaction. Viruses or vectors that have infected/transduced an individual and reached the blood stream will be transported to the liver. In the liver, FIX and/or FX bridge Ads to hepatocytes, so Ads cannot escape from the liver. This Ad:FIX/FX interaction is not beneficial for Ad-based vectors, which cannot reach other organs.
(or tumours). On the other hand, the Ad:FIX/FX interaction is beneficial for the host as wt Ads now can be neutralized by the immune response. Wt Ads that have infected the respiratory tract, eye, intestine, etc. of the host may not be in direct contact with the blood and will only get in contact with low concentrations of FIX and FX. For these viruses, other components, such as DPPC and Lf, may be more important for infection than coagulation factors. Infections of the liver by Ads may result in hepatitis in immunocompromised patients. In these patients, Ad:FIX/FX interactions may be detrimental. In immunocompetent individuals, Ads have never been reported to cause hepatitis. Perhaps Ads can also infect the liver also in immunocompetent individuals but only cause asymptomatic infections.

In summary, the interactions between Ads and FIX/FX may have several consequences:

1. **Wt Ads are more efficient in binding to and infection of cells outside the liver (e.g., airways and intestines)** (Figure 8a). This may explain respiratory, intestinal, and other infections caused by wt Ads.
2. **Ad-based vectors are trapped in the liver instead of reaching their target organ** (Figure 8b). This makes the treatment with low Ad-based vectors less efficient.
3. **Wt Ads and Ad-based vectors are shielded from attack by the host’s innate immune response** (Figure 8c). This may explain infections caused by wt Ads as well as entrapment of Ad-based vectors in the liver.

Future studies, however, are needed to reveal more details of the already established interactions between Ads and coagulation factors, antibodies, complement components, etc. It may also be that Ads can bind to and use other soluble components in body. Future studies of Ad interactions with components in body fluids should also involve more Ad types than those we have included in our study in order to determine whether other Ad types may interact with components in body fluids. Ad types that interact with coagulation factors are not suitable as vectors, as these types are trapped in the liver. In this way, we may gain further knowledge of natural infections by Ads, and of interactions between wt Ads/Ad-based vectors and components in body fluids, and how those interactions may influence Ads ability to infect cells/transduce cells, be protected from immunity or be part of a yet undiscovered function.
Figure 8. Ad:FIX/FX interactions and its consequences: a) FIX/FX bridges wt Ads to HS and thereby promotes infection of epithelial cells from respiratory or G/I-tract (Jonsson et al., 2009, Lenman et al., 2011); b) FIX/FX bridge Ad-based vectors to HSPG on hepatocytes (this interaction home Ad-based vectors to the liver) (Shayakhmetov et al., 2005, Parker et al., 2006) and c) FX shields wt Ads and Ad-based vectors from attack by natural antibodies (Nabs) and complement components (C1q and C4) (Xu et al., 2013; Xu et al., 2013).
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