ADENOVIRUS ENDOCYTOSIS AND ADENOVIRAL GENE TRANSFER IN CARDIOVASCULAR AND DERMATOLOGIC DISEASE MODELS

TANJA RAUMA-PINOLA

Department of Pharmacology and Toxicology, Biocenter Oulu, Department of Internal Medicine, University of Oulu

OULU 2004
TANJA RAUMA-PINOLA

ADENOVIRUS ENDOCYTOSIS AND ADENOVIRAL GENE TRANSFER IN CARDIOVASCULAR AND DERMATOLOGIC DISEASE MODELS

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium of the Department of Pharmacology and Toxicology, on September 10th, 2004, at 12 noon.
Rauma-Pinola, Tanja, Adenovirus endocytosis and adenoviral gene transfer in cardiovascular and dermatologic disease models
Department of Pharmacology and Toxicology, Biocenter Oulu, University of Oulu, Department of Internal Medicine, University of Oulu, P.O.Box 5000, FIN-90014 University of Oulu, Finland
2004
Oulu, Finland

Abstract
Adenoviral gene transfer is a valuable tool in molecular biology research. In order to be an efficient and safe vector, adenovirus structure and infection mechanism as well as molecular biology of the used transgene need to be well studied. The aim of this study was to evaluate the role of adenovirus as a gene transfer vector from several perspectives. Adenovirus uses receptor-mediated endocytosis in order to enter the target cell. The effect of Rab5 GTPase on adenovirus entry and gene transfer efficiency was examined first. Next, adenovirus was used as an investigatory tool in the cardiovascular research, focused on clarifying the role of adrenomedullin (AM) in heart and vascular remodeling. Finally, a model of adenoviral gene transfer into skin fibroblasts was used.

The role of Rab5 GTPase in the adenovirus endocytosis was examined in HeLa cells using Cy3-labeled adenovirus, and gene transfer efficiency using β-galactosidase encoding adenovirus. Rab5 increased both adenovirus uptake and gene transfer, whereas dominant negative Rab5S34N decreased both endocytosis and gene transfer. The data indicate that Rab5 is needed in mediating the adenovirus uptake into the target cell.

In the rat heart, adenovirus-mediated AM gene transfer transiently improved systolic function both in vivo and in vitro. AM caused activation of translocation of protein kinases Cε and δ, whereas phosphorylation of p38 mitogen activated protein kinase was decreased in the left ventricle. AM significantly attenuated the development of angiotensin II-induced cardiac hypertrophy. In rats with myocardial infarction, AM enhanced dilatation of left ventricle and thinning of anterior wall. The role of AM in neointima formation was evaluated in rat artery after endothelial injury. Intravascular AM gene transfer decreased neointimal growth and increased neointimal myofibroblasts apoptosis. These results show that AM regulates left ventricular systolic function and remodeling in the heart, and plays a role in pathological vascular remodeling.

Adenovirus-mediated lysyl hydroxylase (LH) gene transfer into skin fibroblasts of type VI Ehlers-Danlos syndrome patient and rat skin increased functional LH production, elevated LH activity, and human LH mRNA production both in vitro and in vivo. LH gene replacement therapy may thus lead to possibilities to improve skin wound healing in Ehlers-Danlos syndrome patients.

Keywords: adenovirus, adrenomedullin, gene transfer, lysyl hydroxylase, Rab5
To my brother
Acknowledgements

This work was carried out at the Department of Pharmacology and Toxicology, University of Oulu, during the years 1997-2004. I wish to thank Professor Olavi Pelkonen, the Head of the Department of Pharmacology and Toxicology, for providing excellent research facilities in his department. His positiveness and broad experience of science have created an inspiring atmosphere to work in.

I want to express my deep thanks to Professor Heikki Ruskoaho for giving me an opportunity to work in his research group. I appreciate his friendly advice, wide knowledge, continuous optimism, and his capability to lead research group wisely. I own my special gratitude to my supervisor MD, PhD Timo Hautala, whose patient and excellent guidance has been essential for the completion of this thesis. His expertise, valuable conversations, and inspired attitude to the research work have been significant for training me to the scientific world.

I also wish to thank the Professors of the Department of Physiology, Juhani Leppäluoto and Olli Vuolteenaho, for their methodological experience and instructive advice. I am very grateful to Docent Ken A. Lindstedt and PhD Mikko Turunen for their valuable comments on this thesis. Professor Raii Myllylä, Docent Paavo Pääkkö, Docent Ylermi Soini, and Docent Juha Tuukkanen are warmly acknowledged for their collaboration and valuable discussions. I thank Anna Vuolteenaho for revising the language and Liisa Kärki and Seija Leskelä for preparing posters related to this work.

Kati Viitala, Pirjo Korpi, Sirpa Rutanen, Tuulikki Kärnä, Marja Arbelius, Kaisa Penttilä, Ulla Hirvonen, Tuula Inkala, Riitta Vuento, Ulla Weckström, Erja Tomperi, Mirja Vähera, and Liisa Äijälä are greatly acknowledged for their skillful technical assistance and cooperation during these years. I warmly thank Raija Hanni, Esa Kerttula, Kauno Nikkilä, Terttu Keränen, and Marja Räinä for helping me in many ways during these years. I owe my thanks to the whole personnel of the Department of Pharmacology and Toxicology.

I wish to express my thanks to all my co-authors and colleagues. I sincerely thank Hanna Leskinen, whose cooperation and expert knowledge have been valuable. I warmly thank Maria Suo-Palosaari for her friendship and useful advice concerning the thesis process. Jaana Rysä, Nina Hautala, Sampsu Pikkarainen, Heikki Tokola, Risto Kerkelä, Jarkko Pihula, Hannu Romppanen, Raisa Serpi, Marja Luodonpää, Sanna Kumpumäki,
Mika Ilves, Paavo Uusimaa, István Szokodi, Pietari Kinnunen, and Minna Marttila are greatly acknowledged for their friendly help and fruitful collaboration during the research years. I thank Olli Tenhunen, Tuomas Peltonen, Antti Ola, Jani Aro, the new roommates Marja Paso, Anna-Maria Kubin, Elina Koivisto, Leena Kaikkonen, and the other younger colleagues of our group for the good atmosphere to work in.

I am warmly grateful to all my good friends. I thank my Veteli friends Johanna Klemola, Minni Koskinen, Mirva Käänsälä, Merja Lappi, Tanja Pakkala, and Matleena Saari, my biochemist friends Anu Mursula, Pia Nyberg, Mira Pekkala, Maarit Rossi, and Anna Rytkönen, and the other friends from Oulu, Terhi Gärding, Johanna Heinonen, Saija Mustonen, and Maria Rantala, for the numerous great and fun experiences together.

Especially, my parents Eliisa and Raimo Rauma are warmly acknowledged for their loving care and support. They have always encouraged me and given me an excellent basis for my life. I owe special thanks to my brother Timo Rauma for his wise comments, good sense of humor, and for the years we were allowed to grow up together. I thank my little sister Päivi Rauma just for being there and having brought so much brightness and joy into my life. I wish to express my warm thanks to my grandparents Taimi Övermark, Veikko Övermark, Vieno Rauma, and Teimo Rauma for their continuous support and encouragement for my studies.

Finally, my deepest thanks go to my dear husband and best friend Tero Pinola. The love and happiness we share together makes everything worthwhile. I want to thank him for his understanding, intelligence, his great skill to make me laugh numerous times per day, and simply for making my life wonderful.

This work was generously supported by the Finnish Foundation for Cardiovascular Research, the Finnish Cultural Foundation, the Finnish Medical Foundation, Emil Aaltonen Foundation, Aarne Koskelo Foundation, the Research and Science Foundation of Farmos, Pharmacal Research Foundation, Maud Kuistila Memorial Foundation, the University Hospital of Oulu (KEVO funding), Sigrid Juselius Foundation, Finnish Gene Therapy Society, Duodecim Society of Oulu, and Biocenter Oulu.

Oulu, July 2004

Tanja Rauma-Pinola
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic</td>
</tr>
<tr>
<td>AM</td>
<td>adrenomedullin</td>
</tr>
<tr>
<td>AMBP</td>
<td>adrenomedullin binding protein</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackievirus and adenovirus receptor</td>
</tr>
<tr>
<td>CAS</td>
<td>Crk-associated substrate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cox-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CRL1195</td>
<td>hydroxylysine-deficient human skin fibroblasts</td>
</tr>
<tr>
<td>CRLR</td>
<td>calcitonin receptor-like receptor</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>developed pressure</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>EDS</td>
<td>Ehlers-Danlos syndrome</td>
</tr>
<tr>
<td>EEA</td>
<td>early endosome antigen</td>
</tr>
<tr>
<td>EF</td>
<td>ejection fraction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial cell nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate-dextran</td>
</tr>
<tr>
<td>FS</td>
<td>fractional shortening</td>
</tr>
<tr>
<td>GAP</td>
<td>guanine triphosphatase-activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HE</td>
<td>hematoxylin-eosin</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>I/M</td>
<td>intima/media</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>Ir</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>ITRs</td>
<td>inverted terminal repeats</td>
</tr>
<tr>
<td>IVS</td>
<td>interventricular septum</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LAD</td>
<td>left anterior descending</td>
</tr>
<tr>
<td>LH</td>
<td>lysyl hydroxylase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccaride</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>LVEDD</td>
<td>left ventricle end-diastolic dimension</td>
</tr>
<tr>
<td>LVEDP</td>
<td>left ventricle end-diastolic pressure</td>
</tr>
<tr>
<td>LVESD</td>
<td>left ventricle end-systolic dimension</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>mesangial cell</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity on infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>PAMP</td>
<td>proadrenomedullin N-terminal 20 peptide</td>
</tr>
</tbody>
</table>
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDGF  platelet-derived growth factor
PFA  paraformaldehyde
pfu  plaque-forming unit
PI3K  phosphatidylinositol 3-kinase
PKA  protein kinase A
PKC  protein kinase C
PKG  protein kinase G
Pr  protease
PS  penicillin-streptomycin
PTA  percutaneous peripheral angioplasty
PTCA  percutaneous coronary angioplasty
PW  posterior wall
rAM  rat adrenomedullin
RAMP  receptor activity modifying protein
RGD  arginine-glysine-aspartic acid
RIA  radioimmunoassay
RNA  ribonucleic acid
RSV  rous sarcoma virus
RT-PCR  reverse transcriptase polymerase chain reaction
SAP  shrimp alkaline phosphatase
s.c. subcutaneously
SD-rat  Sprague Dawley rat
SEM  standard error of mean
sma  smooth muscle actin
SMC  smooth muscle cell
SNARE  soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TGF-β  transforming growth factor beta
TIMP  tissue inhibitor of metalloproteinase
tk  thymidine kinase
TNF  tumor necrosis factor
TP  terminal protein
TVD  threonine-valine-aspartic acid
VEGF  vascular endothelial growth factor
VSMC  vascular smooth muscle cell
X-gal  5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
List of original papers

This thesis is based on the following articles which are referred to in the text by Roman numerals:


Contents

Abstract
Acknowledgements
Abbreviations
List of original papers
Contents

1 Introduction ...................................................................................................................17
2 Review of the literature .................................................................................................19
  2.1 Adenovirus biology.................................................................................................19
    2.1.1 Structure of adenovirus....................................................................................19
    2.1.2 Adenovirus life cycle.......................................................................................20
      2.1.2.1 Adenovirus endocytosis............................................................................21
      2.1.2.2 Rab GTPases.............................................................................................23
      2.1.2.3 Other small GTPases ................................................................................25
    2.1.3 Recombinant adenoviral vectors......................................................................26
  2.2 Adenovirus as a tool for cardiovascular research ...................................................28
    2.2.1 Adenoviral gene transfer into myocardium .....................................................28
    2.2.2 Adenoviral gene transfer into vascular wall ....................................................30
    2.2.3 Adrenomedullin ...............................................................................................32
      2.2.3.1 Structure and distribution of AM..............................................................32
      2.2.3.2 Biological effects of AM...........................................................................33
      2.2.3.3 Regulation of AM synthesis and signal transduction mechanisms ...........34
  2.3 Adenoviral gene transfer into skin..........................................................................37
    2.3.1 Gene transfer in wound healing.......................................................................37
    2.3.2 Other skin gene transfer targets.......................................................................39
3 Aims of the research......................................................................................................41
4 Materials and methods...................................................................................................42
  4.1 Preparation of plasmid constructs (I)......................................................................42
  4.2 Adenovirus vectors ..................................................................................................42
  4.3 Animal studies ..........................................................................................................43
    4.3.1 Gene transfer into myocardium (II).................................................................44
    4.3.2 Gene transfer into artery wall (III).................................................................44
4.3.3 Gene transfer into rat skin (IV) ................................................................. 45
4.3.4 Myocardial infarction (II) ....................................................................... 45
4.3.5 Angiotensin II infusion with osmotic minipumps (II) .............................. 45
4.3.6 Echocardiography (II) ........................................................................... 46
4.3.7 Isolated perfused rat heart preparation (II) .............................................. 46
4.3.8 Measurement of gene transfer efficiency by X-Gal staining ................. 47
4.3.9 Histological stainings (III) ..................................................................... 47
4.4 Cell culture studies .................................................................................... 48
4.4.1 Cell cultures and β-galactosidase assays (I) ............................................ 48
4.4.2 Fluorescent microscope analysis .............................................................. 49
4.4.3 Measurement of $[Ca^{2+}]$ ................................................................... 50
4.5 Isolation and analysis of cytoplasmic RNA (II-IV) ....................................... 50
4.6 Western blot analysis (I,II,IV) ................................................................. 52
4.7 Radioimmunoassay of ir-AM ................................................................. 53
4.8 Assays for lysyl hydroxylase (IV) ............................................................ 53
4.8.1 LH activity assay ................................................................................ 53
4.8.2 LH amino acid analysis ...................................................................... 54
4.9 Statistical analysis .................................................................................. 54
5 Results ......................................................................................................... 55
5.1 The role of Rab5 GTPase in adenovirus endocytosis (I) ............................ 55
5.1.1 Effects of adenovirus on $[Ca^{2+}]$, and protein tyrosine phosphorylation 55
5.1.2 Effects of Rab5 on adenoviral gene transfer .......................................... 56
5.1.3 Rab5 enhances virus endocytosis ....................................................... 56
5.2 Adenovirus-mediated AM gene transfer into rat myocardium (II) ............. 57
5.2.1 AM gene expression in the left ventricle .............................................. 57
5.2.2 Effects of AM on cardiac contractility .................................................. 58
5.2.3 PKC and MAPK activation after AM gene transfer ............................. 59
5.2.4 AM decreases angiotensin II-induced cardiac hypertrophy ................. 59
5.2.5 AM regulates cardiac remodeling after myocardial infarction ............. 60
5.3 Adenoviral AM gene transfer into rat carotid artery (III) ......................... 62
5.3.1 The effect of AM on neointimal hyperplasia after vascular injury ......... 62
5.3.2 Effects of adenoviral AM gene transfer on neointimal apoptosis and stem-like cells recruitment .................................................. 62
5.4 Adenoviral gene transfer restores LH activity in type VI EDS (IV) .......... 63
5.4.1 LH activity in human skin fibroblasts ............................................... 63
5.4.2 Adenoviral LH gene transfer into rat skin ........................................... 64
5.4.3 Human LH mRNA production after LH gene transfer ....................... 64
6 Discussion .................................................................................................. 65
6.1 Intracellular factors mediating adenovirus entry .................................... 65
6.2 The cardiac effects of intramyocardial AM gene transfer ......................... 67
6.3 Intravascular AM gene transfer after endothelial injury ......................... 70
6.4 Adenoviral gene transfer into the skin fibroblasts .................................. 72
7 Summary and conclusions ....................................................................... 74
References
Introduction

Gene transfer is an important method in molecular biology research. The administration of genes requires vehicles that guide the gene to the target cell. The binding of the genetic material to the cell, its internalization, transport to the nucleus, and expression all constitute potential limitations of this process (Boulikas 1998). Recombinant viruses are highly efficient vectors that exploit their natural mechanisms of cell entry. Generally, genetically modified viruses are replication defective and should, therefore, be non-pathogenic. However, their use may still be limited by their potential toxicity or inherent immunogenicity, which indicates the need for further studies of the basic biology of viral vectors.

Adenovirus is the most widely used viral vector for gene transfer. Among over 50 different serotypes, the closely related serotypes 2 and 5 have been most intensively studied. Human adenovirus is a non-enveloped, icosahedral virus with linear, double-stranded DNA. It uses receptor-mediated endocytosis in order to enter the target cell. Adenovirus interacts with two types of receptors, coxsackie and adenovirus receptor (CAR) and αv-integrin receptors (Wickham et al. 1993, Bergelson et al. 1997), and numerous cellular factors and pathways are needed for virus internalization. Adenovirus can infect a broad variety of cell types and tissues in both dividing and non-dividing cells. It can be grown to high titer, which may lead to efficient gene transfer in the target tissue. Disadvantages include transient transgene expression lasting two to three weeks, tissue toxicity, and immune reactions of the host (for review, see Russell 2000).

Cardiovascular disorders including atherosclerosis, restenosis, angiogenesis, myocardial ischemia, and coronary artery disease are potential targets for gene transfer studies (for review, see Ylä-Herttuala & Martin 2000). Adenovirus has provided both a powerful investigatory tool and allowed new insights into the molecular mechanisms of cardiovascular diseases. Adenoviral gene transfer to blood vessel wall and heart myocardium is a convenient method for testing experimentally the local effects of various genes. Recombinant adenovirus is an effective gene delivery vector also in dermatology. Adenoviruses can infect human skin cells with efficiency as high as 95% (Kozarsky & Wilson 1993, Mulligan 1993). The molecular basis of wound healing, squamous cell carcinoma, and melanoma are the most common subjects for the skin gene transfer studies.
In this study, the role of adenovirus as a gene transfer vector was explored. Initially, the effects of Rab5 GTPase on the adenovirus endocytosis and gene transfer efficiency were studied. Next, the use of adenovirus as an investigatory tool in the cardiovascular research was examined, focused on clarifying the role of AM in the heart and vascular remodeling. Finally, as an example of adenovirus use in dermatological research, adenoviral gene transfer into skin fibroblasts was employed.
2 Review of the literature

2.1 Adenovirus biology

Adenoviruses are a significant cause of acute respiratory, ocular, and gastrointestinal diseases in humans. They have been intensively characterized since their initial description in the early 1950s (Rowe et al. 1953). Among over 50 different adenovirus serotypes (Table 1), the closely related serotypes 2 and 5 (the species C adenoviruses) have been most intensively studied in terms of their structure, life cycle, genome organization, and sequence. Nevertheless, there is still a lack of understanding of many mechanisms, e.g. viral entry, that operate in the adenovirus-infected cells.

Table 1. Taxonomy of adenoviruses (modified from Bailey & Mautner 1994)

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
</tr>
<tr>
<td>B</td>
<td>3, 7, 11, 14, 21, 34, 35</td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6</td>
</tr>
<tr>
<td>D</td>
<td>8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 48, 49</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
</tr>
<tr>
<td>F-G</td>
<td>40, 41</td>
</tr>
</tbody>
</table>

2.1.1 Structure of adenovirus

Adenoviruses are nonenveloped deoxyribonucleic acid (DNA) viruses of about 70 - 90 nm diameter. The adenovirus particle is composed of an outer capsid and an inner DNA-associated core with linear DNA, two terminal proteins and condensing proteins V and VII. The chromosome also contains about ten copies of the cysteine protease p23 and it is linked to the outer capsid by protein VI. Adenovirus capsid has icosahedral symmetry consisting of three major proteins, hexon, penton base and a knobbed fiber. Each virion
contains 240 copies of hexon, which is the major coat protein. The 12 vertices of adenovirus contains a complex, known as the penton, which is composed of the 320 kDa penton base and the 182 kDa fiber protein (Stewart et al. 1991, Stewart et al. 1993). The fiber protein mediates attachment to cells by a COOH-terminal domain (knob). The adenovirus capsid consists also of a number of other minor proteins, such as VI, VIII, IX, IIIa, and IVa2.

The virus genome is a linear, 36 – 38 kb long double-stranded DNA encoding over 70 gene products. It has a terminal protein (TP) attached covalently to the 5’ terminus (Rekosh et al. 1977). DNA replication requires sequences within the inverted terminal repeats (ITRs) as origins of replication at both DNA termini (Hay et al., 1995). The virus DNA is intimately associated with the highly basic protein VII and a small peptide termed mu (Anderson et al. 1989). Protein V is packaged with this DNA–protein complex and appears to provide a structural link to the capsid via protein VI (Matthews & Russell 1995). The virus also contains a virus-encoded protease (Pr) (Weber 1976, Webster et al. 1989), which is necessary for processing some of the structural proteins to produce mature infectious virions.

The viral genome contains five early transcription units (E1A, E1B, E2, E3, and E4), two early delayed (intermediate) transcription units (pIX and IVa2) and five late units (L1–L5). The E1 gene products are primarily associated with modulating cellular metabolism to make the cell more susceptible to virus replication. The E1 proteins regulate host cell defence mechanisms, immune systems, and apoptosis. They are also expressed to alter expression of host genes and to activate the other adenoviral early activated genes E2, E3, and E4. The E2 gene products provide the machinery for replication of the viral DNA (Hay et al. 1995) and they ensure transcription of the late genes. The E3 genes provide a compendium of proteins that influences the host defence mechanisms. One of these E3 gene products has been termed the adenovirus death protein, since it facilitates late cytolysis of the infected cell and thereby releases progeny virus more efficiently (Tollefson et al. 1996). The gene products derived from the E4 cassette mainly facilitate virus messenger RNA metabolism (sometimes in association with E1B gene products) (Goodrum & Ornelles 1999, Weigel & Dobbelstein 2000) and provide functions to promote virus DNA replication and shut-off of host protein synthesis (Halbert et al. 1985). Adenoviruses also transcribe a set of ribonucleic acids (RNAs) that are not translated, which may play a role in combating the cellular defence mechanisms.

Thereafter, late transcription ensues, with five cassettes of transcripts (termed L1 to L5) resulting from a complex series of splicing events. These genes mostly encode structural proteins for the capsid and the internal core. Late activated genes synthesis leads to the encapsidation and maturation of virus particles in the nucleus.

### 2.1.2 Adenovirus life cycle

The adenovirus infectious cycle can be defined into two phases. The early phase covers the entry of the virus into the host cell and the passage of the virus genome to the nucleus, followed by the selective transcription and translation of the early activated genes. These early events modulate the functions of the cell in order to facilitate the replication of the
virus DNA (the late phase) and to allow transcription and translation of the late activated genes. This leads to the assembly of the structural proteins in the nucleus and the maturation of infectious virions. The early phase in a permissive cell may take about 6–8 hours, while the late phase is normally much more rapid, yielding mature virus in another 4–6 hours (Russell 2000).

2.1.2.1 Adenovirus endocytosis

Uptake of adenovirus into cells by high-affinity binding receptor-mediated endocytosis involves at least two viral proteins, the fiber and the penton base (Fig. 1). The adenovirus fiber protein mediates attachment to cells via interaction with a 46 kDa cell receptor designated CAR (Tomko et al. 1997, Bergelson et al. 1997) and the adenovirus penton base protein mediates internalization via interaction with αv integrins (Wickham et al. 1993). CAR serves as the receptor for many, but not all, adenovirus serotypes (Roelvink et al. 1998). At present, all adenovirus serotypes except those belonging to subgroup B (serotypes 3 and 7) or subgroup D (serotypes 8 and 37) recognize CAR (Roelvink et al. 1999, Arnberg et al. 2000). The cytoplasmic domain of CAR is not required for virus attachment or infection (Wang & Bergelson 1999), suggesting that cell signaling through this receptor may not be involved in viral entry.

Fig. 1. The entry pathway of adenovirus into a cell (modified from Nemerow et al. 2000, Smith & Helenius 2004). Ad, adenovirus; CAR, coxsackie and adenovirus receptor.
αv-integrins play a role in intracellular signal transduction, controlling gene expression, and cell division (Schwartz et al. 1995). Ligand binding to αv-integrins can trigger a transient increase in intracellular calcium concentration ([Ca^{2+}]) which may regulate integrin uptake and recycling (Schwartz & Denninghoff 1994, Lawson & Maxfield 1995). Ligand binding to αv-integrins has also been found to stimulate phosphorylation of several proteins, including focal adhesion kinase pp125FAK (Miyamoto et al. 1995), p130CAS (Crk-associated substrate), and paxillin (Vuori & Ruoslahti 1995, Petch et al. 1995). These intracellular events following integrin ligand binding lead to focal adhesion site formation and reorganization of the actin cytoskeleton. Actin filaments may provide the mechanical force required for endosome formation. Alternatively, actin networks may serve as structural platforms that stabilize the half-life of signaling molecules in the cell.

Increased knowledge of the general αv-integrin biology has initiated studies on the role of the described pathways in adenoviral entry.

In an efficient internalization of adenovirus into the cell, the adenovirus penton base protein binds to integrins αvβ3 and αvβ5 through a conserved arginine-glycine-aspartic acid (RGD) sequence (Bai et al. 1993, Wickham et al. 1993, Stewart et al. 1997). This interaction occurs in many, but not all, cell types or adenovirus serotypes. For example, in the hepatocytes αv integrins have a minimal role (Hautala et al. 1998). The adenoviral penton base-αv integrin interaction leads to phosphatidylinositol 3-kinase (PI3K) activation, which is essential for viral entry (Li et al. 1998b). PI3K may in turn regulate endocytosis via Rab5 GTPase (Li et al. 1995). Rac and Cdc42, the members of the Rho family of small GTPases act downstream of PI3K to promote adenovirus endocytosis (Li et al. 1998a). A major downstream target of the PI3K and Rho GTPase signaling pathway is the actin cytoskeleton (Li et al. 1998a). Dynamin GTPase (Wang et al. 1998) and association of p130CAS with PI3K have been found to mediate adenovirus endocytosis in some cell types (Li et al. 2000a). Instead, we and others have found that pp125FAK, whose activation is usually associated with activation of p130CAS, is not crucial for this process (Rauma et al. 1999; Li et al. 2000a). The adenovirus infection also induces the Raf-1/mitogen activated protein kinase (MAPK) pathway, leading to increased interleukin-8 (IL-8) production (Bruder & Kovesdi 1997).

Following the attachment step, adenovirus and cell integrins are rapidly internalized into clatrin-coated vesicles (Wang et al. 1998) (Fig. 1). Exposure of the virus to mildly acidic conditions in the early endosome triggers partial disassembly of the viral capsid and concomitant disruption of the endosomal membrane, allowing the virus to be released into the cytoplasm. αvβ3-integrin has been shown to be required for adenovirus-mediated membrane permeabilization at pH 6.2. Penton base interaction with αv integrins is also required for activation of the 23-kDa cysteine protease, an adenovirus enzyme that participates in virus uncoating. A threonine-valine-aspartic acid (TVD) motif in the cytoplasmic tail of the β3-integrin subunit was recently shown to specifically regulate adenovirus-mediated membrane permeabilization and endosome disruption (Wang et al. 2000). Incoming adenovirus capsids utilize microtubule motors in order to traffic to the nuclear pore complex (Suomalainen et al. 1999, Nakano & Greber 2000). The nuclear import machinery, including importin-α and -β and hsp70, is also believed to promote docking of the adenovirus capsid at the nuclear pore complex (Saphire et al. 2000). Interestingly, the known nuclear import factors alone are not sufficient to promote
adenovirus DNA delivery into the nucleus, and thus other, as yet unidentified host cell proteins may participate in this process.

2.1.2.2 Rab GTPases

Because adenovirus entry into the target cell occurs via receptor-mediated endocytosis, the factors involving endocytosis in general are also possible regulators of adenoviral gene transfer. Rab GTPases belong to the superfamily of small GTP-binding proteins (G proteins), which is composed of more than 50 members and has been divided into five families: Rab, Rho, Arf/Sar, Ran and Ras (Takai et al. 2001). The proteins exist in two interconvertible forms: the guanosine diphosphate (GDP) bound inactive and the guanosine triphosphate (GTP) bound active forms. Active GTPases interact with their specific downstream targets and perform their cellular functions, whereas GTP-hydrolysis and liberation of phosphate inactivates the GTPases. The GTP-GDP exchange reactions are regulated by guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs) (Lamarche & Hall 1994, Narumiya 1996).

Rab proteins are a family of more than 60 small GTPase regulatory proteins, and members of this family have key regulatory roles in most membrane-transport steps (Zerial & McBride 2001). Rab proteins control a variety of important cellular processes, such as endocytosis, trafficking, endosome fusion, and exocytosis. The first rab gene (YPT1) was identified by Gallwitz and colleagues as an open reading frame between actin and tubulin genes in Saccharomyces cerevisiae (Gallwitz et al. 1983). Four years later, the first homologs of the yeast SEC4/YPT1 in mammals were cloned and termed rab (ras-like in rat brain) genes (Touchot et al. 1987, Martinez & Goud 1998). Rab GTPases are post-translationally modified; the addition of two carboxyl-terminal geranylgeranyl (20-carbon) groups allows them to associate tightly with membranes. Rab GTPases localize to specific compartments of both the endocytic and exocytic pathways (Fig. 2). Moreover, a given organelle may contain several species of Rab proteins (Sonnichsen et al. 2000, Zerial & McBride 2001). For example, vesicles mediating endoplasmic reticulum-to-Golgi transport contain at least two Rab GTPases, Rab1 and Rab2 (Plutner et al. 1991, Tisdale et al. 1992, Davidson & Balch 1993), whereas Rab4, Rab5, Rab11, Rab15, and Rab 22 localize to early endosomes (Gorvel et al. 1991, Bucci et al. 1992, van der Sluijs et al. 1992, Sonnichsen et al. 2000, Zuk & Elférink 2000, Kauppi et al. 2002). Rab4 and Rab11 have also been found to be associated with perinuclear recycling endosomes (Daro et al. 1996, Ren et al. 1998, Casanova et al. 1999, Sonnichsen et al. 2000). Rab7 and Rab9 localize to late endosomes and lysosomes (Lombardi et al. 1993). Given the low similarity (< 55% identical) within each pair, it is likely that each of these proteins serves a different intracellular function.
Rab5, one of the most well-characterized Rabs, is involved in the formation and function of the sorting endosome. Rab5 together with early endosome antigen 1 (EEA1) (McBride et al. 1999, Dumas et al. 2001, Lawe et al. 2002) and soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) regulate fusion between primary endocytic vesicles and sorting endosomes (Mills et al. 1999, Clague 1999, Woodman 2000). More recently, it has been appreciated that the effects of Rab5 are not limited to regulation of fusion, but that it also has a role in working as a signaling GTPase involved in actin remodeling and controlling endosome dynamics by recruiting microtubule motors to the endosomes (Sonnichsen et al. 2000, Zerial & McBride 2001, Murray et al. 2002, Lanzetti et al. 2004). Rab5 is localized to the plasma membrane, clathrin-coated vesicles, and early endosomes (Bucci et al. 1992). Its membrane association depends on a hydrophobic isoprenoid moiety close to its C-terminus, but it is also regulated by a GTPase cycle. Thus, in its GTP-bound state Rab5 is membrane-associated, while GDP-bound Rab5 is found in a cytosolic complex with the GDI. GTP-bound Rab5 promotes
endosome fusion by recruiting cytosolic components of the fusion apparatus. Such effectors which are essential for endosome fusion are Rabaptin-5 and EEA1 (Stenmark et al. 1995, Mills et al. 1998, Simonsen et al. 1998).

2.1.2.3 Other small GTPases

The other small GTP-binding proteins regulate a wide variety of cell functions, and they may also have a role in adenovirus endocytosis. In addition to the Rab family, the Sar1/ADP-ribosylation factor (Arf) family also regulates vesicle trafficking. The budding process of endocytosis is mainly regulated by Sar/Arf proteins (Chavrier & Goud 1999). Mammalian Arf proteins are structurally grouped into three classes: class I including Arf1, Arf2, and Arf3; class II including Arf4 and Arf5; and class III including Arf6 (Moss & Vaughan 1998). Of these Arf proteins, Arf1 has been the most extensively characterized, and is established to be involved in the budding of vesicles from the Golgi. Arf6 is involved in recycling of endosomal vesicles and regulates receptor-mediated endocytosis (D'Souza-Schorey et al. 1995). Arf6 is also implicated in remodeling of the cytoskeleton underlying the plasma membrane. Arf6 is localized to the plasma membrane, especially to the budding endosome process (for review, see Takai et al. 2001). Activation of Arf6 induces remodeling of the actin cytoskeleton and cell spreading (D'Souza-Schorey et al. 1997). Recent studies indicate that Arf6 is involved in remodeling of the actin cytoskeleton and cell motility downstream of Rac1 (Radhakrishna et al. 1999). Sar1 proteins are homologous to Arf with 35% identity. They are associated with the endoplasmic reticulum and are involved in the formation of transport vesicles from the endoplasmic reticulum (Barlowe et al. 1994) (Fig. 3).

The Rho family proteins are well known for their effects on the actin cytoskeleton, and are activated in response to a variety of extracellular stimuli. Several Rho family members are localized to vesicular compartments, and increasing evidence suggests that they play important roles in the trafficking of vesicles on both endocytic and exocytic pathways. In particular, RhoA, RhoB, RhoD, Rac and Cdc42 have been shown to affect various steps of membrane trafficking (Ellis & Mellor 2000) (Fig. 3). RhoA induces actomyosin-based contractility, leading to the formation of stress fibers in many types of adherent cells, and/or cell retraction. RhoA has also been shown to participate in the early stage of receptor-mediated endocytosis (Lamaze et al. 1996). Both Rac and Cdc42 stimulate actin polymerization: Rac to induce broad plasma membrane extensions known as lamellipodia and membrane ruffles, and Cdc42 to induce the extension of finger-like plasma membrane extensions called filopodia or microspikes (Ridley 2001). Rac and Cdc42 are also known to be involved in the early endocytosis steps mostly via regulation of the actin cytoskeleton (Ellis & Mellor 2000). RhoD regulates the rate of traffic of vesicles along cytoskeletal track, increasing the efficiency of sorting (Allan & Schroer 1999). RhoB GTPase also localizes to endocytic vesicles and is thought to be involved in the sorting of internalized material to the lysosome (Ellis & Mellor 2000).
Fig. 3. Model for other G-proteins functions in receptor-mediated endocytic pathway (modified from Ridley 2001). ARF, ADP-ribosylation factor; EE, early endosome; LE, late endosome; Lyso, lysosome.

The members of the Ras family are localized to the plasma membrane, endosomes, endoplasmic reticulum (ER), and Golgi (Hancock 2003). They directly bind to and activate Raf protein kinase (Dickson et al. 1992), which then induces gene expression through the MAPK cascade in response to various extracellular signaling molecules (Dent et al. 1992). Ras proteins have been found to regulate not only cell proliferation but also differentiation (Bar-Sagi & Feramisco 1985), morphology (Bar-Sagi & Feramisco 1986), and apoptosis (Kauffmann-Zeh et al. 1997). Ras proteins regulate these functions mainly through gene expression. The mutations of Ras proteins genes and their regulator genes have been associated with human cancers (Almoguera et al. 1988). The answer to whether non-plasma-membrane-localized Ras has a specific role remains uncertain (for reviews, see Takai et al. 2001, Hancock 2003).

The Ran (Ras-related nuclear protein) is localized either in the cytosol or in the nucleus. The Ran family function in nucleocytoplasmic transport (Melchior et al. 1993, Moore & Blobel 1993) and microtubule organization (Carazo-Salas et al. 1999).

### 2.1.3 Recombinant adenoviral vectors

Recombinant adenoviruses are commonly used vectors for experimental gene transfer. Examples of other viral gene transfer vehicles are shown in Table 2. Adenoviruses can infect a variety of both dividing and non-dividing cells. They can also be grown to high titer (10^{10-12} plaque-forming units (pfu)/ml). Disadvantages include only a transient expression of transduced gene and immune reactions of the host. Because adenoviruses do not integrate into host genome, the transgene expression usually peaks within one
week and is limited to only two or three weeks (French et al. 1994a, Channon et al. 1998, Rekhter et al. 1998). The adenoviral proteins or the transgene product itself may elicit an immune response, with the result of inflammation and loss of expression of the therapeutic gene. Cellular and humoral immune response against the virus may neutralize the vector when it is administered repeatedly (Zabner et al. 1994b). The immunological problems have led to engineering vectors that have minimized expression of viral antigens.

Table 2. Examples of viral gene transfer vectors (Crook & Akyurek 2003)

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Genome</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>dsDNA</td>
<td>High transduction efficiency, infection of non-dividing cells</td>
<td>Transient gene expression, host immune responses</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>RNA</td>
<td>Long-term gene expression</td>
<td>Low gene transfer efficiency, inability to infect non-dividing cells, mutagenesis</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>ssDNA</td>
<td>Long-term transgene expression, non-pathogenic</td>
<td>Limited size of insert, protracted production protocol</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>RNA</td>
<td>Long-term gene expression, infection of non-dividing cells, high gene transfer efficiency</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>dsDNA</td>
<td>Infection of non-dividing cells, high transduction efficiency in some cell types</td>
<td>Host immune responses, transient gene expression</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>dsDNA</td>
<td>Almost unlimited size of insert, non-mammalian vector</td>
<td>Transient gene expression</td>
</tr>
<tr>
<td>Ebstein-Barr virus</td>
<td>dsDNA</td>
<td>High transduction efficiency</td>
<td>Mutagenesis</td>
</tr>
</tbody>
</table>

The E1 and/or E3 genes are deleted from the first generation adenovirus vector and replaced by the foreign gene rendering them replication defective. The virus can be propagated in vitro in 293 human embryonic kidney cells that allow stable expression of the E1A and E1B adenoviral genes (Graham et al. 1977). Removal of the E1 region had the advantage of impairing the transcription of the E2 genes (which are E1 dependent) and consequently the replication of virus DNA and the production of the virus capsid proteins. The E3 region of adenovirus encodes products associated with host defense mechanisms, and thus, the E3 region is not only often deleted to enlarge the packageable size limit for foreign genes, but also to diminish the immune response. Deletions of the E1 and E3 regions permit insertion of approximately 8.2 kb of foreign DNA (Bett et al. 1993).

In the second-generation vectors some or all of the E2 genes are excised (Lusky et al. 1998, Moorhead et al. 1999) hence producing less immune response. The third generation vectors have been constructed by deleting more virus genes, and the latest of these have all or nearly all the virus genes removed. So-called ‘gutless’ vectors (Mitani et al. 1995,
Kochanek et al. 1996) originally retained only the ITR and packaging sequences and required helper virus and appropriate complementing cells for propagation, followed by careful purification. The advantages of ‘gutless’ vectors are larger insert capacity (up to 37 kb) and smaller cellular immune response. The disadvantage is difficult and slow production.

Strategies for increasing the effectiveness of adenoviral gene transfer have been studied widely. Such mechanisms are for example developing virus retargeting mechanisms, improving adenovirus endocytosis efficiency, minimizing immune and apoptotic responses, influencing factors that affect delivery of transgenes in the cell, and ensuring the expression of the transgene (for review, see Russell 2000). Progress has been made for example in the development of tissue-specific adenoviral vectors for arterial gene transfer (Turunen et al. 2002). Transgene expression with viral heterologous promoters such as cytomegalovirus (CMV) and rous sarcoma virus (RSV) results in ubiquitous expression in many tissues, whereas cell-specific transcriptional gene regulation generally limits recombinant gene expression to specific cells (Kim et al. 1997). Adenoviral vectors programmed by vascular smooth muscle cell (VSMC) specific promoters (SM22α) have been demonstrated to drive transgene expression exclusively in the arterial and venous smooth muscle cells (SMCs) in vitro (Kim et al. 1997, Akyurek et al. 2000). Similar results have been demonstrated by specifically targeting vascular endothelial cells (Nicklin et al. 2001).

Biodistribution of adenoviral vector has been evaluated in some in vivo models. After local intravascular administration of adenovirus, transgene has been measured mostly in hepatocytes, circulating monocytes and testis, and after periadventitial gene transfer in the local lymph nodes, liver and testis, in addition to the target vessel (Hiltunen et al. 2000). Intravenous injection of adenovirus has been found to induce transgene expression essentially in the liver, adrenal glands, lungs, pancreas and spleen (Wood et al. 1999, Groot-Wassink et al. 2002). Adenoviral biodistribution has also been examined after dermal administration using biocompatible matrix (Gu et al. 2004). In this model, biolocalization of the vector was seen only in the local lymph nodes in addition to the target area three weeks after the first administration of the vector (Gu et al. 2004).

2.2 Adenovirus as a tool for cardiovascular research

2.2.1 Adenoviral gene transfer into myocardium

Experimental models for heart failure, myocardial ischemia, and coronary arterial disease are potential disease models in which adenoviral vectors may be employed. The delivered genes can be targeted to study mechanisms of cardiac remodeling, enhancement of contractility, or neovascularization of ischemic tissues. Examples of the latest applications of adenovirus use in cardiac remodeling research are the findings that targeted overexpression of growth hormone by adenoviral gene transfer following myocardial infarction resulted in reduced ventricular dilatation and increased local wall thickness in rats (Jayasankar et al. 2004), and that adenoviral gene transfer of apoptosis
repressor with caspase recruitment domain decreased apoptosis, left ventricular dilatation and wall thinning in a rabbit model of regional ischemia/reperfusion (Chatterjee et al. 2003). Kallikrein, studied by adenovirus-mediated gene delivery, has also been found to have a role in cardiac remodeling, by attenuating cardiac hypertrophy and fibrosis, and by inhibiting myocardial apoptosis through the Akt-mediated signaling pathway in the rat heart after myocardial infarction (Agata et al. 2002).

When choosing the suitable virus vector for myocardial gene transfer, the target cell, as well as the efficiency, level, and the duration of transgene expressions must be considered. Only a few of the currently available virus vectors achieve efficient, high-level transgene expression in postmitotic cells, such as cardiomyocytes. Adenovirus has been the most common vector used in cardiac studies because of its ability to transduce non-replicating cells and its large packaging capacity. The short half-life of gene expression is a major drawback. However, this short-term delivery of gene products could be sufficient and appropriate for treatments under which long-term exposure would lead to toxicity, such as in the case of angiogenic factors. Two other potential virus vectors are adeno-associated virus (AAV) (Svensson et al. 1999) and lentivirus vectors (Sakoda et al. 1999, Fleury et al. 2003). Both these viruses can integrate into the host cell genome causing long-term expression of therapeutic transgene up to several months. The limited size of DNA insert has become one consideration of using AAV and lentivirus vectors (Feldman & Steg 1997, Romano et al. 2000).

Gene delivery routes are important in achieving efficient gene transfer and extending duration of transgene expression in vivo (Fig. 4). Direct injection is the most frequently used method for cardiac gene transfer. Injection of adenovirus into the ventricular wall has been shown to induce a significant expression of transgene. However, the expression is focal, and the injections within the myocardium cause needle damage, inflammation, and fibrosis to the areas of injection (Lin et al. 1990, Guzman et al. 1993b, Barr et al. 1994, French et al. 1994b). Intravenous injection of recombinant viral vectors causes systemic infection and only low efficiency of gene transfer (Stratford-Perricaudet et al. 1992). Pericardial injection of an adenovirus has produced high concentrations of transgene, but only in the pericardial cell layers (Lamping et al. 1997, Fromes et al. 1999). This method also provides more widespread distribution of transgene expression compared to direct injection (Wang et al. 1996). Intracoronary catheter delivery achieves transduction of 30% of the myocytes in the distribution of the coronary artery (Barr et al. 1994). Other gene delivery strategies into coronary artery and vessel wall (for review, see Ylä-Herttuala & Alitalo 2003) are described in the next paragraph.
2.2.2 Adenoviral gene transfer into vascular wall

To date, recombinant adenoviral vector is the most efficient method of gene transfer into the arterial wall. It has both provided a powerful investigatory tool and allowed new insights into the molecular mechanisms of atherosclerosis, thrombosis and stenosis. Adenoviral gene transfer to blood vessel wall is convenient for testing experimentally the effects of various genes in a local vascular compartment. Atherosclerosis, restenosis after percutaneous coronary angioplasty (PTCA), percutaneous peripheral angioplasty (PTA) or stent implantation, arterial thrombosis, and angiogenesis are potential targets for gene transfer studies.

A number of useful gene delivery methods exist for vascular gene transfer studies. Intravascular systems include direct intravascular delivery, via a gene transfer catheter (Laitinen et al. 1998), or a stent coated with slow-releasing gene transfer matrix (for review, see Baek & March 1998). Various types of catheter, such as microporous, hydrogel-coated, and channel balloon, are available (Bailey 1996). Extravascular systems include adventitial gene delivery route via an adventitial collar (Laitinen et al. 1997), biodegradable gel (Simons et al. 1992), or direct injection in adventitia (Morishita et al. 1994). Other gene delivery systems such as ex vivo transduction and transplantation of SMCs (Kankkonen et al. 2004), ex vivo transduction of vein grafts, and intramuscular injection, have also been used (Tsurumi et al. 1996). Injection of biodegradable microspheres or nanoparticles coated with recombinant growth factors or expression vectors have also been used (Arras et al. 1998)
Anatomical barriers lower the intra-arterial transfection efficiency. Such barriers include continuous endothelium, an intact internal elastic lamina, atherosclerotic lesions, and lipid-rich atheroma (Laitinen et al. 1998). Atherosclerotic lesions may contain only a limited number of transfectable cells, dense fibrous tissue, and calcification (Stary et al. 1995). In arteries with an intact internal elastic lamina, local intravascular infusion of virus can target gene transfer to the developing neointima, particularly if gene transfer is delayed for several days following injury (Guzman et al. 1993a). The introduction of virus into the deeper cell layers of the media and adventitia of the vessel wall thus requires disruption of the barriers. Viral access to deeper cell layers is primarily via fissures and dissection planes, although some diffusion may also occur.

The pathophysiology of restenosis after vascular injury is one of the most common research approaches in which adenoviral gene transfer has been utilized. Injury to the arterial wall exposes subendothelial structures to circulating blood elements, leading to immediate inflammatory response (Serruys et al. 1988). Later, a number of growth factors cause migration of medial SMCs or adventitial myofibroblasts towards the inner layers of vascular wall and initiates cell proliferation (Shi et al. 1996, Li et al. 2000b, Siow et al. 2003). The highest proliferation activity of the neointimal cells is within a few days after the injury. Endothelial layer regrows over most of the injured arterial segment by 12 weeks in the rat carotid artery (Clowes et al. 1983). The origin of the neointimal cells, however, is controversial. In addition to the findings that the intimal cells may originate from adventitial fibroblasts (Li et al. 2000b, Siow et al. 2003), it has been suggested that they are derived from a preexisting and distinct subpopulation of medial SMCs (Li et al. 2001). Medial SMC are traditionally suggested in general to undergo a phenotypic modulation prior to migration, proliferation, and matrix synthesis (Campbell & Campbell 1990). It has also been hypothesized that neointimal cells originate from differentiated bone marrow hematopoietic stem cells (Han et al. 2001, Sata et al. 2002), or circulating macrophages differentiated into myofibroblasts (Bayes-Genis et al. 2002). These arguments are based on insights that intimal SMCs differ from medial SMCs in phenotype and gene-expression patterns (Zohlnhofer et al. 2001a) and that neointimal SMCs have been shown to express a number of hematopoietic lineage markers (Zohlnhofer et al. 2001b).

Because the majority of neointimal proliferation takes place early after vascular injury, short-term transient transgene expression may be sufficient to reduce neointima formation. The components of restenosis, including proliferation and migration of SMCs and myofibroblasts, re-endothelization, apoptosis, formation of connective tissue, angiogenesis, recruitment of inflammatory and hematopoietic stem cells, platelet adherence and aggregation, thrombus formation, and vascular remodeling could be potential targets of a gene transfer approach (Crook & Akyurek 2003). A great variety of different genes including vascular endothelial growth factors (VEGFs), nitric oxide synthase (NOS), thymidine kinase, p53, and tissue inhibitor of metalloproteinase (TIMP) have already been used in gene transfer studies for preventing restenosis (for review, see Ylä-Herttuala & Martin 2000).
2.2.3 Adrenomedullin

2.2.3.1 Structure and distribution of AM

In 1993, Kitamura and colleagues discovered a peptide from pheochromocytoma which increased cAMP levels in a platelet assay (Kitamura et al. 1993a). Pheochromocytomas are tumors derived from the adrenal medulla, and thus the new peptide was named adrenomedullin (AM). AM consists of 52 amino acids in humans and 50 amino acids in the rat and belongs to the calcitonin gene-related peptide (CGRP) family, which also includes calcitonin, CGRP, and amylin (for review, see Richards et al. 1996). The human preproAM is 185 amino acids in length and is then processed into a 164-amino acid peptide, called proAM, which includes the AM sequence (Kitamura et al. 1993b) (Fig. 5). In addition to AM, proAM contains a unique 20 amino acid peptide in the N-terminal region. This peptide is termed proadrenomedullin N-terminal 20 peptide (PAMP) (Kitamura et al. 1994b) and it has been observed to have a dose-dependent hypotensive effect in rats (Kitamura et al. 1993a).

Fig. 5. Schematic drawing on the structure of human adrenomedullin (AM) precursors preproadrenomedullin (preproAM) and proadrenomedullin (proAM) (modified from Samson 1999). PAMP, proadrenomedullin N-terminal peptide.

AM has been found in several tissues by detecting tissue AM mRNA and immunoreactive- (ir) AM levels. In humans, AM mRNA is highly expressed in the adrenal medulla, but equally high levels are also found in cardiac ventricle, lung and kidney (Kitamura et al. 1993b). In agreement with the mRNA results, the highest concentration of ir-AM is found in the adrenal gland. Ir-AM is also detected in heart atrium and ventricle, pancreas, small intestine, plasma, lung, aorta, kidney, liver, spleen, brain, and thyroid gland (Kitamura et al. 1993a, Ichiki et al. 1994). Since the concentration of ir-AM in ventricle, lung and kidney was reported to be much lower than in adrenal medulla (Kitamura et al. 1993a), the AM synthesized in these tissues may be rapidly released into the blood or degraded. However, these organs are not the main sources of circulating AM (Nishikimi et al. 1994). AM is suggested to be synthesized to act preferably as a local autocrine/paracrine factor. AM is detected in plasma at a concentration of 2-10 pM (Kitamura et al. 1994a, Sato et al. 1995) and the plasma half-life of AM is estimated to be about 22 minutes (Meeran et al. 1997)
Cultured vascular endothelial cells (ECs) actively synthesize and secrete AM, the mRNA level being at least 20 times higher than that of adrenal gland, lung and atrium (Sugo et al. 1994a). VSMCs also produce and secrete AM, but their secretion rate is estimated to be at most only one sixth of that of ECs (Sugo et al. 1994b, Isumi et al. 1998). Purified rat cardiac ventricular myocytes (Nishimori et al. 1997, Horio et al. 1998) and non-myocytes (Horio et al. 1998) actively express and release AM. In addition, AM is also produced in fibroblasts (Isumi et al. 1998), monocytes, and macrophages (Kubo et al. 1998, Nakayama et al. 1999).

2.2.3.2 Biological effects of AM

AM is a multifunctional peptide with major roles in the paracrine control of vascular function and in regulation of diuresis/natriuresis (Richards et al. 1996, Samson 1999) (Table 3). AM dilates arteria both in endothelium-dependent and independent manner and decreases systemic arterial pressure. Intrarenally administered AM increases natriuresis by vascular and tubular mechanisms. Although the plasma levels of AM are normally low, they increase in septic shock, pulmonary hypertension, congestive heart failure, myocardial infarction, renal failure, hypertension, and diabetes, suggesting that AM may have a pathophysiological role in these disorders (Hinson et al. 2000).

As well as being a powerful vasodilator and natriuretic, AM also regulates cell growth depending on the cell type. AM inhibits the migration and proliferation of rat VSMC stimulated with fetal calf serum (FCS) and platelet-derived growth factor (PDGF) (Horio et al. 1995, Kano et al. 1996). These authors also showed that AM inhibits angiotensin II (Ang II) induced migration of human coronary artery muscle cell (Kohno et al. 1997). AM stimulates EC growth and inhibits EC apoptosis (Kato et al. 1997, Sata et al. 2000). In rat mesangial cells (MCs), AM has been demonstrated to decrease proliferation, to increase apoptosis (Parameswaran et al. 1999), and to reduce mitogenesis (Chini et al. 1997). In vivo, local AM overexpression in rat balloon angioplasty model (Agata et al. 2003) or systemic excess of AM in transgenic mouse model (Imai et al. 2002) has been shown to inhibit intimal growth in injured arteries in vivo, suggesting that AM may have a vasculoprotective role. These results are in disagreement with an earlier observation of Shimizu et al., who reported that AM antagonist CGRP(8-37) inhibits neointimal hyperplasia after balloon injury. They suggested that endogenous AM in the injured tissue may promote the proliferation of VSMC (Shimizu et al. 1999).

<table>
<thead>
<tr>
<th>Tissue or cell type</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasculature</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vasodilatation, hypotension</td>
</tr>
<tr>
<td></td>
<td>inhibition of VSMC proliferation and migration</td>
</tr>
<tr>
<td></td>
<td>stimulation of EC proliferation and inhibition of EC apoptosis</td>
</tr>
<tr>
<td></td>
<td>stimulation of angiogenesis</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td></td>
<td>increase of cardiac output</td>
</tr>
<tr>
<td></td>
<td>inotropic effects</td>
</tr>
<tr>
<td></td>
<td>inhibition of cardiomyocyte protein synthesis and hypertrophy</td>
</tr>
<tr>
<td></td>
<td>inhibition of cardiac fibroblast proliferation</td>
</tr>
<tr>
<td></td>
<td>down-regulation of ANP gene expression</td>
</tr>
<tr>
<td></td>
<td>coronary artery vasodilatation</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibition of aldosterone secretion</td>
</tr>
<tr>
<td></td>
<td>inhibition of cortisol secretion</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
</tr>
<tr>
<td></td>
<td>diuresis, natriuresis</td>
</tr>
<tr>
<td></td>
<td>MC contraction</td>
</tr>
<tr>
<td></td>
<td>inhibition of MC proliferation, increase of MC apoptosis</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibition of ACTH secretion</td>
</tr>
<tr>
<td>CNS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibition of water intake and salt appetite</td>
</tr>
</tbody>
</table>

ACTH, adrenocorticotropin; ANP, atrial natriuretic peptide; CNS, central nervous system; EC, endothelial cell; MC, mesangial cell; VSMC, vascular smooth muscle cell.

In vivo, AM infusion has been found to increase cardiac output (Parkes 1995, Charles et al. 1997, Parkes & May 1997, Rademaker et al. 2002). In vitro, AM has been reported to have a positive inotropic effect on isolated perfused rat heart (Szekodi et al. 1998) and isolated rat papillary muscles (Ihara et al. 2000). AM has also been found to have negative inotropic effects (Ikenouchi et al. 1997) or no effects at all (Stangl et al. 2000). AM attenuates myocardial remodeling by inhibiting protein synthesis and gene expression of hypertrophy-associated genes in cardiomyocytes and proliferation of cardiac fibroblasts (Tsuruda et al. 1998, Tsuruda et al. 1999, Luodonpää et al. 2001). In human transplanted hearts ventricular AM levels have been shown to be associated with myocyte hypertrophy (Tsuruda et al. 2003). In studies with knockout mice, Ang II infusion caused more severe cardiac hypertrophy in AM knockout mice than in wild type mice (Niu et al. 2003, Niu et al. 2004). Myocardial infarction produced by ligation of left anterior descending coronary artery (LAD) has been found to increase AM mRNA levels, expression of AM receptors and receptor activity modifying protein (RAMP) 2 (Oie et al. 2000) as well as AM protein synthesis in the left ventricle (Nagaya et al. 2000) (Table 2).

2.2.3.3 Regulation of AM synthesis and signal transduction mechanisms

AM secretion, especially in cardiovascular tissues, is regulated mainly by mechanical stressors, such as shear stress and stretching of cardiomyocytes. Also inflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF), and
lipopolysaccharide (LPS), hormones such as Ang II and endothelin (ET)-1, and metabolic factors such as hypoxia, ischemia, and hyperglycemia regulate AM secretion (Sugo et al. 1994b, Sugo et al. 1995a, Sugo et al. 1995b, Nishimori et al. 1997, Cormier-Regard et al. 1998, Isumi et al. 1998, Horio et al. 1998).

In plasma, AM is bound to AM binding protein-1 (AMBP-1), identified as complement factor H (Elsasser et al. 1999, Pio et al. 2001). AMBP-1 does not change the affinity of AM receptors for AM, but has sequences which may bind to cell surface adhesion molecules and could therefore bring AM near its receptors and raise the effective concentration of AM (Elsasser et al. 1999). AMBP-1 may also create a locally contained reservoir of AM at high concentration. By analogy with other binding proteins, it is possible that AMBP-1 inhibits the degradation of AM.

AM receptors have been characterized as consisting of two associated proteins: calcitonin receptor-like receptor (CRLR) and RAMP-2 or RAMP-3 (McLatchie et al. 1998). CRLR is the seven-transmembrane-domain G-protein-coupled receptor and the adjoined RAMP is a single-transmembrane-domain protein. A receptor component protein is also needed to establish the connection with the downstream signal transduction pathway. CRLR with RAMP-2 or RAMP-3 makes AM-specific receptors AM\textsubscript{1} and AM\textsubscript{2}, respectively, while CRLR with RAMP-1 makes CGRP-specific CGRP\textsubscript{1} receptors (McLatchie et al. 1998, Fraser et al. 1999, Foord & Marshall 1999). AM has also some affinity for CGRP\textsubscript{1} receptor.

The effects of AM may be mediated by cAMP-protein kinase A (PKA), which is typical for the calcitonin family of peptides (Kitamura et al. 1993a, Muff et al. 1995), and cGMP-protein kinase G (PKG) signaling pathways (Fig. 6). Via cAMP-PKA, AM has been demonstrated to decrease proliferation and to increase apoptosis in rat MCs (Parameswaran et al. 1999) and to reduce MAPK activity and mitogenesis in rat VSMCs and rat MCs (Chini et al. 1997). AM gene transfer, however, has been reported to increase cGMP, but not cAMP, levels in balloon-injured arteries (Agata et al. 2003). Endothelial cell nitric oxide synthase (eNOS) has been implicated in AM-mediated antiapoptotic effects in ECs (Sata et al. 2000). AM causes the vasodilation of ECs both using the direct activation of eNOS, and the PI3K/Akt pathway to activate endothelial NOS (eNOS) (Nishimatsu et al. 2001). The PI3K/Akt pathway has also been found to mediate angiogenesis promoting effects (Kim et al. 2003) (Fig. 6).
In the heart, the signal transduction pathways mediating AM effects are not well understood either. AM increases cardiac contractility via cAMP-independent mechanisms, which include mobilization of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores, activation of PKC and activation of L-type Ca\(^{2+}\) channels (Szokodi et al. 1998). PKC has been found to act as an important mediator underlying multiple aspects of myocardial function, including Ca\(^{2+}\)-induced inotropy and cardiac hypertrophy (Cain et al. 1999). Different PKC isoforms seem to have different cardiac effects. PKC\(\varepsilon\) and PKC\(\delta\) have been shown to have opposing cardioprotective actions, but parallel hypertrophic effects (Chen et al. 2001). Activation of PKC\(\varepsilon\) mediates cardioprotection from ischemia-caused cell damage, whereas PKC\(\delta\) increases cell damage (Dorn et al. 1999, Chen et al. 2001, Gray et al. 2004). Systolic function is improved after ischemia/reperfusion by PKC\(\varepsilon\), while PKC\(\delta\) mediates opposing effects on systolic and diastolic functions (Hahn et al. 2002, Inagaki et al. 2003). In cardiomyocytes, the effects of AM on MAPK pathways are not known. However, these pathways (p44/42 MAPK, p38 MAPK and c-Jun N-terminal kinase (JNK)) are found to have a significant role in regulating development of cardiac hypertrophy (Molkentin & Dorn II 2001). The PI3K/Akt pathway is an anti-apoptotic signal that protects cardiomyocytes against ischemia-reperfusion injury and promotes cardiomyocytes’ survival (Fujio et al. 2000, Okumura et al. 2004) (Fig. 6). AM gene transfer has been found to reduce the ratio of infarct size to ischemic area at risk and to protect against arrhythmia and apoptosis via activation of the Akt-pathway after ischemia/reperfusion (Kato et al. 2003, Yin et al. 2004). More studies with different
experimental models are still needed to clarify AM signal transduction pathways in different cardiovascular disease models.

2.3 Adenoviral gene transfer into skin

The skin is an attractive target for therapeutic gene transfer because of easy access. Difficulties include inducing sustained expression of the desired gene *in vivo*, the challenge of targeting genes to long-lived stem cells, and the difficulty in achieving specific and uniform transfer to different compartments of the skin. Human skin is built of three main layers: epidermis, corium of dermis and subcutis (for review, see Kanitakis 2002). The epidermis is the topmost and thinnest skin layer. It consists of three types of the cells: undifferentiated, proliferating keratinocytes, melanocytes, and Langerhans cells. The keratinocytes are formed in the basal layer and move up slowly, and epidermal gene therapy is mainly focused on the keratinocytes. The melanocytes produce melanin pigment grains, and the Langerhans cells are part of the immune system (Trainer & Alexander 1997). The epidermis as a whole forms a natural barrier against chemical substances and physical influences. The corium dermis is a thick layer, which consists of collagen and elastin fibers, lymph and blood vessels as well as nerve fibers, sweat and sebaceous glands, and hair follicles. The subcutis is the deepest layer of the skin, composed primarily of fat cells. It has an important function as a warmth isolating layer as well as energy storage and buffer (for review, see Kanitakis 2002).

Recombinant adenovirus is an effective vector for skin gene transfer (Setoguchi *et al.* 1994, Rauma *et al.* 2001). Adenoviruses can infect human skin cells with efficiency as high as 95% (Kozarsky & Wilson 1993, Mulligan 1993, Kremer & Perricaudet 1995). When injected subcutaneously, gene expression is seen both in the epidermis and dermis, encompassing keratinocytes, sebaceous glands, smooth muscle cells, fibroblasts, and adipocytes. However, it has been suggested that the presence of tissue macrophages may limit the use of adenovirus as an *in vivo* gene vector via the innate (i.e. non-antigen-specific) immune system in host defenses (Wolff *et al.* 1997).

2.3.1 Gene transfer in wound healing

Wound healing is a dynamic process involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells. The process of wound healing is divided into three phases, which are shown in Table 4 (for reviews, see Clark 1996, Singer & Clark 1999). Gene transfer in wound healing holds a significant promise, mainly as a tool for understanding the role of growth factors in wound healing. The goal of adenovirus mediated gene transfer is temporary high level transgene expression in cells participating in the wound healing response.
Table 4. The process of wound healing (modified from Clark 1996, Singer & Clark 1999).

<table>
<thead>
<tr>
<th>Phase of healing</th>
<th>Time after injury</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>Early (0-3 days)</td>
<td>Infiltration of neutrophils, liberation of growth factors</td>
</tr>
<tr>
<td></td>
<td>Late (0.3-10 days)</td>
<td>Infiltration of monocytes and macrophages</td>
</tr>
<tr>
<td>Tissue formation</td>
<td>3-18 days</td>
<td>Epithelialization, granulation tissue formation, neovascularization</td>
</tr>
<tr>
<td>Tissue remodeling</td>
<td>8 days -</td>
<td>Extracellular matrix remodeling, cell maturation, cell apoptosis</td>
</tr>
</tbody>
</table>

Growth factor proteins display potent tissue repair activities. PDGF stimulates fibroblast proliferation and chemotaxis, as well as the synthesis of extracellular matrix (Kaplan et al. 1979, Seppä et al. 1982, Heldin et al. 1989). Adenovirus-mediated PDGF gene transfer has been shown to increase granulation tissue formation and keratinocyte migration over the wound bed, accelerating wound closure (Liechty et al. 1999). Hepatocyte growth factor (HGF) has also been found to significantly enhance wound healing and inhibit overscarring (Ha et al. 2003). Adenovirus-mediated VEGF(165) gene transfer enhances wound healing by promoting angiogenesis in CD1 diabetic mice (Romano et al. 2002). The other possible transgenes in wound healing process are fibroblast growth factor (FGF), insulin-like growth factor II (IGF), and transforming growth factor β (TGF-β) (Sprugel et al. 1987, Lynch et al. 1989, Mustoe et al. 1991, Pierce et al. 1991, Lynch et al. 1991, Klingbeil et al. 1991, Pierce et al. 1992, Uhl et al. 1993).

It has been proved that fibroblasts, keratinocytes, and endothelial cells with transgene expression exhibit normal proliferative potential after adenoviral gene transfer (Sylvester et al. 2000). Besides direct local injection of recombinant adenovirus, matrix-mediated delivery of the transgene to the wound has also been used to prolong the availability of the vector to incoming repair cells (Chandler et al. 2000, Gu et al. 2004). Immobilizing matrices enable the controlled delivery of tissue promoting genes. When delivered to subcutaneously implanted sponges, a PDGF-B-encoding adenovirus formulated in a collagen matrix enhanced granulation tissue deposition, wound closure, and complete wound healing without excessive scar formation (Doukas et al. 2001). Further enhancements of the tissue repair response have been achieved by combining matrix-enabled gene transfer with molecular targeting, in which the DNA vector is conjugated to a growth factor ligand. One ligand family, highly effective at targeting adenoviral vectors, is the family of FGFs. The FGFs allow a high degree of targeting specificity due to their cognate high affinity FGF receptors, which are expressed on cells undergoing repair and regeneration. These promising results support the clinical evaluation of gene activated matrices for the treatment of chronic dermal wounds (Chandler et al. 2000).

Severe burns and chronic ulcers have been treated using keratinocyte-containing skin substitutes. These skin substitutes can be enhanced by genetic modification. Exogenous retrovirus-mediated expression of IGF has been shown to promote keratin growth in vitro and stimulate proliferation in vivo, without altering epidermal differentiation (Eming et al. 1996). Lymphangiogenesis and angiogenesis are induced by subcutaneously injected recombinant adenovirus expressing VEGF-C (Enholm et al. 2001). Adenovirus-mediated angiopoietin-1 gene therapy has been used to enhance postoperative skin flap survival by promoting therapeutic angiogenesis and decreasing the necrotic area (Jung et al. 2003).
Another area of interest is the modulation of TGF-β levels in preventing scarring and fibrosis during wound healing (O’Kane & Ferguson 1997).

### 2.3.2 Other skin gene transfer targets

Squamous cell carcinoma, consisting of transformed keratinocytes, is a common skin malignancy. Adenovirus-mediated transfer of a sequence encoding the herpes simplex thymidine kinase ‘suicide’ gene, followed by treatment with gancyclovir, achieved regression of squamous head and neck tumours. (O’Malley et al. 1995). Similarly, adenoviral delivery of the wild-type p53 gene also caused regression of these tumours as followed by activation of apoptosis (Liu et al. 1995). Targeted inhibition of human collagenase-3 (MMP-13) expression using adenoviral gene transfer has been found to inhibit squamous cell carcinoma growth and invasion in vivo (Ala-aho et al. 2004). ONYX-015 is an E1B-55kDa gene-deleted adenovirus found to replicate in and lyse p53-deficient cancer cells but not cells with functional p53. The human adenovirus E1B gene encodes a 55-kDa protein that inactivates the cellular tumor suppressor protein p53. Injection of this mutant virus into p53-deficient human cervical carcinomas grown in nude mice caused reduction in tumor size and regression of 60 percent of the tumors (Bischoff et al. 1996). It has also been reported that tumor-selective replication and necrosis induction occur within squamous cell carcinomas of the head and neck in patients; normal tissues have not been adversely affected clinically (Kirn et al. 1998, Kirn 2001). However, although selective tumor necrosis has been demonstrated in several clinical trials, objective responses with this virus as a single agent have been uncommon (≤ 10%) (Kirn 2001). When intratumoral ONYX-015 injection is given in combination with cisplatin and 5-fluorouracil chemotherapy, more encouraging antitumoral activity has been demonstrated in patients with recurrent squamous cell cancer of the head and neck (Khuri et al. 2000).

Melanoma is used as a model for tumor-directed gene therapeutic treatments due to its accessibility in the skin. Bonnekoh et al. showed that adenovirus is an efficient in vivo gene delivery system for treating experimental human melanomas. They observed an approximately 40-50% reduction in melanoma volume in comparison to controls in their nude mouse model experiments using adenovirus-mediated herpes simplex virus thymidine kinase gene (HSV-tk) transfer and administration of ganciclovir (Bonnekoh et al. 1996). Utilizing an ex vivo cytokine vaccine approach or an in vivo strategy using a combination of cytokine/HSV-tk suicide gene delivery and treatment with ganciclovir, they observed an up to 88% reduction in the B16 melanoma volume (Bonnekoh et al. 1998). Direct injection of adenoviruses encoding p53 alone or in combination with antisense cyclin D1 has also been successfully used to induce apoptosis and reduce tumor size in human melanoma (Sauter et al. 2002). Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 (TIMP-3) has been shown to inhibit invasion and to induce apoptosis in melanoma cells (Ahonen et al. 1998). Cyclooxygenase-2 (cox-2) expression has been analysed as a potential target for transcriptional targeting of adenoviral vector-based melanoma gene therapy (Nettelbeck et al. 2003).
Genetic vaccines against chronic infections with viral agents such as the human immunodeficiency virus (HIV), herpes simplex virus (HSV), and hepatitis C virus (HSV) are potential gene transfer applications (for reviews, see McDonnell & Askari 1996, Lisziewicz et al. 2003, Duenas-Carrera 2004). These vaccines contain genes for an antigenic portion of a virus, such as the core protein or the envelope protein, and the DNA is packaged into recombinant viral vectors, for example a retrovirus, vaccinia virus, or adenovirus (Jin et al. 2002). Naked DNA vaccination without a vector system is also widely studied. The main objective is to augment virus-specific host immune responses. Ongoing research shows promise in prophylactic and therapeutic vaccination for viral infections, but further studies are necessary before vaccines for HSV, HPV, and HIV become commercially available.
3 Aims of the research

The aims of the present study were to evaluate the mechanisms of adenovirus endocytosis, to clarify the role of adrenomedullin in cardiovascular disease models using adenovirus as an investigatory tool, and to apply adenovirus-mediated lysyl hydroxylase gene transfer into skin fibroblasts.

Specifically the aims were:

1. to study the $\alpha_v$ integrin-mediated signaling pathways and the effect of Rab5 GTPase on adenovirus entry and gene transfer efficiency.
2. to investigate the role of adrenomedullin in the heart function and myocardial remodeling in normal rat heart, during angiotensin II infusion and after myocardial infarction using adenovirus-mediated intramyocardial gene transfer.
3. to evaluate the significance of adrenomedullin in neointima formation after endothelial injury in rat artery using adenovirus-mediated intravascular gene transfer.
4. to evaluate the possibility of increasing functional lysyl hydroxylase production and lysyl hydroxylase activity in type VI Ehlers-Danlos syndrome patient fibroblasts and in rat fibroblasts in vitro as well as in rat skin in vivo.
4 Materials and methods

4.1 Preparation of plasmid constructs (I)

pGEM-1-Rab5 and pGEM-1-Rab5S34N were kind gifts from Dr. Marino Zerial (EMBL Laboratories, Heidelberg, Germany.) The cDNA fragment encoding rab5 protein was digested with BamHI (Promega Co., San Diego, CA, USA) and the cDNA fragment encoding Rab5S34N was digested with EcoRI (Promega). Both cDNAs were incubated with Shrimp Alkaline Phosphatase (SAP) (USB, Amesham Life Science) according to manufacturer’s instructions and cloned to BamHI or EcoRI and SAP digested expression plasmid pcDNA3.1(+). The CAR and αv-integrin cDNAs were kind gifts from Dr. Erkki Ruoslahti (Burnham Institute, La Jolle, CA). cDNA fragments encoding these proteins were ligated into the expression plasmid pcDNA3.1(+) (Invitrogen, San Diego, CA). As a control plasmid for endocytosis efficiency, pure pcDNA3.1 without any cDNA fragment was used. Plasmid DNA maxi-prep was prepared using QIAfilter Plasmid Maxi Kit (Qiagen Inc., Chatsworth) according to the manufacturer’s instructions. All nucleotide sequences were confirmed with automated nucleotide sequencing utilizing ABI PRISM BigDye Terminators Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3700 DNA Analyzer. Oligonucleotides for ABI sequencing were purchased from Sigma-Genosys (Cambridge, England, UK).

4.2 Adenovirus vectors

Ad5RSVβgal and Ad2CMVβgal were generated as described in detail previously (Davidson et al. 1994, Zabner et al. 1994a).

For construction of Ad5RSVrAM, a full-length coding region of rat AM cDNA containing poladenylation signal was subcloned into adenovirus shuttle plasmid. This shuttle plasmid and the Ad5 backbone, sub360, were cotransfected into HEK293 cells. Ad5RSVrAM was purified by conventional techniques (Jones & Shenk 1979, Rowe et al. 1983) by the University of Iowa Gene Transfer Vector Core. Cell lysates from infected
cells were evaluated for AM production by radioimmunoassay as described later. Recombinant Ad5RSVrAM was expanded and concentrated using CsCl centrifugation by the AIV Institute, University of Kuopio, Finland.

For generation of Ad5RSVLH, a 3040 nucleotide BamHI-EcoRI fragment of human LH cDNA LH3A clone (Hautala et al. 1992) containing polyadenylation signal was subcloned into adenovirus shuttle plasmid. This shuttle plasmid and the Ad5 backbone, sub360, were cotransfected into HEK293 cells. Ad5RSVrAM was purified by conventional techniques (Jones and Shenk, 1979; Rowe et al. 1983) by the University of Iowa Gene Transfer Vector Core. Cell lysates from infected cells were evaluated for LH activity as described later. The Ad5RSVrAM was plaque-purified and concentrated using CsCl centrifugation.

### 4.3 Animal studies

**Table 5. Summary of the animal experimental protocols**

<table>
<thead>
<tr>
<th>Study</th>
<th>Virus</th>
<th>Target tissue</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Ad5RSVrAM</td>
<td>SD-rat myocardium</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td></td>
<td>Ad5RSVβgal</td>
<td></td>
<td>Ang II infusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Echocardiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Isolated perfused rat heart</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Northern blot</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Morphology of hearts</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>X-gal staining</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Western blot</td>
</tr>
<tr>
<td>III</td>
<td>Ad5RSVrAM</td>
<td>SD-rat carotis communis</td>
<td>Endothelial injury</td>
</tr>
<tr>
<td></td>
<td>Ad5RSVβgal</td>
<td></td>
<td>Intima/media ratio</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>X-gal staining</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Quantitative RT-PCR analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Apoptosis staining</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Primitive cell staining</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Myofibroblast staining</td>
</tr>
<tr>
<td>IV</td>
<td>Ad5RSVLH</td>
<td>SD-rat skin</td>
<td>X-gal staining</td>
</tr>
<tr>
<td></td>
<td>Ad5RSVβgal</td>
<td></td>
<td>β-gal activity assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LH activity assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LH mRNA analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Western blot</td>
</tr>
</tbody>
</table>

Ad, adenovirus; rAM, rat adrenomedullin; Ang II, angiotensin II; β-gal, β-galactosidase; LH, lysyl hydroxylase; RNA, ribonucleic acid; RSV, rous sarcoma virus; SD-rat, Sprague-Dawley rat; X-gal, X-galactosidase

Male Sprague-Dawley (SD) rats were obtained from the Center for Experimental Animals at the University of Oulu, Finland. The rats were housed in plastic cages in a room with a controlled humidity of 40 % and temperature of +22°C. A controlled
environmental 12-hour light-dark cycle was maintained. All the experimental designs were approved by the Animal Use and Care Committee of the University of Oulu. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The summary of the animal experimental protocols of the studies have been shown in Table 5.

4.3.1 Gene transfer into myocardium (II)

Adenoviral AM gene transfer into the left ventricular free wall was done by the method recently described (Szatkowski et al. 2001) with appropriate modifications. Male SD-rats, weighing 250 to 300 g, were anesthetized with medetomidine hydrochloride (Domitor) 250 µg/kg intraperitoneally (i.p.) and ketamine hydrochloride (Ketamine) 50 mg/kg, i.p. A left thoracotomy and pericardial incision was performed. 5 x 10⁸ infectious units of recombinant adenovirus with rat AM coding sequences (Ad5RSVrAM) or control virus (Ad5RSVlacZ) in a 50 µl volume was injected directly into the left ventricular free wall using a Hamilton precision syringe. The heart was repositioned in the chest, the rats were briefly hyperventilated, and the incision was closed. After operation the anesthesia was partially antagonized with atipamezole hydrochloride (Antisedan) 1.5 mg/kg, i.p. and the rats were given buprenorphine hydrochloride (Temgesic) (Schering-Ploug, Brussels, Belgium) 0.2 mg/kg subcutaneously (s.c.) for analgesia.

Echocardiography was performed before, 1 day, 3 days, 1 week and 2 weeks after operation. Part of the animals was sacrificed at each time point, hearts were removed and the chambers separated. Left atrial, right atrial, left ventricular and right ventricular tissue samples were weighed, immersed in liquid nitrogen and stored at -70°C for later analysis.

4.3.2 Gene transfer into artery wall (III)

Male SD-rats, weighing 300 to 400 g, were used. The animals were anesthetized with fentanyl citrate 0.315 mg/ml, midazolam 5 mg/ml, and aqueous solution (1:1:2) 3.3 ml/kg i.p. The right common carotid artery was exposed, and two ligatures were tied tightly around the artery, separated by a distance of approximately 1.5 cm. A 30-gauge needle was inserted into both upper and lower end of the isolated segment, and all blood was rinsed with 0.9 % NaCl from inside the isolated segment. A gentle stream of air (25 ml/min) was passed along the lumen of the vessel, through the segment, for 3 minutes to produce drying injury of the endothelium (Fishman et al. 1975). The holes were then ligated and the segment was filled with recombinant adenovirus (Ad5RSVrAM or Ad5RSVlacZ) in a volume of 50 µl (1 x 10¹⁰ pfu/ml) for 20 minutes. The ligatures were cut and the circulation was released. The rats received buprenorphine hydrochloride (Temgesic) 0.2 mg/kg s.c. twice, 6 and 12 hours after the procedure, for analgesia.
4.3.3 Gene transfer into rat skin (IV)

To determine LH gene transfer efficiency in rat skin, SD-rats were anesthetized with intraperitoneal injection of fentanyl citrate 0.315 mg/ml, midazolam 5 mg/ml, and fluaniisone 10 mg/ml (1:1:1). 5 x 10^8 infectious units of Ad5RSVLH virus in 50 µl volume were injected into the abdominal dermis of rats. The rats were sacrificed on days 0, 7, 14, or 28 after injection and skin samples were collected. The samples were used for LH activity assay, mRNA preparation, or amino acid analysis.

4.3.4 Myocardial infarction (II)

Myocardial infarction was produced by ligation of the LAD coronary artery by a method based on that previously described (Pfeffer et al. 1979) with appropriate modifications. Male SD-rats weighing 250-300 g were anesthetized with medetomidine hydrochloride (Domitor) 250 µg/kg i.p. and ketamine hydrochloride (Ketamine) 50 mg/kg i.p. The rats were connected to a respirator through a tracheotomy and ventilated at a rate of 55 - 60 breaths per minute. A left thoracotomy and pericardial incision was performed. A solution containing recombinant adenovirus (Ad5RSVRAM or Ad5RSVLacZ) was injected directly into the left ventricular free wall using a 100 µl Hamilton precision syringe. After that the LAD coronary artery was ligated about 3 mm from its origin. The heart was repositioned in the chest and the incision was closed. After operation the anesthesia was partially antagonized with atipamezole hydrochloride (Antisedan) 1.5 mg/kg, i.p. and the rats were hydrated with 10 ml physiological saline solution given subcutaneously. For postoperative analgesia, buprenorphine hydrochloride (Temgesic) 0.2 mg/kg s.c. was administered. The sham operated rats underwent the same surgical procedure without any gene transfer or ligation of the LAD coronary artery.

Echocardiography was performed before, 1 week and 2 weeks after operation. Part of the animals was sacrified at each time point, hearts were removed and the chambers separated. Left atrial, right atrial, left ventricular and right ventricular tissue samples were weighed, immersed in liquid nitrogen and stored at -70°C for later analysis.

4.3.5 Angiotensin II infusion with osmotic minipumps (II)

Ang II (33.3 µg/kg/h) was administered by using subcutaneously implanted osmotic minipumps (Alzet model 2001 for 1 week infusion, pumping rate 1 µl/h, volume 220 µl, B & K Universal AB, Sollentuna, Sweden). SD-rats weighing 250-300 g were anesthetized with medetomidine hydrochloride (Domitor) 250 µg/kg i.p. and ketamine hydrochloride (Ketamine) 50 mg/kg, i.p. The minipump was placed subcutaneously into the back of the SD-rat and the adenoviral gene transfer was performed as described. After operation the anesthesia was partially antagonized with atipamezole hydrochloride (Antisedan) 1.5 mg/kg, i.p. and the rats were given buprenorphine hydrochloride (Temgesic) 0.2 mg/kg s.c. for analgesia.
Echocardiography was performed before, 3 days and 1 week after operation. Part of the animals was sacrificed at each time point, hearts were removed and the chambers separated. Left atrial, right atrial, left ventricular and right ventricular tissue samples were weighed, immersed in liquid nitrogen and stored at -70°C for later analysis.

4.3.6 Echocardiography (II)

Transthoracic echocardiograms were performed using a commercially available Acuson Ultrasound System (Sequoia™ 512) and a 15-MHz linear transducer (15L8) (Acuson, MountainView, CA, USA). The echocardiographist was blinded for the study. Before examination, rats were sedated with ketamine 50 mg/kg and xylazine 10 mg/kg i.p. and the chest was shaved. The rats were placed in the supine position and normal body temperature was maintained during the examination by a warming pad and a lamp. Warmed acoustic gel was applied to the transducer and chest.

Using two-dimensional imaging, a short-axis view of the left ventricle at the level of the papillary muscles was obtained, and the two-dimensionally guided M-mode recording through the anterior and posterior walls of the left ventricle was obtained. Left ventricle (LV) end-systolic (LVESD) and end-diastolic (LVEDD) dimensions as well as interventricular septum (IVS) and posterior wall (PW) thickness were measured from the M-mode tracings. LV shortening fraction (FS), a measure of left ventricular systolic function, and ejection fraction (EF) were calculated from the M-mode LV dimensions using the following equations: LVFS (%) = [(LVEDD-LVESD) / LVEDD] x 100, EF (%) = [(LVEDD)³–(LVESD)³ / LVEDD³] x 100. An average of three measurements of each variable was used.

4.3.7 Isolated perfused rat heart preparation (II)

Adenovirus-mediated gene transfer was performed as described earlier. Three days after gene transfer, rats were decapitated and hearts were quickly removed and arranged for retrograde perfusion by the Langendorff technique as described previously (Szokodi et al. 2002). The hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer, pH 7.40, equilibrated with 95% O₂-5% CO₂ at 37°C. Hearts were perfused at a constant flow rate of 15 ml/min with a peristaltic pump (Minipuls 3, model 312) resulting in similar perfusion pressure values in control and adrenomedullin transfected hearts (69 ± 4 versus 74 ± 3 mmHg, P=NS). The heart rate was maintained constant (300 ± 1 beats / min) by atrial pacing using a Grass stimulator (model S88, 11 V, 0.5 ms). Isovolumic left ventricular pressure was measured using a fluid-filled balloon, which was placed in the left ventricular chamber and connected to a pressure transducer (Isotec, Hugo Sachs Elektronik, Germany). The balloon was large enough so that negligible pressure resulted when the balloon alone was filled up to the maximum volume used. Analog signals were digitized at a sampling frequency of 1 kHz. All the data were recorded and analyzed with an IBM PC-compatible computer using Ponemah data acquisition software (Gould Instrument System Inc, OH, USA). The following parameters were obtained: peak
systolic left ventricular pressure (DP), maximum and minimum values of the first derivative of isovolumic pressure (dP/dtmax, dP/dtmin), and time constant of exponential pressure decay (τ). After a 20-minute equilibration period, the balloon was inflated in 10 µl steps to achieve LVEDP of 1, 5, 10, 15 and 20 mmHg. Parameters of left ventricular contractility were obtained when a new steady-state was reached.

4.3.8 Measurement of gene transfer efficiency by X-Gal staining

Rat heart, artery, or skin samples were removed two days after infection with Ad5RSVβgal or control virus. The samples were rinsed in phosphate buffered saline (PBS), and fixed in PBS containing 4 % paraformaldehyde (PFA) (Sigma) for 10 min at room temperature. Samples were washed twice in 0.15 M sodium phosphate buffer (pH 7.2) for 15 min, and incubated with 1 mg/ml X-gal (5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) in reaction buffer (2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆.3H₂O, 1 x PBS) at 37°C for 3 hours. Some tissue samples were embedded in OCT compound, frozen, sectioned and analyzed with a light microscope for blue β-galactosidase expression.

4.3.9 Histological stainings (III)

To analyze carotid arteries after AM gene transfer, rats were sacrificed 14 days after procedure. The transfected segments were removed, washed with saline and divided into three parts. The parts were fixed in 10 % formalin overnight, embedded in paraffin, sectioned, and stained with hematoxylin-eosin or Verhoeff (elastin) stainings. Intima/media (I/M) ratios were calculated using a light microscope (40x, Olympus BH-2, Olympus, Japan). Ratios from at least three randomly selected sections were analyzed, and means of the measurements in each rat were calculated.

In order to detect apoptotic cells, in situ labeling of the 3’-ends of the DNA fragments generated by apoptosis-associated endonuclease was performed using an ApopTag in situ apoptosis detection kit (Chemicon International, Temecula, USA) according to the manufacturer’s instructions. The slides were lightly counterstained with hematoxylin. To estimate the apoptotic index (the percentage of apoptotic events in neointima), all neointimal apoptotic cells and bodies in tissue sections of all three arterial parts were counted (40x), and this figure was divided by the number of all neointimal cells. The average count for each rat was used. Evaluation of the stainings was performed independently by two persons.

For immunohistochemical stainings of stem cell and myofibroblast markers, formaline fixed, paraffin embedded carotid artery sections (5 µm) were placed on poly-l-lysine coated (Sigma Chemicals, St Louis, MO, USA) glass slides, air-dried overnight, and stained within a few days. The sections were then dewaxed in xylene and rehydrated in graded alcohol. Before the staining process, the tissue sections were subjected to antigen retrieval in a microwave oven in Tris-EDTA (pH 9) by boiling for 15 min. Endogenous peroxidase and non-specific binding were blocked by incubating the slides in Peroxidase-
Blocking Solution (DakoCytomation, Denmark) for 30 minutes. Antibodies against c-Kit (Santa Cruz Biotechnology, Santa Cruz, USA), human multidrug resistance-1 (MDR1) gene product (Signet Laboratories Inc., Dedham, MA, USA), α-smooth muscle actin (α-sma) (Sigma Bio-Sciences, St Louis, MO), and desmin (DakoCytomation) were used with dilutions 1:400, 1:50, 1:100, and 1:50, respectively. For c-Kit and MDR1 stainings, the ChemMate™EnVision™DetectionKit, Peroxidase/DAB (DakoCytomation) was then used, and for α-sma and desmin stainings, the Histomouse™-Max Kit (Zymed Laboratories, Inc., San Francisco, CA, USA) was used according to the manufacturer’s instructions. The sections were lightly counterstained with hematoxylin. Negative control stainings were carried out using washing solution instead of primary antibody. Evaluation of all the stainings was performed independently by two persons.

4.4 Cell culture studies

4.4.1 Cell cultures and β-galactosidase assays (I)

Monolayers of the HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL, Grand Island, New York, USA) containing 10 % fetal bovine serum (FBS) (Gibco Europe, Paisley, Scotland, UK), and 1 % penicillin-streptomycin (PS) (Sigma, St. Louis, MO, USA) in a humidified incubator with a 5 % CO₂ atmosphere at 37°C. For the expression of the protein interest, HeLa cells were split 18-24 hours before transfection so that on the day of transfection they were about 80 % confluent. The cells were washed in Dulbecco’s PBS (Gibco-BRL) and transfected with plasmid DNA containing the gene of interest using Lipofectin transfection reagent® (Gibco-BRL) according to the manufacturer’s instructions. The ratio on Lipofectin Reagent to plasmid DNA was 6. The cells were incubated for 6 hours at 37°C in 5 % CO₂ humidified incubator, washed with PBS, and grown for another 18-24 hours in DMEM containing 10 % FBS and 1 % PS.

To test the effect of Rab5 on recombinant adenovirus gene transfer, HeLa cells on 24-well plates (Falcon 3047) (Falcon, Plymouth, England, UK) were transfected (100,000 cells/well) with 6 µg of Lipofectin and 1 µg of plasmid. 24 hours after transfection, the cells were incubated with 50 pfu/cell of Ad2CMVβgal for 15 min in a 5 % CO₂ humidified environment at 37°C. β-galactosidase activity was assayed with the Luminescent β-galactosidase Detection Kit II (Clontech Laboratories Inc., Palo Alto, CA, USA) and luminometer (Luminoscan RS, Labsystems Oy, Helsinki, Finland) 24 hours after infection. Cells were removed from dishes by incubation with 120 µl of lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2 % Triton X-100, and 1 mM dithiothreitol) for 15 min, followed by scraping. A 10-µl aliquot from each 24-well plate was used for each assay. The protein concentration in each sample was measured with Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) and spectrophotometer (Shimadzu, UV-160A).

To investigate the role of pp125FAK in adenovirus infection, pp125FAK deficient cells (-/-) and pp125FAK positive control cells (Ilic et al. 1995) were infected with 10 pfu/cell of
Ad2CMVβgal at 37°C for 30 min. β-galactosidase activity was measured 24 hours later using a Luminescent β-galactosidase Detection Kit II and a luminometer.

Hydroxylysine-deficient human skin fibroblasts (CRL1195) (Sussman et al. 1974) were obtained from the American Type Culture Collection (Manassas, VA). These cells and locally established rat and control human skin fibroblasts were cultured in DMEM supplemented with 10 % FBS, 28 µM ascorbate, 2 mM glutamate, 100 U/ml penicillin, and 100 µl/ml streptomycin. The cells were infected by recombinant Ad5RSVLH or Ad5RSVβgal (50 or 100 pfu/cell), and LH activity assay and detection of mRNA were done 48 hours after infection as described later.

### 4.4.2 Fluorescent microscope analysis

Measurement of fluid phase endocytosis was done by determining endocytosis of Fluorescein Isothiocyanate-dextran (FITC) (FD-10S; Sigma) to show the functionality of the Rab5 expression constructs used. At 24 hours after transfection HeLa cells (300,000 cells/dish) cultured on glass coverslips placed on 60 mm dishes were incubated with serum-free DMEM containing 330 µg/ml of dextran-FITC for 15 min at 37°C. The samples were prepared for microscopy assay by washing with PBS, fixing in 4 % PFA in PEM buffer (Pipes (piperazine-N,N'-bis[2-ethanesulfonicacid]) (Sigma) 100 mM, EGTA 5 mM, MgCl₂ (Sigma) 2 mM, pH 6.8) for 15 min at room temperature, and mounting on glass slides containing 90 % glycerol. Fluorescence intensity was determined with videomicroscopy as described later.

To test the transfection efficiency of Lipofectin Reagent, HeLa cells cultured on glass coverslips were transfected with 1 µg plasmid pEGFP-C1 (Clontech), using 6 µg Lipofectin Reagent. 24 hours after transfection the HeLa cells were washed with PBS, fixed in 4 % PFA in PEM buffer for 15 min, and mounted on glass slides in PBS containing 90 % glycerol. Transfection efficiency was estimated by the number of cells positive for the green fluorescent protein detected with fluorescence laser scanning confocal microscopy as described later.

For measurement of the endocytosis of Cy3-labeled adenovirus, recombinant adenovirus particles were fluorescently labeled by stirring 3 x 10¹¹ virus particles with 200 µl of Fluorolink Cy3 (Amesham Life Science Inc., Arlington Heights, IL) in 100 mM sodium carbonate buffer, pH 9.3, for 30 min at room temperature with protection from light. Cy3-labeled adenovirus was purified with dialysis (Slide-A-Lyzer, Pierce Chem. Co., Rockford, IL). Dialysis buffer (150 mM NaCl, 20 mM MgCl₂, 10 mM Tris-HCl, pH 7.8 and 10 % glycerol) was traded in on a new one after one hour. HeLa cells were cultured on glass coverslips (300,000 cells/60-mm-diameter dish) and transfected (3 µg of plasmid and 18 µg of Lipofectin) with CAR and/or wild-type Rab5, Rab5S34N, or a control plasmid. 24 hours after transfection the HeLa cells were infected by Cy3-labeled adenovirus (50 multiplicity of infection, MOI) for 15 min at 37°C. Distribution and intensity of endocytosed virus was determined in HeLa cells fixed in 4 % PFA by fluorescence laser scanning confocal microscopy.

The FITC, pEGFP-C1, and Fluorolink Cy3 labeled cells were studied with fluorescence laser scanning confocal microscopy (Leica Aristoplan CLSM, Leica...
Lasertechnics BmbH, Heidelberg). The microscope was equipped with 75 mW ArKr Laser (Omnichrome, Chino, CA), and 488 nm and 568 nm excitation wavelengths were used, respectively. Leitz PL APO 63x 1.4Na oil immersion objective was used.

The fluorescence intensities of the labeled cells were determined using videomicroscopy. An inverted Nikon TMD Diaphot microscope was equipped with Nikon Fluor 40x 1.5Na oil immersion objective and Nikon B-2A filter sets for FITC and Cy3, respectively. Emission light was collected using 1X camera tubus and Hamamatsu C4880 cooled slow scan CCD camera with 2x binning. Appropriate ND filters were used in front of the 100W Xenon light source to minimize photobleaching. The image data were collected to PC and analyzed with the image analysis software MCID-M2 (Imaging Research Inc. St. Catharines, Canada). Relative optical densities were acquired in the same session from all the samples with the same camera settings and the same segmentation parameters in the analysis software. Endocytose efficiency was quantified by comparing the proportion of bright cells to the total number of the calculated cells. The average of the estimated counts of samples (measured on same occasion) was calculated. The cells whose estimated counts were higher than the average were counted. The results were proportioned to 100 cells. The estimated counts are the estimated number of targets, derived by dividing large targets into smaller ones of a specified size.

4.4.3 Measurement of [Ca^{2+}]

To investigate the [Ca^{2+}] after adenovirus binding, HeLa cells were loaded with fura-2-acetoxyethyl ester (fura-2 AM) (Molecular Probes, Eugene, Oreg.) 5 mg/ml. [Ca^{2+}], was measured before and after virus binding with a Photoscan 2 spectrofluorometer (Photon Technologies International, New Brunswick, N.J.).

As a further test of the potential role of Ca^{2+} in viral infection, the effect of 0 to 50 nM calmidazolium (Calbiochem, San Diego, Calif.) was measured. The cells were preincubated in medium with calmidazolium for 30 min, and then 10 pfu/cell of Ad2CMV-βgal was added in the continued presence of inhibitor for 30 min. β-galactosidase activity was measured using a Luminescent β-galactosidase Detection Kit II and a luminometer.

4.5 Isolation and analysis of cytoplasmic RNA (II-IV)

Total RNA was isolated from SD-rat left ventricles by guanidine thiocyanate followed by CsCl gradient centrifugation (Chirgwin et al. 1979). In Northern blot hybridization the size and amount of specific mRNA molecules in total RNA preparations was determined (Alwine et al. 1977). For the RNA Northern blot analysis, 20 µg samples of the RNA from the left ventricles were separated by electrophoresis on agarose-formaldehyde gel and transferred to nylon membranes. A rat AM cDNA probe, an atrial natriuretic peptide (ANP) cDNA probe, a brain natriuretic peptide (BNP) cDNA probe, and a full-length cDNA probe complementary to rat 18 S ribosomal RNA (Lee et al. 1988) were labeled with \[^{32}P\]-deoxyCTP with Quick Prime Kit (Pharmacia LKB Biotechnology, Uppsala,
Sweden). The membranes were hybridized overnight at +42°C in 5 x SSC (saline sodium citrate, 1 x SSC = 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate, pH 7), 0.5 % sodium dodecyl sulphate (SDS), 5 x Denhardt’s solution, 50 % formamide and 100 µg/ml sheared herring sperm DNA. After hybridization, the membranes were washed in 0.1 x SSC, 0.1 % SDS three times for 20 min at +61°C and exposed to phosphor imager screen (Molecular Dynamics, Sunnyvale, CA, USA) at room temperature. Phosphor imager screens were scanned with Phosphor Imager. The hybridization signals of rAM, ANP and BNP were normalized to that of 18S to correct the potential differences in loading and/or transfer.

In order to measure rAM mRNA from rat carotid artery pieces, rats were sacrified, and the vessels were removed two days after Ad5RSVrAM transduction. Total RNA was extracted from pieces of transfected vascular segments using GenElute™ Mammalian Total RNA Kit (Sigma) following the instructions of the manufacturer. 0.5 µg of total RNA was used for cDNA synthesis using First-strand cDNA Synthesis Kit (Amesham Biosciences, Piscataway, USA) according to manufacturer’s instructions. rAM and 18S RNA levels were measured with Real-Time Quantitative RT-PCR analysis using Taqman chemistry on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers and probes for detection were designed with Primer Express (Applied Biosystems) program and default settings. The sequences of the forward and reverse primers for rAM mRNA detection were CATTGAACAGTGCGGCGAGT and CAGGGTGCGAGCTCTG, respectively, and the fluorogenic probe was 5'-Fam-CCCATTGGCGCTGCGGA-Tamra-3'. The results were normalized to 18S RNA quantified from the same samples using the forward and reverse primers TGGTGCAAGCTGAACTTAAAG and AGTCAAATTAAGCCG CAGGC, respectively. The probe for the 18S amplicon was 5'-Vic-CCTGGTGCCCTTTCCGTA-Tamra-3'.

For detection of mRNAs for LH, total RNA was treated with Dnase (Pharmacia Biotech) for 1 hour in order to remove contaminating DNA, after which 1 µg of RNA was reverse transcribed according to the manufacturer’s instructions (First Strand DNA Synthesis Kit, Pharmacia Biotech). LH sequences were amplified using oligonucleotide primers 5'-GGCATGGGGATGAAGCTCT and 5'-GGTTGGTCAGGAACATGAAC. This sequence primer is identical in rat and human cDNA, and both generate a 661 nt polymerase chain reaction (PCR) product. The PCR mixture consists of 1 µM of each primer, 20 µM of deoxyribonucleoside triphosphates, 1 IU DNA polymerase (DynazymeII, Finnzymes, Helsinki, Finland), and buffer [1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris-HCl (pH9.0)]. Amplification was performed at 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s for a total of 30 cycles in a thermocycler (Minicycle, MJ Research, Waltham, MA). The rat cDNA sequence has a unique HindIII site while the human one has none. To distinguish between rat and human cDNA, the PCR products were incubated at 37°C with HindIII (Pharmacia Biotech) for 2 h and separated on 1.5 % agarose gel. The 661 nt product was considered the human sequence and the 155 and 506 nt products were considered the rat sequence. The amplification protocol was not optimized for quantitative analysis.
4.6 Western blot analysis (I, II, IV)

In order to investigate the possible signal transduction pathways after adenoviral AM gene transfer into rat myocardium, tissues were homogenized in the buffer containing 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA (ethane glycol-bis(β-aminoethylether)-N,N',N'-tetraacetic acid) (Sigma), 1 mM β-glycerophosphate, 1 mM Na3VO4, 2 mM benzamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 1 mM dithiothreitol (DTT), 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin. Cytosolic and particulate fraction were separated by centrifugation. The resulting supernatant was labeled the cytosolic fraction, whereas the pellet was resuspended with buffer containing 1 % Triton X-100 and sonicated. The sonicated pellet was centrifuged and the supernatant was labeled the particulate fraction.

For Western blot analysis, 15-30 µg protein was run on SDS-PAGE and transferred to nitrocellulose filters. The membranes were blocked in 5 % non-fat milk and incubated with primary antibodies. After incubation with horseradish peroxidase-linked secondary antibody, the protein amount was detected by enhanced chemiluminescence using hyperfilm MP (Amesham, Bucks, UK). To study PKC activation the translocation of PKC subtypes from cytosolic to particulate fraction was determined. Rabbit anti-PKCα (Sigma P4334), anti-PKCε (Sigma P8458), or anti-PKCδ (Sigma P8333) were used as primary antibodies for these studies. MAPK activity was determined by detecting phosphorylation of p44/42 MAPK, p38 MAPK or JNK. Anti-phospho-p44/42 MAPK (Cell Signaling #9106), anti-p44/42 MAPK (Cell Signaling #9102), anti-phospho-p38 MAPK (Chemicon AB3828), anti-p38 MAPK (Cell Signaling #9212), anti-phospho-JNK (Santa Cruz Biotechnology sc-6254), and anti-SAPK/JNK (Cell Signaling #9252) were used as primary antibodies for these studies. Akt activity was determined by detecting phosphorylation of Thr308 or Ser473 sites by using two different phospho-Akt antibodies (Cell Signaling #9275 and #9271). Anti-Akt antibody (Cell Signaling #9271) was also used.

To test the effect of adenovirus on pp125FAK, p130CAS, or paxillin phosphorylation, HeLa cells in suspension as well as serum-starved HeLa cells (0.3 % FBS for 36 hours) attached to culture dishes were used. Cells (2 x 10⁶) were incubated with 50 pfu/cell of Ad2CMVβgal or Ad5RSVβgal for 0 to 30 min at 37°C, washed with PBS, and suspended in 500 ml of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 0.1 % sodium dodecyl sulfate (SDS), 1 % deoxycholate, 50 mM NaF, 0.5 mM Na3VO4, 0.1 U/ml of aprotinin, 10 mg/ml of leupeptin, and 4 mg/ml of pepstatin). Monoclonal anti-pp125FAK, anti-p130CAS, or antipaxillin antibodies (2 mg; Transduction Laboratories, Lexington, Ky.) were added to the supernatants and incubated with shaking on ice for 1 hour. GammaBind G Sepharose (30 ml; Pharmacia Biotech, Uppsala, Sweden) was added, and incubation on ice was continued for 2 hours, after which the precipitate was carefully washed, solubilized in 2 x sample buffer, boiled for 3 min, and run on an SDS–8 or 12 % polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride filters (Millipore). Filters were blocked for 2 hours (3 % bovine serum albumin in 10mM Tris, pH 7.5, 100 mM NaCl, 1 % Tween 20), incubated with PY20H peroxidase-conjugated antiphosphotyrosine antibody (Transduction Laboratories), washed, and the bound antibody was detected by chemiluminescence
(Pierce Chemical Co., Rockford, Ill.). Equal protein loading in pp125FAK, p130CAS, and paxillin phosphorylation experiments were verified by stripping and reblotting the filters with the appropriate monoclonal antibody.

4.7 Radioimmunoassay of ir-AM

AM production was evaluated by radioimmunoassay in order to ensure the quality of prepared Ad5RSVrAM. HeLa cells were transfected with 10, 50, or 100 pfu/cell of Ad5RSVrAM or Ad5RSVβgal adenovirus, the culture medium was collected, and analyzed for ir-AM production. Radioimmunoassay was also used in order to measure the transgene expression for rat carotid samples two days and two weeks after endothelial injury and gene transfer (Study III). The supernatants of homogenized tissue samples were diluted with 0.1% trifluoroacetic acid (TFA) and extracted with Sep-Pak C18 cartridges (Waters). The extracts were dried and redissolved in radioimmunoassay buffer. The AM content was examined with specific radioimmunoassay as follows (Romppanen et al. 1997): Synthetic rat AM 1-50 standards (Phoenix Pharmaceuticals, Mountain Wiew, CA, USA) and the cell culture medium were incubated for 16–24 hours at +4°C with rabbit anti-rat AM serum (Phoenix Pharmaceuticals), 125I-rat AM 1-50, prepared with chloramine-T iodination and purified with Sephadex G-25 (Pharmacia, Uppsala, Sweden) gel filtration followed by Symmetry Shield RP8 (Waters, Milford, MA, USA) reverse phase HPLC, was added, and the incubation was continued for another 16-20 hours. The free and bound fractions were separated by double antibody precipitation. The rat AM antiserum does not cross-react with rat AM 1-20, human amylin or endothelin-1. The sensitivity of the assay was 1 fmol/tube and the intra- and interassay coefficients of variation were <10 % and <15 %, respectively.

4.8 Assays for lysyl hydroxylase (IV)

4.8.1 LH activity assay

Cultured human skin fibroblasts (5 x 10⁶) or rat skin samples (50-100 mg) were homogenized in a buffer containing NaCl 0.2 M, glycine 0.1 M, Triton X-100 0.1 %, Tris-HCl (pH 7.5) 0.02 M at 4°C and centrifuged at 12,000g for 10 min. A 50 µl fraction of soluble protein of cell lysates or tissue samples was mixed with collagenous peptide substrate ([14C]lysine-labeled protocollagen prepared in cultures of tendon cells (120,000 cpm) or nonradioactive GLKGEPGRKGEKG-peptide in final concentration of 0.5 mg per ml) and brought up to 1 ml volume with reaction buffer [Tris-HCl (pH 7.8) 50 mM, bovine serum albumin 1 mg per ml, catalase 0.1 mg per ml, dithiothreitol 0.1 mM, ascorbate 1 mM, FeSO₄ 0.05 mM, 2-oxo-gluutarate 0.5 mM]. The mixture was incubated for 30 min at 37°C and then for 5 min at 95°C (Kivirikko & Myllylä 1982). A fraction of reaction mixture was hydrolyzed with 6 M HCl and analyzed for amino acid composition.
using Applied Biosystems 421 AminoAcidAnalyzer. In experiments with [¹⁴C]lysine – protocollagen substrate, the amino acid analyzer was connected to a fraction collector, and fractions corresponding to lysine and hydroxylysine residues were measured for radioactivity. The protein concentration in each sample was determined (Bio-Rad Laboratories, Hercules, CA), and LH activity is expressed as percentage of hydroxylated lysine residues normalized for sample protein concentration.

4.8.2 LH amino acid analysis

Rat skin samples were analyzed for amino acid composition at indicated times after Ad5RSVHLH or Ad5RSVβgal injection. The skin biopsies were weighed and cut in small pieces, and the tissues were sealed in tubes under a nitrogen atmosphere for hydrolysis with 6 M HCl and 1 mM β-mercaptoethanol at 110°C for 24 hours. Subsequently, the samples were filtered with a disposable 0.2 µm filter (Schleicher & Schuell), lyophilized, and analyzed with Applied Biosystems 421 AminoAcidAnalyzer. The data are expressed as the number of hydroxylated lysine residues per 1,000 amino acids.

4.9 Statistical analysis

The results are expressed as mean ± SEM. For the comparison of statistical significance between two groups and for analysis of multiple groups, Student’s t-test and one-way analysis of variance (ANOVA) followed by LSD post hoc test were used. P value < 0.05 was considered statistically significant.
5 Results

5.1 The role of Rab5 GTPase in adenovirus endocytosis (I)

5.1.1 Effects of adenovirus on $[Ca^{2+}]_i$ and protein tyrosine phosphorylation

Binding of ligand to $\alpha_v$ integrin transiently increases $[Ca^{2+}]_i$ in several cell types (Lawson et al. 1995, Schwartz et al. 1994). To investigate the effect of adenovirus (100 pfu/cell) on $[Ca^{2+}]_i$, the $[Ca^{2+}]_i$ in HeLa cells loaded with fura-2 before and after virus binding was measured. Ad2CMV$\beta$gal, Ad5RSV$\beta$gal or vehicle control did not alter $[Ca^{2+}]_i$. However, subsequent addition of 1 $\mu$M histamine triggered an immediate increase in $[Ca^{2+}]_i$ in the continued presence of the virus. As a further test of the potential role of Ca$^{2+}$ in viral infection, the effect of 0 to 50 nM calmidazolium, an inhibitor of Ca$^{2+}$-calmodulin, was measured. The cells were preincubated in medium with calmidazolium for 30 min, and then 10 pfu/cell of Ad2CMV$\beta$gal was added in the continued presence of inhibitor for 30 min. Calmidazolium did not inhibit adenovirus-mediated $\beta$-galactosidase gene transfer, and at high concentrations there was a small increase in transgene expression.

Focal adhesion kinase pp125$^{FAK}$ can be autophosphorylated and activated by ligand binding to $\alpha_v$ integrin. pp125$^{FAK}$ activation following $\alpha_v$ integrin ligand binding is associated with increased tyrosine phosphorylation of p130$^{CAS}$ and paxillin. The effect of adenovirus on pp125$^{FAK}$, p130$^{CAS}$ or paxillin phosphorylation was measured by Western blot analysis using HeLa cells in suspension or serum-starved HeLa cells attached to culture dishes. Adenovirus did not increase pp125$^{FAK}$ phosphorylation in HeLa cells in suspension; there was a small decrease instead. Likewise, there was no change in the pp125$^{FAK}$ phosphorylation in attached, serum-starved HeLa cells following Ad2CMV$\beta$gal infection. Similar results were obtained with cells exposed to Ad5RSV$\beta$gal. Incubation with adenovirus had no effect on the total amount of pp125$^{FAK}$. In contrast, as previously reported (Vuori & Ruoslahti 1995), pp125$^{FAK}$ phosphorylation increased when attached HeLa cells were exposed to 10 % FBS, or when a suspension of HeLa cells was allowed...
to attach on vitronectin. In addition, adenovirus had no effect on p130CAS or paxillin phosphorylation. Similar results were obtained with attached, serum-starved cells. The role of pp125FAK in adenovirus infection was tested further with αv integrin-positive pp125FAK/– cells, which are deficient in pp125FAK. β-galactosidase expression was similar in both pp125FAK/– and control cell lines 24 hours after infection with 10 pfu/cell of Ad2CMVβgal. These results agree with the previously published data (Li et al. 1998b) and confirm that pp125FAK activity is not required for adenovirus infection and gene transfer.

5.1.2 Effects of Rab5 on adenoviral gene transfer

To test the hypothesis of whether Rab5 GTPase may play a role in adenovirus entry, HeLa cells were transfected with wild-type Rab5 or dominant-negative Rab5S34N cloned in pcDNA3.1(+) expression plasmid. To confirm that the Rab5 expression constructs are functional, endocytosis of FITC dextran in HeLa cells was determined. At 24 hours after transfection fluorescence intensity was determined with videomicroscopy. Expression of wild-type Rab5 significantly increased (190 % of control value) the uptake of dextran-FITC, whereas dominant-negative Rab5S34N expression decreased it (75 % of control value).

To test the effect of Rab5 on recombinant adenovirus gene transfer, transfected HeLa cells were incubated with 50 pfu/cell of Ad2CMVβgal for 15 min. The adenovirus β-galactosidase activity was assayed with the Luminescent β-galactosidase Detection Kit II and luminometry 24 hours following infection. HeLa cells overexpressing wild-type Rab5 displayed a slightly increased (118 % of control; P=NS) level of adenovirus β-galactosidase expression. HeLa cells expressing Rab5S34N showed reduced β-galactosidase activity compared to the control (78 % of control; P<0.05). The cells were also transfected with a combination of CAR receptor and Rab5, Rab5S34N, or a control plasmid. The data indicate that the S34N-mutated Rab5 significantly decreased (102 % vs. 141 %; P<0.05), while the wild-type Rab5 only slightly increased (141 % vs. 156 %; P=NS) adenovirus-mediated gene transfer in HeLa cells overexpressing CAR (Fig. 7A).

Lipofection efficiency was tested with pEGFP-C1 plasmid (Clontech) coding for green fluorescent protein, and it showed that about 50 % of the HeLa cells were transfected.

5.1.3 Rab5 enhances virus endocytosis

The effect of Rab5 GTPase on the efficiency of adenovirus endocytosis was evaluated using fluorescently Cy3-labeled adenovirus particles. HeLa cells were cultured on microscope coverslips and transfected with CAR and/or wild-type Rab5, Rab5S34N, or a control plasmid. The distribution and intensity of endocytosed virus was determined in fixed HeLa cells by fluorescent laser-scanning confocal microscopy and videomicroscopy of at least 150 randomly selected cells in each condition. The efficiency of endocytosis was estimated by determining the average of the amount of fluorescently
labeled adenovirus in each cell. Overexpression of wild-type Rab5 increased endocytosis of labeled virus in HeLa cells compared to the control (126 % vs. 100 %; \( P < 0.05 \)), and Rab5S34N expression decreased the amount and intensity of fluorescent endosomes (80 % vs. 100 %; \( P < 0.05 \)). The HeLa cells overexpressing CAR had an increased amount of endocytosed Cy3-adenovirus compared to the control (128 % vs. 100 %; \( P < 0.05 \)), indicating that endocytosis depended on virus attachment to the CAR. In the presence of CAR overexpression, Rab5S34N decreased and wild-type Rab5 further increased Cy3-adenovirus endocytosis (108 %, and 169 % vs. 128 %; \( P < 0.05 \)) (Fig. 7B).

Fig. 7. A. Adenovirus-mediated gene expression in HeLa cells or HeLa cells overexpressing CAR. The cells were transfected with wild-type Rab5, dominant-negative Rab5S34N, or control plasmid, incubated later with Ad2CMV\( \beta \)gal, and measured for \( \beta \)-galactosidase activity. \( N = 15 \) in each experiment. Data are expressed as mean ± SEM. *\( P < 0.05 \) compared to the control. B. Videomicroscopic analysis of Cy3-labeled adenovirus uptake by HeLa cells overexpressing Rab5, Rab5S34N, and/or CAR as described. The cells were incubated with Cy3-labeled adenovirus (5,000 particles/cell). The relative amount of fluorescence is indicated for each experiment. \( N > 150 \) cells in each experiment, and the experiments were repeated at least three times. Data are expressed as mean ± SEM. *\( P < 0.05 \) compared to the control.

5.2 Adenovirus-mediated AM gene transfer into rat myocardium (II)

5.2.1 AM gene expression in the left ventricle

Recombinant gene transfer efficiency was evaluated by X-gal staining 2 days after adenoviral LacZ gene transfer. An intensive segmental staining area was seen in the anterior wall of the left ventricle. In light microscope analysis after X-gal and
hematoxylin-eosin (HE) counterstaining X-gal stained cardiomyocytes and also some inflammatory cells were seen, analyzed by our pathologist, indicating slight inflammation after virus infection.

AM gene expression was evaluated by Northern blot analysis 3, 7, and 14 days after adenoviral gene transfer. AM mRNA levels in the left ventricle increased 20.9- (\(P<0.001\)) and 17.2-fold (\(P<0.001\)) at 3 and 7 days after adenoviral AM gene transfer when compared to LacZ gene transfer rats. At 14 days after gene transfer AM mRNA levels had returned towards basal levels (4.8-fold vs. LacZ, \(P=NS\)). Little or no induction in AM mRNA levels was seen in right ventricle, left atrium or right atrium after gene transfer, indicating high but local gene expression around the injection site.

To evaluate the effects of AM gene transfer on cardiac gene expression, ANP and BNP mRNA levels were measured by Northern blot analysis. AM gene transfer decreased ANP mRNA levels in the left ventricle maximally by 55 % (\(P<0.05\)) after a two-week follow-up period. No significant effect on BNP mRNA levels was seen. This finding is in accordance with previous in vitro studies showing that AM inhibits ANP gene expression in cardiomyocytes (Sato et al. 1997) and suppresses ANP secretion from isolated rat atria (Kaufman & Deng 1998).

### 5.2.2 Effects of AM on cardiac contractility

Adrenomedullin gene transfer transiently improved left ventricular systolic function assessed by echocardiography. Fractional shortening increased maximally by 14 % (\(P<0.05\)) and ejection fraction by 8 % (\(P<0.05\)) 3 days after AM gene transfer (Fig 8).

Isolated isovolumic rat heart preparations were used to evaluate left ventricular contractility in AM gene transfected hearts independent of any potential confounding systemic hemodynamic or neurohumoral effects. The maximal derivative of isovolumic left ventricular pressure (\(dP/dt_{max}\)) was similar in the control and the AM gene transfer group at left end-diastolic pressure (LVEDP) of 1 or 5 mmHg. However, when LVDP was increased to 10, 15 or 20 mmHg in AM gene transfected hearts, \(dP/dt_{max}\) was increased by 22 % (\(P<0.05\)), 19 % (\(P<0.05\)) and 16 % (\(P<0.05\)) compared to the lacZ group. Similarly, developed pressure (DP) was enhanced by 18 % (\(P<0.05\)), 15 % (\(P<0.05\)) and 14 % (\(P<0.05\)) in the AM gene transfer group at LVEDP of 10, 15 or 20 mmHg, respectively. In contrast to the enhanced contractility, the diastolic function was not affected by AM gene transfer at any level of LVEDP. The minimal derivative of isovolumic pressure (\(dP/dt_{min}\): -2107 ± 76 vs. -1894 ± 135 mmHg/s, \(P=NS\)) and the time constant of exponential pressure decay (\(\tau\): 22.0 ± 0.8 vs. 20.6 ± 1.3 ms, \(P=NS\)) did not differ significantly between the AM gene transfer group and the LacZ group.
5.2.3 PKC and MAPK activation after AM gene transfer

To study PKC activation, the translocation of PKCα, PKCδ and PKCε from cytosolic to particulate fraction was measured by using Western blot analysis 3 days after adenoviral AM or LacZ gene transfers. Translocation of PKCδ into the particulate fraction was activated by 30 % (P<0.01) and PKCε by 20 % (P<0.01), whereas no effect on PKCα-translocation was seen. p38 MAPK activity evaluated by p38 phosphorylation in Western analysis was decreased by 36 % (P<0.05). No significant change was seen in phosphorylation of p44/42 MAPK or JNK. Neither were there any significant changes in Akt phosphorylation in the left ventricle after AM gene transfer.

5.2.4 AM decreases angiotensin II-induced cardiac hypertrophy

To investigate whether AM attenuates cardiac hypertrophy induced by angiotensin II, osmotic minipumps were used to infuse angiotensin II (33.3 µg/kg/h). Angiotensin II infusion caused cardiac hypertrophy and increased cardiac contractility in echocardiography as previously described (Lako-Futo et al. 2003). Interventricular septum and posterior wall thickness in diastole increased 1.7-fold (P<0.001) and 1.4-fold in 3 days (P<0.001), 1.8-fold (P<0.001) and 1.6-fold in 7 days (P<0.001) and 1.8-fold
(P<0.001) and 1.4-fold (P<0.001) in 14 days during angiotensin II infusion in LacZ virus injected rats (Fig. 9).

AM gene transfer significantly decreased angiotensin II induced hypertrophy in interventricular septum (P<0.01) and posterior wall (P<0.01) when compared to LacZ virus injected rats. Increase in thickness of interventricular septum and posterior wall decreased by 19% (P<0.01) and 21% (P<0.01) in 7 days (Fig. 9). AM had no significant effect on systolic function during angiotensin II infusion.

**Fig. 9.** AM gene transfer attenuates angiotensin II-induced cardiac hypertrophy. Angiotensin II (33.3 µg/kg/h) was infused with osmotic minipumps. A. Thickness of interventricular septum in diastole (IVSd). B. Diastolic left ventricular diameter (LVD). C. Thickness of left ventricular posterior wall in diastole (LVPWd). Open bars represent LacZ and solid bars AM gene transfer group. N=5-11 in each group. Data were analysed with one-way ANOVA followed by LSD post hoc test. *P<0.05 vs. basal, **P<0.01 vs. basal, *** P<0.001 vs. basal. D. Representative pictures of hearts 7 days after gene transfer and angiotensin II infusion. Control heart without angiotensin II infusion in the left, LacZ gene transfer heart with angiotensin II infusion (Ang II) in the middle and adrenomedullin (AM) gene transfer heart with angiotensin II (Ang II) infusion in the right.

### 5.2.5 AM regulates cardiac remodeling after myocardial infarction

The effects of adenoviral AM or LacZ gene transfer on remodeling of myocardium were also evaluated by using myocardial infarction produced by ligation of the LAD coronary
artery at the same time as virus injection. Myocardial infarction caused a marked hypokinesia of anterior wall in echocardiography. Fractional shortening was decreased from 46 ± 3 % to 29 ± 3 % (P<0.001) and ejection fraction from 82 ± 3 % to 60 ± 5 % (P<0.01). Myocardial infarction caused dilatation of the left ventricle. Left ventricular diameter in diastole increased by 10 % (from 7.8 ± 0.3 to 8.6 ± 0.3 mm) (P<0.05) in 7 days after ligation of LAD artery (Fig. 10B).

AM gene transfer increased AM mRNA levels in the left ventricle 9.1-fold (P<0.001) in myocardial infarction. AM gene transfer enhanced dilatation of the left ventricle and decreased anterior wall thickness at 7 days compared to LacZ gene transfer rats with myocardial infarction. After myocardial infarction interventricular septum was 29 % thinner (Fig. 10A) and left ventricular diameter in diastole was 12 % larger (Fig. 10B) in AM gene transfer group compared with the LacZ virus treated group after myocardial infarction (P<0.05). No effect on posterior wall thickness was seen (Fig. 10C). In the AM gene transfer group, fractional shortening and ejection fraction after myocardial infarction decreased.

**Fig. 10.** AM gene transfer regulates cardiac remodeling after myocardial infarction. A. Thickness of interventricular septum in diastole (IVSd). B. Diastolic left ventricular diameter (LVd). C. Thickness of left ventricular posterior wall in diastole (LVPWd). Open bars represent sham-operated group, hatched bars LacZ gene transfer group with myocardial infarction and solid bars AM gene transfer group with myocardial infarction. N=5-11 in each group. Data were analysed with one-way ANOVA followed by LSD post hoc test. *P<0.05 vs. basal, ** P <0.01 vs. basal, *** P <0.001 vs. basal. D. Representative pictures of hearts 14 days after gene transfer and myocardial infarction. Control heart with sham operation in the left, LacZ gene transfer heart with myocardial infarction (AMI) in the middle and adrenomedullin (AM) gene transfer heart with myocardial infarction (AMI) in the right.
5.3 Adenoviral AM gene transfer into rat carotid artery (III)

5.3.1 The effect of AM on neointimal hyperplasia after vascular injury

Production of rAM mRNA in injured vessels increased significantly after adenoviral gene transfer (Ad5RSVrAM) measured by real-time quantitative RT-PCR analysis. The mRNA levels were 1.98 ± 0.37 in Ad5RSVrAM group (n=8) and 1.00 ± 0.26 (P<0.05) in the control group (Ad5RSVlacZ) (n=6) two days after the vascular injury and gene transfer. There was no difference in mRNA between the groups at two weeks. There was a tendency for increase in immunoreactive rAM levels in the Ad5RSVrAM group compared to the control group measured with radioimmunoassay two days after the injury in the rat carotid tissue samples. However, this change (1.5-fold) was not statistically significant. Recombinant gene transfer efficiency was also demonstrated with X-gal staining two days after gene transfer. X-gal positive cells were seen mostly in the adventitia of the Ad5RSVlacZ administrated vessel, although at relatively low efficiency, indicating successful gene transfer.

Intima/media (I/M) ratios were calculated from stained vascular segments in order to estimate intimal hyperplasia two weeks after the endothelial injury when the luminal narrowing is most pronounced (Clowes et al. 1983, Siow et al. 2003). Following adenoviral rAM gene delivery, a 52 % reduction in I/M ratio at the injured site was found compared to the control rats (Ad5RSVlacZ). The I/M ratio in the animals treated with Ad5RSVrAM (n=15) was 0.48 ± 0.18 and that in the control group (n=22) 1.01 ± 0.20 (P<0.05) (Fig. 11A).

5.3.2 Effects of adenoviral AM gene transfer on neointimal apoptosis and stem-like cells recruitment

In order to investigate the possible mechanisms by which AM inhibits neointima formation, paraffin sections of arteries were stained using an ApopTag in situ apoptosis detection kit two weeks after injury and gene transfer. All neointimal apoptotic cells and bodies were counted and proportioned to the number of all neointimal cells. The apoptotic index was significantly higher in the rAM group (2.78 ± 0.5) compared to the control (0.57 ± 0.20, P<0.01), indicating that adenoviral AM gene transfer activates apoptosis of neointimal cells (Fig. 11B).

To examine the type and origin of the neointimal cells, immunohistochemical stainings were used. Stem cell markers c-Kit and MDR1 (Anversa & Nadal-Ginard 2002, Quaini et al. 2002) were used to identify the primitive cells. Very few antigen positive neointimal stem-like cells were seen within the injured vascular sections, and no difference was seen between the AM gene transfer and control groups two weeks after injury. Two markers, α-sm a and desmin, were used to determine whether the neointimal cells were myofibroblasts originating from adventitia or SMCs originating from media. Almost all
neointimal cells were positive for α-sma and negative for desmin, showing that cells were myofibroblasts.

![Graphs showing I/M ratios and apoptotic index](image)

**Fig. 11.** A. Intima/media (I/M) ratios of Ad5RSVlacZ (n=22) and Ad5RSVrAM (n=15) groups two weeks after vascular injury and gene transfer. Data are expressed as mean ± SEM. *P<0.05. B. Apoptotic index of Ad5RSVlacZ (n=3) and Ad5RSVrAM (n=4) groups two weeks after injury and gene transfer. Apoptotic cells and bodies were calculated from neointima and proposed to all neointimal cells after staining with ApopTag in situ apoptosis kit and hematoxylin. Data are expressed as mean ± SEM. **P<0.01.

### 5.4 Adenoviral gene transfer restores LH activity in type VI EDS (IV)

#### 5.4.1 LH activity in human skin fibroblasts

Skin fibroblasts of type VI Ehlers-Danlos syndrome (EDS) patient transduced with the control virus produced 0.4 % ± 0.2 % lysine hydroxylation (10 % - 15 % of normal skin fibroblasts), which equals previously reported LH activity levels (Sussman et al. 1974). The fibroblasts transfected with 50 pfu/cell Ad5RSVLH produced 2.3 % ± 0.1 % hydroxylation (85 % of normal skin fibroblasts), and 100 pfu/cell further elevated the hydroxylation level to 3.4 % ± 0.2 % (128 % of normal skin fibroblasts). The data demonstrate that the Ad5RSVLH significantly increased the LH activity in vitro in EDS VI skin fibroblasts.
5.4.2 Adenoviral LH gene transfer into rat skin

In order to demonstrate the recombinant adenovirus gene transfer into rat skin, 5 x 10^8 pfu of Ad5RSVβgal was injected into abdominal skin and the skin samples were stained for β-galactosidase expression 48 hours later. The skin sections that received Ad5RSVβgal had blue nuclei demonstrating successful adenoviral gene transfer.

For measurement of LH activity in rat skin, 5 x 10^8 pfu of Ad5RSVLH was injected into abdominal skin. The skin samples were collected on days 0, 7, 14, or 28 after the injection and analyzed for amino acid composition. The hydroxylysine content in skin samples was not significantly increased after the gene transfer, although there was a tendency for elevation. The result demonstrates that endogenous LH activity is sufficient to produce fully hydroxylated collagen in rat skin.

5.4.3 Human LH mRNA production after LH gene transfer

Human LH mRNA was analyzed in cultured rat skin fibroblast lysates 48 hours after Ad5RSVLH or Ad5RSVβgal transduction using PCR and HindIII enzyme digestion. HindIII digestion cuts the rat sequence into 155 nt and 506 nt fragments, whereas the 661 nt human sequence amplification product remains undigested. Agarose gel electrophoresis results showed that the 661 nt band for human LH mRNA dominated in Ad5RSVLH transfected rat skin fibroblasts cultures. The 661 nt band was clearly more intense than the 506 band, suggesting strong human LH mRNA expression from the gene transferred with Ad5RSVLH. Control cells that received equal amounts of Ad5RSVβgal showed fragments corresponding to rat sequences.

Rat skin samples were analyzed for LH mRNA after Ad5RSVLH or Ad5RSVβgal injection. Results showed a 661 nt band for human and only a very faint band for rat LH mRNA after Ad5RSVLH injection. Products of several amplifications were pooled to demonstrate the rat LH mRNA in control (Ad5RSVβgal) skin samples. The data demonstrate that Ad5RSVLH gene transfer produces specific human LH expression in vivo. The amplification protocol, however, was not optimized for quantitative analysis.
6 Discussion

6.1 Intracellular factors mediating adenovirus entry

Rab5 is a small GTPase associated with the plasma membrane and early/sorting endosomes. Rab5 controls homotypic early endosome fusion and functions in the formation of early endosomes. Rab5 causes enlargement of early endosomes and increases the rate of endocytosis (Stenmark et al. 1994). Antibodies against Rab5 and dominant-negative Rab5 mutants have inhibitory effects on endosome fusion in vitro (Gorvel et al. 1991, Li et al. 1994, Hoffenberg et al. 1995). Overexpression of a dominant-negative Rab5 mutant causes fragmentation of early endosomes and reduces endocytosis (Bucci et al. 1992, Barbieri et al. 1994). On the other hand, Ceresa et al. have recently reported that although overexpression of the constitutively active form Rab5(Q79L) lead to dramatically enlarged endosomes, the rates of transferrin receptor-mediated endocytosis and recycling were unaffected, suggesting that GTP hydrolysis by Rab5 is rate-limiting for endosome fusion but not for endocytic trafficking (Ceresa et al. 2001). The present data, however, support the hypothesis that adenovirus endocytosis efficiency and gene transfer are regulated by Rab5 GTPase. In the present assay, an approximately 40 to 50% difference in β-galactosidase activity and an approximately 50% difference in fluorescently labeled adenovirus uptake was seen between conditions with the most (wild-type overexpression) and the least (dominant-negative overexpression) Rab5 GTPase activity. A more pronounced effect might be expected if Rab5 activity were completely eliminated.

Interestingly, the increase caused by wild-type Rab5 in the uptake of dextran-FITC was significantly greater than the increase observed in virus uptake or viral β-galactosidase expression. The result implies that endogenous levels of Rab5 may be sufficient for internalization of all bound virus, and the number of receptors is the limiting factor. Rab5 may also stimulate more profoundly the non-specific fluid-phase endocytosis compared to the receptor-mediated endocytosis of adenovirus. Part of the adenovirus entry may even be due to non-specific endocytosis, especially in the cell types that express low levels of CAR receptor (for review, see Meier & Greber 2004). The recycling of several receptors has found to be specifically and strongly regulated by Rab5.
GTPase (Seachrist et al. 2002, Dinneen & Ceresa 2004), but it is not known whether Rab5 has direct interactions with either one of the adenovirus receptors. Because integrins take part in numerous intracellular biochemical processes, including the reorganization of actin, it is likely that Rab5 may have interactions with αv integrin receptors (Juliano & Haskill 1993).

The small GTPases Rac1 and Cdc42, which belong to the Rho-family, have also been found to have a role in adenovirus endocytosis via reorganization of actin cytoskeleton (Li et al. 1998a) (Fig. 12). Actin filaments may provide the mechanical force required for endosome formation. Cortical actin filaments together with integrins have also been found to play a crucial role in detaching fibers at the cell surface (Nakano et al. 2000). Adenovirus entry may also require dynamin, which is a large molecular weight GTPase protein involved in clathrin-mediated internalization of receptors and their ligands from the plasma membrane (Wang et al. 1998). It would be interesting to evaluate the role of the other early endocytosis mediating small GTPases Arf6, RhoA, Rab4, Rab15, and Rab22, which may also be potential regulators of adenovirus entry and gene transfer (Figs. 2 and 3).

Fig. 12. Hypothetical αv-integrin-mediated intracellular mechanisms that may initiate the early endocytosis of the adenovirus (modified from Li et al. 1994, Li et al. 1998a,b, Rauma et al. 1999). *Factors that are found in some experiments not to be required for adenovirus endocytosis. Ad, adenovirus; [Ca2+]i, intracellular calcium concentration; IL-8, interleukin-8; MAPK, mitogen activated protein kinase; CAS, Crk-associated substrate; PI3K, phosphatidylinositol 3-kinase; FAK, focal adhesion tyrosine kinase.
Adenovirus binding to cell surface α<sub>v</sub> integrins has been found to activate PI3K (Li et al. 1998b). It has also been shown that PI3K may regulate endocytosis via Rab5 GTPase (Li et al. 1994). It is possible that adenovirus binding to the α<sub>v</sub> integrin activates PI3K, which in turn regulates Rab5 GTPase and controls adenovirus entry into a cell. GTPases Rac1 and Cdc42 have also been found to mediate the adenovirus entry via PI3K (Li et al. 1998a) (Fig. 12). The results from the present [Ca<sup>2+</sup>] assay show that even if the ligand binding to α<sub>v</sub> integrins can trigger a transient increase in [Ca<sup>2+</sup>], in several cell types (Schwartz & Denninghoff 1994, Lawson & Maxfield 1995), [Ca<sup>2+</sup>] may not play an important role in adenovirus infection. The present study also shows that adenovirus had no effect on pp125<sub>FAK</sub>, p130<sub>CAS</sub>, or paxillin phosphorylation in HeLa cells. These results agree with the previously published data that pp125<sub>FAK</sub> activity is not required for adenovirus infection and gene transfer (Li et al. 1998b). The present results disagree with the later published data that p130<sub>CAS</sub> phosphorylation and association with PI3K is required for adenovirus endocytosis (Li et al. 2000a). The controversial results may be explained by the different cell types and experimental conditions used. The present study was done using HeLa cells, whereas Li et al. used human colon colorectal adenocarcinoma cells (Li et al. 2000a). The phosphorylation of p130<sub>CAS</sub> may also be non-specific and a consequence of many cellular actions. The activation of pp125<sub>FAK</sub> is usually, but not necessarily, associated with the activation of p130<sub>CAS</sub> (Sakai et al. 1997). Thus the controversial results of Li et al., in which p130<sub>CAS</sub> is required for adenovirus endocytosis but pp125<sub>FAK</sub> activity is not (Li et al. 1998b, Li et al. 2000a), may be valid in some cell types.

The data demonstrate that both intracellular and cell surface molecules are important mediators of viral infection. A complex series of intracellular events probably regulates viral endocytosis, exit from the endosome, cytoplasmic transport, and nuclear entry. Additional knowledge of these events will provide a better understanding of adenovirus entry mechanisms.

6.2 The cardiac effects of intramyocardial AM gene transfer

Cardiac remodeling is characterized by structural rearrangement of the left ventricular wall and is regulated by paracrine factors and circulating hormones (for review, see Frey et al. 2004). In order to study the role of AM in myocardial remodeling in the normal and in the failing rat heart and during developing cardiac hypertrophy, intramyocardial adenoviral AM gene transfer was used. The results of the present study show that AM significantly attenuated development of Ang II-induced cardiac hypertrophy. In rats with myocardial infarction, AM enhanced dilatation of the left ventricle and thinning of the anterior wall. On the other hand, AM gene transfer had no significant effect on the normal heart structure when compared to the control group. Significant thickening of interventricular septum, probably due to local inflammation, was seen in both the AM and the LacZ gene transfer group after virus injection.

Previous in vitro studies suggest that AM has effects on cardiac remodeling during development of left ventricular hypertrophy. AM inhibits protein synthesis and expression of hypertrophy-associated genes in cultured cardiac myocytes, and inhibits...
proliferation of cardiac fibroblasts (Tsuruda et al. 1998, Tsuruda et al. 1999, Luodonpää et al. 2001). In vivo ventricular AM levels have been shown to correlate with the extent of left ventricular hypertrophy produced by aortic banding in rats (Morimoto et al. 1999) and to be associated with myocyte hypertrophy in human transplanted hearts (Tsuruda et al. 2003). However, in these in vivo studies the effects of AM on cardiac hypertrophy may be explained by systemic hemodynamic effects of AM. In studies with knockout mice, with more direct effects of AM on regulation of left ventricular hypertrophy, Ang II infusion caused more severe cardiac hypertrophy in AM knockout mice than in wild type mice (Niu et al. 2003, Niu et al. 2004). In accordance, the present study shows that local overexpression of AM in adult heart significantly attenuates left ventricular hypertrophy caused by Ang II infusion, suggesting that AM plays an important role as a defence mechanism against cardiac hypertrophy.

There is also indirect evidence that AM may act as an autocrine or paracrine regulator of left ventricular remodeling after myocardial infarction. Myocardial infarction produced by ligation of LAD artery increases AM mRNA levels, expression of AM receptors (Oie et al. 2000), and increases AM protein synthesis in the left ventricle (Nagaya et al. 2000). Plasma AM concentrations correlate to left ventricular ejection fraction and prognosis after myocardial infarction in patients, but this correlation is less clear than for BNP (Richards et al. 1998). In rat ischemia/reperfusion models, AM infusion reduces myocardial infarct size, left ventricular end-diastolic pressure, left ventricular diastolic dimension, and the number of apoptotic nuclei in myocytes, whereas left ventricular systolic function increases (Okumura et al. 2003, Okumura et al. 2004). Adenovirus-mediated AM gene delivery into the tail vein a week before ischemia/reperfusion injury protects against myocardial infarction, arrhythmia, and apoptosis (Kato et al. 2003). However, in these models, systemic effects of AM may in part explain the beneficial effects seen, and no analysis of the direct effects of AM in the left ventricle could be done.

The present data showed that direct adenovirus-mediated gene transfer into the free wall of the left ventricle enhances dilatation of the left ventricle and thinning of the anterior wall in rats with myocardial infarction produced by ligation of LAD artery. Previously, adenovirus-mediated AM gene transfer into the rat heart has been done by catheter-based strategy five days before acute ischemia/reperfusion (Yin et al. 2004). In opposition to the present study, this previous study suggests that AM has cardioprotective effects on the ischemia/reperfusion model, which are independent of hemodynamic changes (Yin et al. 2004). An explanation for these divergent findings may be the different ischemic injury produced in these models. In the present study, a myocardial infarction model with permanent LAD artery ligation was used, whereas Yin and coworkers used the ischemia/reperfusion model (Yin et al. 2004). These models are known to represent different activation of signal transduction pathways and mechanisms of left ventricular remodeling (Murriel & Mochly-Rosen 2003). Yin and co-workers studied acute effects after two hours’ reperfusion, whereas more long-term changes were looked at in the present study (Yin et al. 2004). Further, in the model of the present study the gene transfer was done at the same operation as the LAD artery ligation, whereas Yin and coworkers carried out the gene transfer five days before ischemia/reperfusion (Yin et al. 2004). Thus, different mechanisms may be involved in different phases of the remodeling process.
An improvement of systolic function both \textit{in vivo} and \textit{in vitro} after direct adenovirus-mediated AM gene transfer into the left ventricle was found in the present study. This is in accordance with earlier \textit{in vitro} studies (Szokodi \textit{et al.} 1998, Ihara \textit{et al.} 2000). In an isolated perfused rat heart model, AM infusion produced a dose-dependent positive inotropic effect (Szokodi \textit{et al.} 1998). A significant increase in contractility was observed 5 minutes after beginning of AM infusion, gaining maximal response in 30 minutes. Similarly, in isolated rat papillary muscle preparation AM increased contractility significantly (Ihara \textit{et al.} 2000). Another study using isolated adult rabbit cardiac ventricular myocytes showed a negative inotropic effect of AM \textit{in vitro} (Ikenouchi \textit{et al.} 1997), and no effect at all was found in one study (Stangl \textit{et al.} 2000). These discrepancies may be explained by different experimental models used. The present study results from \textit{in vivo} AM gene transfer experiments clearly show that AM improves systolic function of the left ventricle.

The signal transduction pathways mediating cardiac effects of AM are not well defined. PKC has been found to function as an important mediator in Ca\textsuperscript{2+}-induced inotropy and cardiac hypertrophy (Cain \textit{et al.} 1999). Different PKC isoforms seem to have different cardiac effects. PKC\textgreek{e} and \textgreek{d} have been shown to have opposing cardioprotective effects but parallel hypertrophic effects (Chen \textit{et al.} 2001). Activation of PKC\textgreek{e} causes cardioprotection, whereas activation of PKC\textgreek{d} increases cell damage caused by ischemia (Dorn \textit{et al.} 1999, Chen \textit{et al.} 2001, Gray \textit{et al.} 2004). In the present study, activation of both PKC\textgreek{e} and PKC\textgreek{d} by overexpression of AM in the left ventricle was found.

The PI3K/Akt-dependent pathway is an anti-apoptotic signal that promotes cell survival (Fujio \textit{et al.} 2000, Okumura \textit{et al.} 2004). AM has been found to attenuate myocardial injury in myocardial ischemia/reperfusion models via the PI3K/Akt-dependent pathway (Kato \textit{et al.} 2003, Yin \textit{et al.} 2004). However, in the present study, local overexpression of AM in the left ventricle had no significant effect on the phosphorylation of Akt. The lack of effects on the Akt-dependent pathway may explain the diverse effect of AM on the left ventricular remodeling after myocardial infarction seen in the study. This finding underlines the importance of studying the effects of local overexpression in different disease models.

MAPK pathways (p44/42 MAPK, p38 MAPK and JNK) are known to regulate development of cardiac hypertrophy (Molkentin & Dorn II 2001). However, their role in cardiac remodeling after myocardial infarction is not known, although inhibition of p38 MAPK may attenuate reperfusion injury and improve cardiac function in a myocardial ischemia/reperfusion model (Ma \textit{et al.} 1999) and activation of p44/42 MAPK signaling may protect the heart from ischemic injury (Lips \textit{et al.} 2004). Activation of p38 MAPK by adenoviral gene transfer in cultured adult rat cardiomyocytes has been found to lead to a significant reduction in baseline contractility (Liao \textit{et al.} 2002). Interestingly, in the present study adenovirus-mediated AM gene transfer decreased p38 MAPK phosphorylation. Inactivation of p38 MAPK signal transduction pathway may mediate positive inotropic effects of AM and be involved in the regulation of left ventricular remodeling during development of hypertrophy.

The results of study II show that AM acts as a regulator of left ventricular systolic function and cardiac remodeling, and suggest PKC\textgreek{e} and PKC\textgreek{d} as well as p38 MAPK as mediators of cardiac effects of AM. Improvement of systolic function and attenuation of
development of left ventricular hypertrophy might be beneficial therapeutic effects of AM. Furthermore, adenovirus showed its potential as a useful tool when studying different mechanisms in ischemic remodeling processes in heart myocardium. Next, the study could be expanded to focus more specifically on the signaling pathways involved in pathological remodeling, or to look at the changes in the gene expression patterns by microarray techniques using intramyocardial adenovirus-mediated gene transfer.

6.3 Intravascular AM gene transfer after endothelial injury

AM has been found to prevent EC apoptosis, to stimulate EC growth (Kato et al. 1997), and to inhibit VSMC proliferation and migration (Horio et al. 1995, Kano et al. 1996, Kohno et al. 1997). Yet, the effect of AM on VSMC or myofibroblast apoptosis is not known (Filippatos et al. 2001). As a growth-regulating factor, it has a potential role in the pathological vascular remodeling associated e.g. with angioplasty (Imai et al. 2002, Agata et al. 2003, Liu et al. 2003) although opposite insights of AM vasculoprotective effects have also been hypothesized (Shimizu et al. 1999). The present study demonstrated for the first time that inhibition of neointimal growth by AM is associated with enhanced neointimal cells apoptosis. The increased apoptosis is at least partly responsible for the reduced neointimal proliferation after endothelial injury.

AM can be detected in plasma at a low concentration (Kitamura et al. 1994a, Sato et al. 1995) and its plasma half-life is estimated to be about 22 minutes (Meeran et al. 1997). The plasma concentrations of AM were also measured in the present study and no significant difference between the AM and the control gene transfer groups was found two days or two weeks after injury (data not shown). Considering the plasma half-life of AM and the low amount of virus, the result was not unexpected. AM is suggested to be preferably synthesized to act as a local autocrine/paracrine factor.

The observed pro-apoptotic effect may be mediated by cAMP-PKA and/or more likely by cGMP-PKG signaling pathway. In rat MCs, AM has been demonstrated to increase apoptosis via cAMP-PKA, (Parameswaran et al. 1999) and MAPK pathway (Chini et al. 1997). eNOS has been implicated in AM-mediated vasodilation, and in addition to the direct activation of eNOS, AM appears to use the PI3K/Akt pathway in ECs to activate eNOS (Nishimatsu et al. 2001). PI3K/Akt pathway has also been found to mediate the angiogenesis promoting effects of AM (Kim et al. 2003). AM gene transfer has been reported to increase cGMP, but not cAMP levels in balloon-injured arteries (Agata et al. 2003). Because cGMP has been found to mediate pro-apoptotic effects in VSMCs (Chiche et al. 1998), cGMP-PKG may be the most likely signaling pathway mediating AM pro-apoptotic effect observed in the present in vivo model.

Growth factors including VEGFs, FGFs, and plateled-derived growth factors (PDGFs) are found to be important in some stages of vascular restenosis (Casscells et al. 1994, Ylä-Herttuala & Alitalo 2003). In the present study, no statistically significant differences were seen in the mRNA levels of VEGF-A or bFGF between the AM gene transfer and the control groups two days or two weeks after injury (data not shown). The result suggests that the neointima formation inhibiting effects of AM are not explained by changes in these growth factors.
The origin of the neointimal cells is not completely understood. Besides the theories that the neointimal cells are a result of migration from the outer layers of the injured vessel (Li et al. 2001, Siow et al. 2003), migration of differentiated hematopoietic stem cells to the vascular injury site may also be possible (Han et al. 2001). Stem cells have been found in the intima layer four weeks after vascular injury in bone-marrow transplanted mice (Sata et al. 2002). At one week, however, migration was not seen (Sata et al. 2002). Surface markers c-Kit, MDR1, and Sca-1 are usually used for the identification of primitive cells. c-Kit is the receptor for stem-cell factor (Jiang et al. 2000), MDR1 is a P-glycoprotein capable of extruding dyes, toxic substances, and drugs (Bunting et al. 2000), and Sca-1 is involved in cell signaling and cell adhesion (Miles et al. 1997). Hu et al. observed that the adventitia of the aortic wall of ApoE-deficient mice contained stem cell markers Sca-1 (21%), c-Kit (9%), and CD34 (15%) (Hu et al. 2004). They also suggested that the Sca-1 positive cells can differentiate into SMCs and endothelial cells in vitro and contribute to atherosclerosis of vein grafts in ApoE-deficient mice (Hu et al. 2004). In the myocardium, the highest number of c-Kit or MDR1 positive cells is seen two to three weeks after injury (Nadal-Ginard et al. 2003). In the present study, a very low number of primitive cells was found in the neointima, and no difference between the AM and the control group was seen using the immunohistochemical stainings for c-Kit and MDR1. Although it is difficult to exclude the possibility of the progenitor cells as an origin of neointimal cells, the present results suggest that in this model the observed neointimal regrowth is more likely to originate from the arterial wall. Tanaka et al. have observed that the contribution of bone marrow cells to vascular remodeling is highly dependent on the type of arterial injury (Tanaka et al. 2003). They induced three distinct types of mechanical vascular injuries in the same mouse whose bone marrow has been reconstituted. Four weeks after endovascular injury, there was a significant number of neointimal and medial cells derived from bone marrow. Instead, there were only few bone marrow-derived cells after perivascular injury or ligation of the common carotid artery. They hypothesized that when the media remain acellular, as seen in the case of violent endovascular injury, it is most likely that bone marrow-derived cells must be recruited to repair the injured artery (Tanaka et al. 2003). However, they did not take into consideration the alternative that the adventitial cells might also have a role in the remodeling process.

Previous studies suggest that in addition to smooth muscle cell proliferation, migration of adventitial myofibroblasts plays a role in neointima formation following vascular injury (Li et al. 2000b, Siow et al. 2003). However, Leon et al. reported on the basis of adventitial cell staining that adventitial cells do not contribute to neointimal mass after balloon angioplasty of the rat common carotid artery (De Leon et al. 2001). They hypothesized that the opposite results are caused by different experimental models (De Leon et al. 2001). In order to identify whether the neointimal and apoptotic cells in the present model were adventitial myofibroblasts or medial SMCs, two immunohistochemical stainings were used. The observation of a-sma positive and desmin negative cells shows that the neointimal cells were myofibroblasts. Furthermore, the air drying method used in this study destroys almost all of the medial SMCs (Fishman et al. 1975), and thus minimizes their possibility to be the origin of the neointimal cells. In the other models with less serious vascular injury procedure, the medial smooth muscle layer may also be the origin of the neointimal cells, and AM may prevent neointimal formation.
after vascular injury also by inhibiting VSMCs proliferation and migration (Horio et al. 1995, Kano et al. 1996, Kohno et al. 1997). As an antiapoptotic and proliferative factor of ECs, AM may also facilitate endothelial regeneration (Agata et al. 2003).

The duration and level of AM overexpression may be significant for its biological effects. It is possible that extended transgene expression produced by alternative recombinant viruses, such as lentiviral vectors, could significantly enhance the AM effects on the neointima formation. In addition, the AM effect in selected models has been shown to be dose dependent (Szikodi et al. 1998). Different animal or cell culture models may also explain the controversial effects of AM observed in previous studies. For example, the rat arteries used in the present study were healthy, whereas atherosclerotic lesions in man may have a marked influence on the vascular remodeling. Furthermore, the air drying method used in our study produces almost complete loss of endothelium (Fishman et al. 1975). In the present model, however, the moderate and short-term AM overexpression produced a significant biological effect. Therefore, the present rodent model can be used as an experimental model for vascular injury regeneration. Further studies, however, are required to elucidate the role of AM in injured atherosclerotic arteries.

At present, antithrombotic drugs are widely in clinical use to prevent restenosis after coronary interventions (for review, see Garas et al. 2001). Restenosis after balloon angioplasty is also routinely treated with stent implantation (Williams et al. 2000). A major limitation of this approach has been in-stent restenosis, which occurs in about 30% of stented coronary arteries. A recent advance is the development of drug-eluting stents that dramatically reduce the incidence of in-stent restenosis to <5% (for review, see Woods & Marks 2004). Two drugs, rapamycin and taxol, have been the lead compounds for testing the idea of an antiproliferative drug-eluting stent. These drugs have been successful largely because of the solid mechanistic understanding of their effects and extensive preclinical examination. As a result of the years of work, the rapamycin (sirolimus)-coated stent has entered the market last year, and the taxol-coated stent appears to follow soon (for review, see Woods & Marks 2004).

This study provides new valuable knowledge of the role and the mechanisms of AM in pathological vascular remodeling. AM is reported for the first time to enhance neointimal myofibroblasts apoptosis after vascular injury. The increased apoptosis may be at least in part responsible for the reduced neointimal growth caused by AM overexpression. The present results suggest that AM or its analogs and signal pathways may be attractive therapeutic targets in the future in treating restenosis or atherosclerosis.

6.4 Adenoviral gene transfer into the skin fibroblasts

Type VI Ehlers-Danlos syndrome (EDS) is a recessively inherited connective-tissue disorder, which results from markedly decreased activity of the LH1 enzyme (Krane et al. 1972, Pinnell et al. 1972, Sussman et al. 1974). LH catalyzes the hydroxylation of lysine residues in collagens, and the formed hydroxyllysine residues are required for cross-link formation and O-linked glycosylation in collagens (Kivirikko & Pihlajaniemi 1998). The decreased LH activity of EDS is caused by many types of mutations in the LH1 gene...
(Hyland et al. 1992, Hautala et al. 1993, Ha et al. 1994). Lack of LH activity in EDS VI patients yields structurally weak collagen molecules causing severe musculoskeletal, cardiovascular and skin disease (Wenstrup et al. 1989, Yeowell & Pinnell 1993, Byers 1994). The skin of patients with LH deficiency is hyperelastic and easily bruisable. Poor wound healing leads to secondary complications. Many patients have also abnormal scarring and follicular papules over the elbows and knees (Pinnell et al. 1972, Sussman et al. 1974). Ascorbic acid treatment of patients with EDS type VI has been shown to partially relieve some of the symptoms (Elsas et al. 1978, Dembure et al. 1987). Administration of hydralazine and ascorbate together has been shown to stimulate LH mRNA and LH activity (Yeowell et al. 1992).

Gene replacement therapy may be feasible and beneficial for some of the local cutaneous symptoms including poor wound healing of EDS VI. Skin is easily accessible and wound healing may benefit from locally administered LH activity. Heterozygous carriers of the defective recessive gene, who have diminished LH activity, are healthy, which suggests that even partial correction of the LH defect in EDS VI patients may be beneficial. In the present study, Ad5RSVLH gene transfer elevated LH activity of EDS patient fibroblasts up to the levels found in normal skin fibroblasts. It is possible that restoration of the LH function leads to correction of mechanical properties of collagens that may correlate with the severity of the disease symptoms.

On the other hand, collagen biosynthesis is characterized by the presence of a large number of cotranslational and post-translational modifications, including intracellular and extracellular reactions (Kivirikko & Myllylä 1982). LH is an intracellular enzyme, and thus the gene of the functional LH enzyme should be transferred into the majority of collagen producing cells in order to achieve an adequate benefit. Furthermore, the major limitation in treatment of these inherited disorders including EDS is their generalized nature, necessitating treatment of most of the cells at an early stage in life.

First generation adenoviral vector has been shown to persist in human skin for at least 28 days with mild/moderate local cellular inflammation (Harvey et al. 1999), and adenovirus-mediated transgene expression has been shown to remain stable in vitro for 2-6 weeks in keratinocytes, melanocytes, and fibroblasts (Feng et al. 1995). In the present study, elevated LH activity was still evident in rat abdominal skin in vivo two weeks after the virus injection. This should allow reasonable time for initiation of successful wound healing. Studies on adenoviral gene transfer have demonstrated loss of the transgene expression due to immune response against the vector or the transgene product. It is probable that LH overexpression will gradually diminish with the vector used in this study. The adenoviral vector can be used to test the principle of LH enzyme replacement, but other vectors, such as AAV or recombinant lentivirus, may provide longer lasting levels of expression. The acute inflammation is usually a disadvantage in gene transfer studies. In the setting of wound healing, however, this limitation may prove to be advantageous. Wound healing is fundamentally an inflammatory response, and adenoviral gene transfer for the induction of endogenous growth factor overexpression may augment inflammation and thereby enhance wound healing (Crombleholme 2000).
7 Summary and conclusions

The aims of the study were to evaluate the mechanisms mediating adenovirus endocytosis, to clarify the role of AM in cardiovascular disease models using adenovirus-mediated gene transfer, and to apply adenoviral LH gene transfer into skin fibroblasts.

1. The role of Rab5 GTPase in the adenovirus endocytosis was examined in HeLa cells using Cy3-labeled adenovirus and recombinant Ad5RSVβgal. Increased uptake of Cy3-labeled adenovirus and higher gene transfer activity were seen in Rab5 overexpressing cells, whereas dominant negative Rab5S34N inhibited adenovirus uptake. In CAR co-overexpressing cells the endocytosis efficiencies were elevated, and the effects of overexpressing Rab5 or Rab5S34N were the same. There was no evidence of [Ca^{2+}]-mediated signaling or of tyrosine phosphorylation of pp125FAK, p130CAS, and paxillin. These results show that Rab5 plays a role in adenovirus entry.

2. Adenovirus-mediated AM gene transfer transiently improved left ventricular fractional shortening and ejection fraction in normal rat heart in vivo. In isolated perfused rat hearts increases in developed pressure and dP/dt_{max} were seen. AM gene transfer caused activation of protein kinases Cε and Cδ, whereas p38 MAPK activity was decreased in the left ventricle. Ang II-induced cardiac hypertrophy was decreased by AM gene transfer, while AM overexpression had no significant effect on systolic function during angiotensin II infusion. In rats with myocardial infarction, AM gene transfer enhanced dilatation of the left ventricle and thinning of the anterior wall. In the AM gene transfer group, fractional shortening and ejection fraction after myocardial infarction were decreased. These results show that AM regulates left ventricular systolic function and cardiac remodeling. Changes in protein kinases Cε and Cδ as well as p38 MAPK activity may mediate the cardiac effects of AM.

3. Intravascular AM gene transfer caused elevation in vascular wall AM expression, significantly less neointimal growth in injured rat artery, and increased the apoptosis index in neointimal myofibroblasts. No difference in stem-like cell recruitment was seen between the AM and control groups two weeks after vascular injury. These data demonstrate that AM overexpression inhibits neointimal growth and enhances myofibroblasts apoptosis after endothelial injury, suggesting that AM plays a role in preventing pathological vascular remodeling.
4. Transduction of human type VI Ehlers-Danlos syndrome fibroblasts and rat fibroblasts with adenovirus encoding functional human LH increased LH synthesis and activity in vitro. Intradermal adenoviral LH gene transfer resulted in LH mRNA production and elevated LH activity in rat skin also in vivo. Thus, LH gene replacement therapy could be possible in some of the local skin symptoms in type VI Ehlers-Danlos syndrome patients.


85


90


91


