Terapia genowa

wykład II

20.06.2010
Gene therapy

- **Enhancing**
  - Enhancement of gene expression
    - Acquired diseases
      - Caused by endogenous and exogenous factors

- **Substituting**
  - Delivery of the missing gene
    - Inherited diseases

- **Suppressive**
  - Inhibition of gene expression
    - Acquired diseases
Therapeutic nucleic acids

DNA
- Genes (DNA vectors)
- Non-coding sequences
  - Antisense oligonucleotides
  - DNA decoys

RNA
- Genes (RNA vectors)
- Non-coding sequences
  - ribozymes
  - siRNA
Strategies of gene therapy – in vivo and ex vivo

**Strategia in vivo**
- Transfer terapeutycznego genu
- Wprowadzenie zmodyfikowanych genetycznie komórek do organizmu

**Strategia ex vivo**
- Hodowla komórek *in vitro*
- Selekcja zmodyfikowanych genetycznie komórek
- Transfer terapeutycznego genu

**Ryc. 2.1. Strategie: in vivo i ex vivo w terapii genowej.**
Strategia *in vivo* polega na dostarczaniu genów terapeutycznych do organizmu miejscowo *in situ* do tkanek (np. domiesińcowo, doguzowo) lub też do krwiobiegu.
Strategia *ex vivo* natomiast polega na pobraniu komórek z organizmu, hodowli i namnażaniu tych komórek w laboratorium. Następnie do wyizolowanych komórek wprowadza się geny terapeutyczne za pomocą dostępnych metod transferu genów. Komórki zmodyfikowane genetycznie genem terapeutycznym selekcjonuje się, po czym wprowadza się je z powrotem do organizmu, z którego zostały wyizolowane.
Vectors

Non-viral/plasmids

"naked" DNA
Lipoplexes
Viroplexes (lipoplexes enhanced in proteins from viral capsids)

viral

RNA
Retroviral (including Lentiviral)
Complexes With chemical vehicles

DNA
Adenoviral
AAV
Herpes
Plasmids

the main tools of gene therapy

Plasmids are always in the beginning…
Organisation of a typical plasmid vector

Plasmids are of bacterial origin, so they have to be modified to act in eucaryotic cells.
A piece of target DNA can be inserted into a plasmid if both the circular plasmid and the target DNA have been cleaved by the same restriction nuclease in such a way as to create sticky ends. The newly created recombinant molecule is stabilized with the DNA ligase enzyme which repairs nicks in the backbone of the DNA molecule.
Isolation of DNA fragments from a mixture by cloning in a plasmid vector
Transformation of bacteria

double-stranded recombinant plasmid DNA introduced into bacterial cell

bacterial cell

cell culture produces hundreds of millions of new bacteria

many copies of purified plasmid isolated from lysed bacterial cells

Figure 8–31. Molecular Biology of the Cell, 4th Edition.
DNA cloning

Cloning into a plasmid
How to make a plasmid working in mammalian cells?
Expression of foreign genes in eucaryotic cells requires the eucaryotic promoter in a plasmid vector

1. Viral promoters: CMV, SV40
2. Eucaryotic promoters
   - constitutive:
     - non-selective: β-globin
   - tissue specific:
     - inducible
3. Complex
Mammalian expression plasmid

cDNA

CMV

polA

f1ori

SV40 ori

neo

amp

Co/E1

pcDNA 3.1
Expression of β-galactosidase in various cells after lipotransfection

Cells from kidney of a monkey

Vascular smooth muscle cells (rat)
Naked plasmid, containing lacZ gene has been injected into the leg muscle of a mouse.
Naked DNA - applications

1. Duchenne muscular dystrophy; patients; mdx mice
2. Transfer of genes encoding secretory proteins, e.g., VEGF
3. Animal models of autoimmune diseases
4. Genetic vaccines
Problems with transfection of naked DNA

1. Cell type dependent – only few cell types can be effectively transfected

2. Maximal expression – after 14 days in skeletal muscles

3. Long-term expression in skeletal muscle – episomal, even up to 2 years

4. Muscle regeneration enables higher expression

5. Promoters – better viral than cell specific?

6. Efficiency is inversely dependent on the animal size...
Viral vectors
Viral vectors used in clinical trials of gene therapy

1. Adenoviruses
2. Retroviruses
   a) oncoretroviruses
   b) lentiviruses
3. Adeno-associated viruses
4. Herpes simplex virus
5. Poxviruses (including vaccinia)
6. Semliki Forest Virus
7. Measles virus
Viral vectors

Integrating
- retroviral
- lentiviral
- AAV

Non-integrating
- Adenoviral
- HSV

Integration depends on:
- LTR sequences and integrase (retroviruses)
- ITR sequences and rep proteins (AAV)
Retroviral expression system
Retroviral infectious cycle

1. Infecting virus
2. Reverse transcription
3. Viral DNA
4. Integration
   - Viral RNA
   - Cell DNA
   - Provirus
   - Cell DNA
5. Viral protein production
6. Viral RNA encapsidation
7. Virus release
Construction of retrovirus vector

1. Construction of retrovirus vector as a recombinant plasmid in *E. coli*

2. Introduction of a plasmid into a packaging cell line

3. Incorporation of vector transcripts into transmissible virus particles

4. Conversion of the transcripts into double-stranded DNA by reverse transcriptase
Structure of a plasmid used for production of retroviral vectors

therapeutic gene
reporter gene
Stages of retroviral construction (1)
## Retroviral vectors – properties

<table>
<thead>
<tr>
<th>Property</th>
<th>Oncoretroviruses</th>
<th>Lentiviruses</th>
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<tbody>
<tr>
<td><strong>Capacity</strong></td>
<td>4-5 kb</td>
<td>8-9 kb</td>
</tr>
<tr>
<td><strong>Ability to integrate</strong></td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Tissue specificity</strong></td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Ability to transfect non-dividing cells</strong></td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Duration of expression</strong></td>
<td>long-term</td>
<td></td>
</tr>
<tr>
<td><strong>Level of expression</strong></td>
<td>short-term</td>
<td></td>
</tr>
<tr>
<td><strong>Safety</strong></td>
<td>risk of insertional mutagenesis</td>
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</tbody>
</table>
Adenoviruses

More than 50 serotypes – type 2 & 5 are used

Genom: 36 kbp, more than 50 proteins
  E1 region– contains genes regulating the expression of genes necessary for viral replication
  E2 i E4 regions – together with E1 are required for viral replication
  E3 region– is not required for replication, modulates response of cell to infections
Adenoviruses

- ~50 serotypes of adenoviruses (for gene therapy type 2 and 5 were used), causing usually mild illness in humans

- Genome consists of 36 kb double-stranded linear DNA with ITR sequences at each end, with:
  - Early genes (responsible for viral gene transcription, DNA replication, host immune suppression and host cell apoptosis
  - Late genes (coding proteins required for virus assembly)

- E1 early gene is essential for the subsequent adenoviral gene expression
Binding and internalization of adenovirus

1) Attachment to cell surface receptor.

2) Receptor-mediated endocytosis
Adenoviral vectors of the first generation

**Great:**
- Very high transduction efficiency
- Broad host and cell type ranges
- Can be prepared in high titers
- Can transduce mitotic and post-mitotic cells
- Do not integrate with genome
- Can harbor ~ 7 kb of transgene

**But:**
- Strong immune response to viral proteins eliminate virally transduced cells within 30 days
- Neutralizing antibody response prevents readministration of adenovirus vector of the same serotype

**Thus:**
*Adenoviral vectors provide the high but transient (<4 weeks) transgene expression*
Adenoviral vectors

Very efficient, transduce many cell types, provide high level of expression

Very immunogenic

High dosage, Multiple injections

systemic inflammatory response, death...
Ornithine transcarbamylase deficiency – gene therapy

Jesse Gelsinger
1999
Types of adenoviral vectors
AAV vectors

adeno-associated viral vectors
Adeno-associated viruses – AAV

Small, non-pathogenic single stranded DNA viruses

For replication require additional genes delivered by other viruses (adenoviruses or herpes simplex viruses)

Genome AAV – 4681 nucleotides, at both ends there are 145 nt-long ITR (inverted terminal repeats)

ITR – necessary in cis – initiation of replication
- packaging signal
- integration into genom
Infectious cycle of AAV
AAV vectors

removal of rap and cap genes
transgene insertion
AAV vectors features

- due to the lack of Rep68 and Rep78 the specific integration into chromosom 19 is lost

- unspecific integration  (low efficacy, about 5-10%)

- episomal expression

- because of non-immunogenic nature the episomal expression in non-dividing cells can be long-term
AAV-2 vectors tropism to skeletal muscles
Different transduction efficiency of AAV-2 viral vectors

A

Endothelial

B

Bronchial epithelial

C

Vascular smooth muscle

D

Skeletal muscle
Features of AAV vectors

Advantages

1. Long term expression
2. High efficiency of transduction of many cell types
3. Non-pathogenic viruses. Low risk of cellular immune response, which is additionally limited by removal of viral sequences

Limitations

1. Unspecific integration
2. Small capacity – maks. 4 kbp
3. Low efficiency of transduction of certain cell types
4. Difficulty of production in sufficient titer for in vivo work
5. Risk of humoral immunity: antibodies detect capsid proteins
Other delivery systems

- herpes virus
- alpha-viruses
- baculoviruses
Problems of viral vectors transduction

1. Toxicity
2. Limited cell specificity
3. Limited capacity
4. Problems with production and storage
5. Risk of recombination
6. Risk of transfer to germ cells
6. High costs
Gene therapy

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Inherited diseases

Acquired diseases
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Hybrydyzacja kwasów naukleinowych jako sposób hamowania ekspresji genów

DNA hybridizes with DNA

DNA hybridizes with RNA

RNA hybridizes with RNA
Inhibitory nucleic acids

1. **Antisense oligonucleotides: short, 12-20 nts.**
   The antisense nucleotides with natural backbones are unstable in physiological conditions. Thus, backbone modified nucleotides are used, that do not recruit RNAse H.

2. **Triple helix-forming oligonucleotides (TFO)**
   Pyrimidine oligodeoxynucleotides that specifically bind to a major groove of polypurine region of dsDNA via the formation of triple helices according to recognition rules established by Hoogsten
   
   Modification: binding of camptothecin to TFO transforms topoisomerase to nuclease - topoisomerase is not able to re-ligate

3. **Ribozymes - short catalytically active RNSs**

4. **Deoxyribozymes (DNAzymes) - short catalytic DNA that cleave sequence-specifically target RNA.**
   More stable than RNA, it is easier to synthesize and to modify them.

   A new approach: see INTAS project

5. **siRNA**
Antisense oligonucleotides

Short fragments of single strand, chemically modified DNA nucleotides (oligonucleotides), complementary to a given mRNA.
Clinical application of antisense oligonucleotides

Vitravene - CMV-induced retinitis

Bcl2 antisense - melanoma
RNA interference

PSTG – post-transcriptional gene silencing

Specific inhibition of gene expression by double-stranded RNA, which stimulates the degradation of a target mRNA
DsRNA is more effective than single stranded


- RNA interference is an inherited mechanism

- Only few dsRNA were required for inhibition of gene expression, indicating for an amplification process
dsRNA inhibits gene expression more effectively than ssRNA

Zarodek nastrzyknięty jednoniciowym antysensowym RNA

Embryo with Visualised mRNA of mex-3

Nicień po podaniu niespecyficznego dsRNA

Dwuniciowe RNA o długości kilkuset nukleotydów

Fire et al., 1998
Nobel 2007

Andrew Fire

Craig Mello
Mechanisms of siRNA

siRNA duplex

→ siRNA-protein complex (siRNP)

RISC

ATP

→ RISC activation

ADP + P_i

siRNA-mediated target recognition

mRNA

m7G

(A)_n

mRNA cleavage

m7G
Clinical trials of siRNA therapy of macular degeneration
Macular degeneration
siRNA against mRNA VEGF

Acuity Pharmaceuticals

Normal Macula

Dry AMD: Drusen formation under the Macula

Wet AMD: Macula with abnormal blood vessels

Carla McCullough, a 79-year-old woman in Cleveland, Ohio - 9 November 2004 - first injection of siRNA
Potential application of siRNA

- therapy of viral infections (HCV, HIV)
- inhibition of angiogenesis - AMD, psoriasis, rheumatoid arthritis, cancer
  - Cancer - inhibition of the expression of oncogenes
- neurodegenerative diseases - Huntington’s diseases
- asthma - inhibition of expression of inflammatory cytokines
Different ways of inhibition of gene expression