NEW ROLES OF ARTIFICIAL MATERIALS IN TISSUE ENGINEERING

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Artificial Materials in Medicine and Biotechnology

- **Devices for diagnostics and therapeutics** (tracers for imaging, carriers for drug and gene delivery)
- **Substrates for cell cultivation** (plastic flask, dishes)
- **Substitution of irreversibly damaged tissues and organs** (vascular prostheses, bone and joint prostheses)
  - Autologous tissue: limited availability, donor site morbidity
  - Heterologous and xenogenous tissue: immune reaction, pathogen transfer
- **Materials should be biocompatible** (tolerated by cells and tissues, non-cytotoxic, non-carcinogenic, appropriate mechanical properties)
  - **Bioinert**: not promoting cell adhesion (articular surfaces, eye lenses, currently used vascular prostheses)
  - **Bioactive** (biomimetic, biospecific, bioanalogous):
    - mimicking the behavior of extracellular matrix molecules, growth factors, hormones, enzymes;
    - promoting specific cell responses in a controllable manner
Cells on Artificial Materials in a Conventional Static Cell Culture System

Tissue culture room with a laminar flow box

Sterilized material samples inserted in polystyrene dishes

Seeded with cells

Cultured in serum-supplemented or serum-free media

Incubated at 37°C in a humidified air atmosphere with 5% CO₂
Primary Cell Cultures and Passaged Subcultures

**Explantation Method**
Vascular Smooth Muscle Cells

Cells migrating from explants of the tunica media cut of the rat aorta on day 2 after seeding (obj. 10x)

Day 7 after seeding: „Hills and valleys“, i.e. monolayered and multilayered areas (obj. 20x)

**Enzymatic Dissociation**
Vascular Endothelial Cells

Cells obtained by collagenase digestion of the tunica intima of porcine v. iugularis, day 1 after seeding (obj. 10x)

Day 7 after seeding: Confluent monolayer, cobblestone-like pattern (obj. 20x)

**Passaging:** After reaching confluence, the cells can be detached by trypsin and EDTA and re-seeded in other culture vessels or on the tested artificial materials.
Commercially Available Cell Lines

Bovine pulmonary artery endothelial cells, line CPAE

Human osteoblast-like cells, line MG-63
Derived at osteosarcoma surgery in a 13-old boy

Immunofluorescence of osteocalcin

Olympus IX 50, Obj. 4x
Obj. 20x
Molecular Mechanism of Cell Adhesion to Artificial Materials

Cell

Adsorbed extracellular matrix molecules:

- fibronectin, vitronectin, collagen, laminin
- from the serum of the culture media or body fluids
- They can also be synthesized by adhering cells

Artificial material (synthetic polymers, glass, metals, ceramics, carbon composites etc.)
Cells Bind Specific Sites on the Adsorbed Extracellular Matrix Molecules
(e.g., certain amino acid sequences of the adsorbed proteins)

Sequences typical for a certain protein:
- RGD..... vitronectin, fibronectin
- REDV....fibronectin
- DGEA....collagen
- YIGSR, IKVAV.... laminin
- KRSR....heparin binding domain

Sequences preferred by a certain adhesion receptor:
- RGD, KQAGDV.....integrin $\alpha_\gamma\beta_3$
- REDV.... integrin $\alpha_4\beta_1$
- DGEA....integrin $\alpha_2\beta_1$
- YIGSR, IKVAV.... $\alpha_6\beta_1, \alpha_7\beta_1$
- KRSR...non-integrin adhesion receptor

Sequences preferred by a certain cell type:
- KQAGDV.. smooth muscle cells
- REDV.... endothelial cells
- YIGSR, IKVAV....neurons
- KRSR...osteoblasts
**Adhesion Receptors on the Cell Membrane**

- **Adhesion molecules of the integrin superfamily**
- **Non-integrin adhesion molecules**, e.g., proteoglycans (in addition to amino acid sequences, such as KRSR, they can bind saccharide ligands, e.g., galactose)

**Model of the integrin receptor:**

- Binding site for an amino acid sequence (RGD)
- Extracellular domain
- Intracellular domain

Types of integrin receptors and their ligands

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Subunits</th>
<th>Ligands</th>
<th>Binding Site</th>
<th>Presence in vascular smooth muscle</th>
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<td>β1</td>
<td>α1</td>
<td>Laminin, collagens</td>
<td>DGEA</td>
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<tr>
<td>α2</td>
<td>Laminin, collagens</td>
<td>RGD</td>
<td>+</td>
<td></td>
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<td>Fibronectin, laminin, collagens</td>
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<td>Fibronectin, VCAM-1</td>
<td>EILDV</td>
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<td>Fibronectin</td>
<td>RGD</td>
<td>+</td>
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<td>Laminin</td>
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<td>α9</td>
<td>Tenascin</td>
<td></td>
<td>+</td>
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<td>RGD</td>
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<td>β2</td>
<td>αLFA-1 ICAM-1, ICAM-2</td>
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<td>Fibrinogen</td>
<td>GPRP</td>
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<td>αIib Fibronectin, fibrinogen, vitronectin, von Willebrand factor, thrombospondin</td>
<td>RGD, KQAGDV</td>
<td>-</td>
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<tr>
<td></td>
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<td>RGD</td>
<td>+</td>
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<tr>
<td>β4</td>
<td>α6 Laminin</td>
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<td>β7</td>
<td>α4 VCAM-1, fibronectin</td>
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<td>αIEL</td>
<td>Vitronectin</td>
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</table>

Cell surface adhesion molecules

(a) **Cadherins**: cell-cell; homophilic; Ca$^{2+}$; actin cytoskeleton; domains

(b) **Immunoglobulins**: cell-cell (e.g., endothelium-immunocompetent cell); homophilic or heterophilic (with integrins); ICAM-1, VCAM-1

(c) **Selectins**: cell-cell (e.g., endothelium-immunocompetent cell); heterophilic (with a glycosylated protein); ELAM-1

(d) **Integrins**: cell-matrix; cell-cell (e.g., integrins on leukocytes bind immunoglobulins on endothelial cells)

Hynes R.O.: Cell adhesion: Old and new questions. Millenium Issue, TBC•TIBS •TIG, 1999
Adhesion receptors are recruited into specific nano- or microdomains on the cell membrane, called focal adhesion plaques (focal adhesion sites, focal adhesions):

Here the receptors communicate with many structural and signalling molecules (e.g. talin, vinculin, focal adhesion kinase), through which they are linked to actin cytoskeleton, associated with various enzymes, cellular organelles and nucleus:

By this way, the signal from extracellular environments, represented by an artificial material, is delivered to the cellular genome, and can influence its expression (proteosynthesis) and cell behavior.
Regulation of Protein Adsorption and Cell Adhesion

Physical and chemical properties of the material surface, e.g.

- Presence of certain chemical functional groups
- Polarity, wettabity
- Electrical charge, conductivity
- **Surface topography** (size, shape and distance of irregularities, importance of **nanotopography**)
- **Mechanical properties** of the material surface (rigidity, flexibility)

These properties influence the **type, amount, spatial conformation, flexibility** and **reorganization** of the adsorbed ECM molecules, and thus the **accessibility of ligands** on these molecules by cell adhesion receptors.
Effects of Wettability on Cell Adhesion (I)

Polyethylene irradiated with O⁺ ions (energy 30 or 150 keV, 10^{13}-10^{15} ions/cm²)

Differential FTIR spectra:
Increase in absorbance between 1650-1750 cm⁻¹:
- Presence of oxygen-containing chemical functional groups (carbonyl, carboxyl, ester, hydroxyl)
- Increased wettability

Static water drop contact angle

Hydrophobic surface

Hydrophilic surface

Polyethylene, O⁺, 150keV
Collagen Adsorption and Cell Adhesion to Ion-Modified Polyethylene

Unmodified polyethylene

10^{14} O^+ ions/cm^2, energy 30 keV

With collagen

Without collagen

Adsorbed with collagen IV conjugated with Oregon Green 488, Molecular Probes, Eugene, OR, USA; concentration 0.02 mg/ml (10 µg/cm^2), incubation for 24 h at room temperature.

Nikon, CCD camera, Image Pro Plus 3.0 software

Adsorption of collagen IV on PE/O^+

Intensity of fluorescence (ref. U)

Mean ± SEM from 4-9 experiments, Student t-test for unpaired data. Ion energy 30 keV
Vascular Smooth Muscle Cells on Ion-Irradiated Polyethylene

**Unmodified polyethylene**

- **Day 1 after seeding**
  - $3 \times 10^{13}$ ions $\text{C}^+$ per cm$^2$; 150 keV

- **Day 3 after seeding**
  - $3 \times 10^{14}$ ions $\text{O}^+$ / cm$^2$; 150 keV
VSMC on Ion-Irradiated Polymers Are More Active in Synthesis of Focal Adhesion and Contractile Proteins

Focal adhesion plaques

Contractile protein α-actin

Rat aortic smooth muscle cells in cultures on ion-irradiated polyethylene

Paxillin, α-actinin

α-actin, SM1,2-myosins, calponin

ELISA per mg of protein, mean ± SEM (4 exp.), Student t-test for unpair. data, *: p<0.05 **: p<0.01
Regionally-Selective Cell Adhesion on Surfaces Micropatterned with Ion- or UV-Irradiation

Polyethylene irradiated with Ar+ ions (150 keV, 10^{13} ions/cm^2) through a metallic mask (holes 100 µm in diameter, center-to-center distance 200 µm)

Polytetrafluoroethylene (PTFE) irradiated with UV light (Xe2*-excimer lamp, 30 min, ammonia atmosphere) through a metallic mask (holes 100 µm in diameter, center-to-center distance 300 µm)

Rat vascular smooth muscle cells, fixed and stained with hematoxylin and eosin

Human embryonic kidney cells, native cultures

Influence of Wettability on Cell Adhesion (II)

Surfaces patterned with hydrophilic and hydrophobic microdomains, prepared by plasma polymerization of acrylic acid and 1,7-octadiene

Vascular smooth muscle cells

6 hours after seeding

Vascular endothelial cells, von Willebrand factor

5 days after seeding

 Adsorbed with collagenem IV conjugated with Oregon Green 488, 10 µg/cm², 24 h, room temperature)

E. Filová, N.A. Bullett, L. Bačáková, J.W. Haycock, A. Shard, V. Lisa: Regionally-selective cell adhesion and growth on microdomains prepared by plasma polymerisation of acrylic acid and 1,7-octadiene. Biomaterials 2007, under revision
Effects of Microtopography on Cell Adhesion

Composites with carbon matrix reinforced with carbon fibres, MG 63 bone-derived cells

Untreated CFRC (Ra= 6.5 ± 1.8 µm)

Adsorbed with fluorescence-labelled collagen IV

Immunofluorescence of vinculin in MG 63 cells

Polished and coated with pyrolytic graphite (Ra= 0.24 ± 0.09 µm)

Influence of Nanotopography on Cell Adhesion

(is beneficial, because the nanostructure mimics the architecture of the natural ECM)

Hydrocarbon plasma polymers with nanoclusters of Ti, seeded with endothelial CPAE cells (Imunofluorescence of talin)

Nanocrystalline diamond, seeded with human osteoblast-like MG 63 cells (Propidium iodide staining)

Continuous and micropatterned fullerene C$_{60}$ layers, seeded with MG 63 cells (Stained with LIVE/DEAD kit)

Human Osteoblast-like MG 63 Cells on Composites of a Terpolymer Mixed with Carbon Nanotubes

Immunofluorescence of β-actin or propidium iodide staining

Collaboration with AGH Univ. of Science and Technology, Krakow, Poland

Concentration of Focal Adhesion Proteins in MG 63 Cells Grown on SWCNT-Polymer Composites (per mg protein, ELISA, day 8)

Mean ± SEM from 3 independent experiments, Student t-test for unpaired data, * p>0.05 in comparison with the value on the pure terpolymer. Day 8 after seeding.
Concentration of markers of differentiation and immune activation in MG 63 cells on SWCNT-polymer composites (measured per mg of protein, ELISA; day 8 after seeding)

Mean ± SEM from 3 independent experiments, Student t-test for unpaired data, * p>0.05 in comparison with the value on the pure terpolymer. Day 8 after seeding.
Rigidity versus Flexibility of Cell Adhesion Substrates

Polyacrylamide gel + covalently bound collagen

Hard gel, $E = 40$ kPa

Rat aortic smooth muscle cell line A7r5 transfected with DNA constructs encoding GFP-paxillin or GFP-actin, 4 hours after seeding

Soft gel, $E = 1$ kPa

GFP-paxillin


GFP-β-actin
Innovation of Clinically Used Vascular Prostheses by Protein Immobilization and Endothelization

A knitted polyethylene terephthalate prosthesis, fabricated in VÚP Company, Brno, CR

Day 7 after seeding (17,000 cells/cm²)

Collagen I (Col I), collagen IV (Col IV), laminin (Lm), fibronectin (Fn), fibrin (Fb), heparin (H) or their combinations, e.g.

Col I + Fn
Col I + Lm
Fb + Lm
Fb + Col I

(A patent submitted in collaboration with the Inst. of Macromolecular Chemistry, Acad. Sci. CR, Dr. E. Brynda)

Immunofluorescence of talin in endothelial cells on fibrin assemblies

Water contact angle 119°
Dynamic Cell Culture Systems in Our Lab

Oligene GmbH (now Provitro GmbH), Berlin

Sterile manipulation in laminar flow box

Schema of the system

„Tube chamber“ for tubular structures (vascular prostheses)

Pump and „Flow chamber“ for planar samples
Dynamic Cell Culture Systems, Provitro GmbH, Germany continued

„Perfusion chamber“ for 3D porous or fibrous scaffolds

Rotary Cell Culture System, Synthecon, Luxembourg
Synthetic Biodegradable Polymers Endowed with Ligands for Cell Adhesion Receptors

**Ligands** (e.g., oligopeptide GRGDSG with its cooperating sequence PHSRN)

**Polyethylene oxide (PEO):**
- extremely hydrophilic
- do not allow protein adsorption and aberrant cell adhesion
- flexible chains ensure accessibility of ligands to cell adhesion receptors

**Poly-DL-lactide (PDLLA):** copolymerized with PEO

**Poly-L-lactide (PLLA):** bulk material of the newly constructed bioartificial tissue, e.g. vascular wall

Material prepared in cooperation with the Institute of Macromolecular Chemistry, Acad. Sci. CR, Prague, Czech Rep.
Adhesion of Vascular Smooth Muscle Cells on Polylactides and PEO

Immunofluorescence staining of vinculin, a protein of focal adhesion plaques, day 3 after seeding, medium with 10% of fetal bovine serum

- **Cell adhesion is similar as on conventional cell culture substrates (polystyrene dish, glass)**
- **Mediated by adsorption of proteins from the serum of the culture medium** (vitronectin, fibronectin)

- **Extremely hydrophilic PEO does not allow adsorption** of serum proteins
- **Cells are not able to adhere and to develop focal adhesion plaques**
Adhesion and Growth of VSMC on Polymers with RGD

Day 3 after seeding, medium with 10% of serum

Immunofluorescence of talin

Incorporation of BrdU

PDLLA-PEO-5% GRGDSG

Non/adhesive copolymer PDLLA-PEO functionalized with Gly-Arg-Gly-Asp-Ser-Gly (GRGDSG), a ligand for integrin adhesion receptors on cells

Cell adhesion markedly improved

Cells were viable, synthesized DNA and proliferated

Conclusion & Further Perspectives

**Innovation of currently used tissue replacements:**
- Introduction of a cell component (endothelization of vascular prostheses produced in the VÚP Company, Brno, CR)
- Higher attractiveness for cells (better osseointegration of bone implants produced in Beznoska Ltd., Kladno, CR, or dental implants)

**Construction of new advanced bio-artificial tissue replacements:**
- Biodegradable (polyesters, chitosan, recombinant proteins)
- Three-dimensional (porous and fibrous scaffolds)
- Hierarchically micro- and nanostructured (including application of nanofibres produced by Elmarco and Technical University Liberec)
- Regionally selective and preferential adhesion of certain cell types (functionalization with appropriate ligands for cell adhesion receptors)

**Colonization of materials with bone marrow stem cells and differentiation of these cells in desired cell types**

**Fundamental scientific research** (e.g., non-integrin-mediated cell-matrix adhesion)
Thank you very much for your attention!

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