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Cell Adhesion on Artificial Materials for Tissue Engineering

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Summary

Advanced interdisciplinary scientific field of tissue engineering has been developed to meet increasing demand for safe, functional and easy available substitutes of irreversibly damaged tissues and organs. First biomaterials were constructed as "two-dimensional" (allowing cell adhesion only on their surface), and durable (non-biodegradable). In contrast, biomaterials of new generation are characterized by so-called three dimensional porous or scaffold-like architecture promoting attachment, growth and differentiation of cells inside the material, accompanied by its gradual removal and replacement with regenerated fully functional tissue. In order to control these processes, these materials are endowed with a defined spectrum of bioactive molecules, such as ligands for adhesion receptors on cells, functional parts of natural growth factors, hormones and enzymes or synthetic regulators of cell behavior, incorporated in defined concentrations and spatial distribution against a bioinert background resistant to uncontrolled protein adsorption and cell adhesion.

Key words

Biomaterials • Bioinert • Bioactive • Integrins • Adhesion Oligopeptides

Introduction

In recent years, artificial materials are of growing importance in medicine and biology. A modern scientific interdisciplinary field known as *Tissue Engineering* has been developed to design artificial biocompatible materials to substitute irreversibly damaged tissues and organs. In addition, biomaterials are important for fundamental scientific research as relatively simple and physicochemically well-defined artificial templates of extracellular matrix (ECM), allowing studies of ECM signals controlling cell adhesion, spreading, growth, differentiation, functioning, viability, matrix degradation etc. (Park *et al.* 1998, 2003, Banerjee *et al.* 2000, Kubies *et al.* 2000, Irvine *et al.* 2001, Rypáček *et al.* 2001, Mann and West 2002, Wang *et al.* 2002, Bačáková *et al.* 2003a, VandeVondele *et al.* 2003).

In construction of biocompatible artificial implants, there are two main strategies for modulating the cell-material interactions. One is to create an inert surface not allowing the adsorption of proteins and adhesion of cells, and thus preventing activation of the immune system, blood coagulation, thrombosis, extracellular matrix deposition and other interactions between material

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and surrounding environments. This type of biomaterial has been used for construction of heads and cups of joint prostheses (Cook *et al.* 1997), intraocular lenses (Smetana and Vacík 1997, Han *et al.* 2003) or blood-contacting devices, such as heart valves, two-dimensional smooth bioinert vascular prostheses, catheters for hemodialysis or vesicles for therapeutic drug delivery (Cook *et al.* 1997, Kim and Kim 2002, Bernacca *et al.* 2002, Ahmed *et al.* 2003, Photos *et al.* 2003).

The other, more general and advanced strategy aims at creation of materials promoting attachment, migration, proliferation, differentiation, long-term viability and cell functioning (such as contraction or secretion of extracellular matrix) in a controllable manner, if possible. These materials can be constructed "two-dimensionally", i.e. as surfaces colonized by cells, such as heart valves or vascular prostheses lined by contiguous, mature, naturally thromboresistant, non-immunogenic and semipermeable endothelial layer (Bordenave *et al.* 1999, Kim *et al*. 1999, Bačáková *et al*. 2000b, Heitz *et al*. 2003), bone implants inducing formation of mineralized osseous tissue only at the interface of native tissue and artificial material (Bačáková *et al.* 2001b,c, 2003b), or skin substitutes containing polymeric sheet with a feeder layer of fibroblasts covered by keratinocytes (Dvořánková *et al*. 2003). However, the most advanced recent trend in tissue engineering aims at creation of so-called "hybrid bioartificial organs". This strategy is used e.g. for construction of artificial vessels, bone, cartilage and parenchymatous organs like pancreas or liver. The artificial component of these constructs is designed as a three-dimensional scaffold promoting controlled ingrowth and maturation of cells. It could be colonized under *in vitro* conditions with patient's own cells obtained by biopsy prior to the planned surgery, or even with stem cells guided to a certain differentiation pathway. In ideal case, the artificial support should be reorganized and resorbed by growing cells and gradually replaced by the newly formed extracellular matrix and differentiated cells, i.e. fully functional native tissue existing in the organ prior to damage (Cook *et al.* 1997, Park *et al.* 1998, 2003, Kim *et al.* 1999, Banerjee *et al.* 2000, Irvine *et al.* 2001, Tassiopoulos and Greisler 2000, Liu *et al*. 2002, Mann and West 2002, Noth *et al*. 2002, Wang *et al.* 2002, Lutolf *et al.* 2003, VandeVondele *et al.* 2003). From this point of view, the bioartificial construct could not be considered as a durable substitute of the damaged or lost tissue but as a temporary structure promoting its regeneration.

Molecular mechanisms of cell adhesion on artificial materials

Direct non-receptor-mediated cell-material binding

Non-receptor-mediated cell adhesion on artificial materials means non-specific cell-material interactions *via* so-called weak chemical bonding, such as hydrogen bonding, electrostatic, polar or ionic interactions between various molecules on cell membrane and functional chemical groups on the polymers, i.e. without presence of extracellular matrix proteins or their functional parts (for review see Bačáková *et al*. 2000a,b). In contrast to integrin-mediated cell adhesion, this type of interactions cannot ensure the transmission of adequate signals from extracellular environments into cells and survival of anchorage-dependent cells. If the cells are not able to synthesize and deposit their own ECM molecules in a relatively short time (usually in 24 to 48 h after seeding), or they do not have some of these molecules attached on cell membrane, they undergo apoptosis (Huang *et al*. 1998, Garcia *et al.* 1999, Groth *et al*. 1999, Moiseeva 2001).

Receptor-mediated binding though ECM molecules or their parts

Functional receptor-mediated and signal-transmitting cell adhesion on a conventional biomaterial is mediated by extracellular matrix (ECM) molecules, such as fibronectin, vitronectin, collagen or laminin (Tang *et al.* 1998, Garcia *et al.* 1999, Groth *et al*. 1999, Moiseeva 2001). These molecules can be adsorbed on the material surface from the surrounding environment, i.e. cell culture media *in vitro* or body fluids *in vivo*. The anchorage-dependent cells bind specific amino acid sequences of these molecules through integrin receptors. The minimum adhesion motif on ECM molecules should contain at least three amino acids, which are often represented by Arg-Gly-Asp (RGD). These adhesion motifs cooperate with synergistic amino acid sequences, e.g. Pro-His-Ser-Arg-Asn (PHSRN), which helps to maintain appropriate spatial conformation of these ligands as well as integrin receptors (Humphries *et al*. 2000, Susuki *et al.* 2002). In more sophisticated biomaterials of new generation, both receptor-binding and synergistic oligopeptides can be attached directly to an artificial material, as will be discussed more deeply below.

Integrins are heterodimeric transmembrane glycoproteins consisting from one alpha and one beta

chains. Their function is dependent on calcium, which binds on the alpha subunit. About 16 different subunits alpha and 8 subunits beta were described (Glukhova and Koteliansky 1995, Horton 1997, Hemler 1998, Hynes 1999, Moiseeva 2001, Aplin 2003). However, these numbers are not definitive, because integrin receptors are still under intensive research and new subunits are being revealed. Various combinations of alpha and beta chains result in constitution of receptors with preferential affinity to certain ECM molecules. For example, integrin $\alpha_2\beta_1$ recognizes the amino acid sequence Asp-Gly-Glu-Ala (DGEA) on collagen, $\alpha_5\beta_1$ the sequence RGD on fibronectin, and $\alpha_v\beta_3$ also is a receptor for RGD but on vitronectin. The possibility of binding RGD to different receptors is probably caused by a different spatial conformation of RGD due to the vicinity of different amino acids or existence of different synergistic sequences on various ECM molecules. On the other hand, one type of integrin can bind more ligands, for example, the integrin $\alpha_5\beta_1$ can bind the sequence RRETAWA (Arg-Arg-Glu-Thr-Ala-Trp-Ala) in addition to the RGD. In other words, the integrin receptors are not strictly specific for a certain ligand, and their affinity to this ligand can be considered as preferential only. If the preferred ligand is absent or non-accessible, these receptors could bind another type of oligopeptide or ECM molecule. Thus the integrins are sometimes called as "the most promiscuous receptors" (Glukhova and Koteliansky 1995, Horton 1997, Hemler 1998, Hynes 1999, Humphries *et al.* 2000, Moiseeva 2001, Aplin 2003).

After ligand binding, the integrin receptors are recruited into distinct dot-like or streak-like nano- or microdomains on cell membrane, called "focal adhesion sites", "focal adhesion plaques" or simply "focal adhesions". In these regions, the integrins communicate with many specific structural and signaling molecules. The former are represented by proteins of membrane-associated cytoskeleton, also called "focal adhesion proteins", such as talin, α-actinin, filamin, paxillin or vinculin (Horton 1997, Hemler 1998, Hynes 1999, Moiseeva 2001, Aplin 2003). These proteins act as linkers between the integrin receptors and the cytoplasmatic actin cytoskeleton, which is associated with nuclear membrane, membranes of cellular organelles as well as with various enzymes, and thus influences intracellular processes important for cell behavior, including transport and secretion of various molecules, endocytosis, decision between cell proliferation and differentiation or apoptosis (Ingber *et al.* 1995, Huang *et al*. 1998, Moiseeva 2001, Wang *et al.* 2001, Aplin 2003). The signaling molecules are represented e.g. by cytohesin-1, focal adhesion kinase (FAK), integrin-linked kinase (ILK), mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), β_3-endonexin, integrin cytoplasmic-domain-associated protein-1 (ICAP-1), receptor for activated protein kinase C (Rack-1), calcium- and integrin-binding protein (CIB) or small GTPases. Both structural and signaling molecules play a decisive role for further behavior of cells after contacting a biomaterial, i.e. switching between cell proliferation and differentiation, survival or apoptosis and other functions (Hemler 1998, Hynes 1999, Huang *et al*. 1998, Moiseeva 2001, Wang *et al.* 2001, Aplin 2003).

Recent investigations have shown that other receptors than integrins can also take place in cell-matrix adhesion, e.g. proteoglycan-based receptors (Dee *et al.* 1998, Park *et al.* 1998, Moiseeva 2001, Aplin 2003). For example, heparan sulphate proteoglycan on osteoblasts recognizes a bone-specific oligopeptide Lys-Arg-Ser-Arg (KRSR) (Dee *et al.* 1998) or asialoglycoprotein receptor on hepatocytes binds a galactose ligand (Park *et al.* 1998). Both these receptor-ligand systems are considered to be unique for osteoblasts and hepatocytes, respectively. Specific receptor-ligand systems could take an important place in creation of nanospheres for site-specific drug or gene delivery using organ-selective targeting molecules, or in construction of microarchitectured scaffolds for spatial organization of diverse cell types (Dee *et al.* 1998, Park *et al*. 1998).

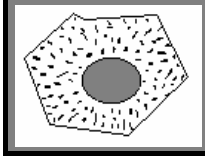
Creation of bioinert, cell non-adhesive surfaces

The surfaces preventing cell adhesion have been generated using various natural or synthetic molecules, such as antiadhesive protein albumin (Wang *et al.* 2002), hydrogels based on hyaluronic acid (Park *et al.* 2003) or poly(hydroxyl ethyl methacrylate) (Smetana and Vacík 1997, Han *et al.* 2003), polyvinyl alcohol, polyacryl-amide, dextran (VandeVondele *et al.* 2003), and particularly poly(ethylene glycol) (PEG; Banerjee *et al.* 2000, VandeVondele *et al.* 2003) or poly(ethylene oxide) (PEO; Irvine *et al.* 2000, Kim and Kim 2002, Wang *et al.* 2002). Due to their poor mechanical properties, the later two compounds have often been applied in a form of a pendant side chains attached to a backbone, represented e.g. by latexes, (Banerjee *et al.* 2000), poly(methyl

methacrylate) (Irvine *et al.* 2001), polyurethane (Wang *et al.* 2002), poly (L-lysine) (VandeVondele *et al.* 2003), and particularly polylactides (Kubies *et al*. 2000, Rypáček *et al.* 2001, Bačáková *et al*. 2003a, Filová *et al.* 2003, 2004a, Proks *et al*. 2003). The pendant side chains of PEO or PEG resist the protein adsorption and receptor-mediated cell adhesion by their extreme hydrophilia, i.e. their high molecular mobility in water (Wang *et al.* 2002, Kim and Kim 2002). In recent years, the main significance of this type of antiadhesive surfaces is the possibility to functionalize the ends of pendant PEO or PEG chains with ligands for cell adhesion receptors and their cooperating molecules, as will be discussed more deeply below.

On the other hand, the extremely hydrophobic surfaces are also endowed with anti-adhesive properties. Although these surfaces allow adsorption of cell adhesion-mediating proteins, even in relatively large quantities, these proteins become very rigid and reorganization-resistant, so that their specific amino acid sequences are not accessible for integrin receptors on cells (Garcia *et al.* 1999, Groth *et al*. 1999; for review see Bačáková *et al*. 2000a,b, 2001a).

Table 1. Correlation between the extent of cell adhesion and the subsequent cell behavior.

Cell behavior/ Cell spreading	Viability	Migration	Proliferation	Differentiation
Small 	↓	↓	↓	↓
Medium 	↑	↑	↑	↓
High 	↑	↓	↓	↑

Creation of cell-interactive surfaces

Adsorption of entire extracellular matrix molecules

The adsorption of cell adhesion-mediating ECM molecules in appropriate amount, spectrum, spatial conformation, flexibility and accessibility for integrin receptors is markedly influenced by physical and chemical properties of the material surface layer, such as wettability, electrical charge, surface roughness and topography, mechanical properties (rigidity or flexibility), crystallinity, porosity, solubility, pH or presence of certain atoms or chemical functional groups, e.g. carbon, amine groups or oxygen groups (Cook *et al.* 1997, Smetana and Vacík 1997, Garcia *et al.* 1999, Groth *et al.* 1999, Bačáková *et al.* 2000a,b, 2001a,b,c, Švorčík *et al.* 2001, 2002, 2004, Walachová *et al.* 2002, Heitz *et al*. 2003).

The optimum protein adsorption and cell adhesion is usually achieved at mild, intermediate values of the surface wettability. However, some materials used in tissue engineering, mainly synthetic polymers as polyethylene, polyurethane, polypropylene or polystyrene, are too hydrophobic in their pristine unmodified state. Relatively safe, effective and low-cost methods for adjustment of their surface wettability are represented by physical methods, such as bombardment with ions, irradiation with ultraviolet light or exposure to plasma discharge. These procedures lead to splitting of chemical bonds between carbon and non-carbon atoms, mainly hydrogen, followed by the release of non-carbon

atoms. The unsaturated carbon-carbon bonds and radicals on carbon chain react with oxygen, and the newly formed oxygen-containing groups (i.e. carbonyl, carboxyl, ester, hydroxyl groups) increase polymer surface hydrophilia. In addition, the beam modification of polymer chains produces conjugated double bonds which provide a higher electrical conductivity of the polymer surface. The material is relatively enriched with carbon, which is known to have beneficial effect on cell adhesion (Bačáková *et al.* 2000a,b, 2001a,b,c, Švorčík *et al.* 2001, 2002, 2004, Walachová *et al.* 2002, Heitz *et al.* 2003) (Figs 1A and 1B).

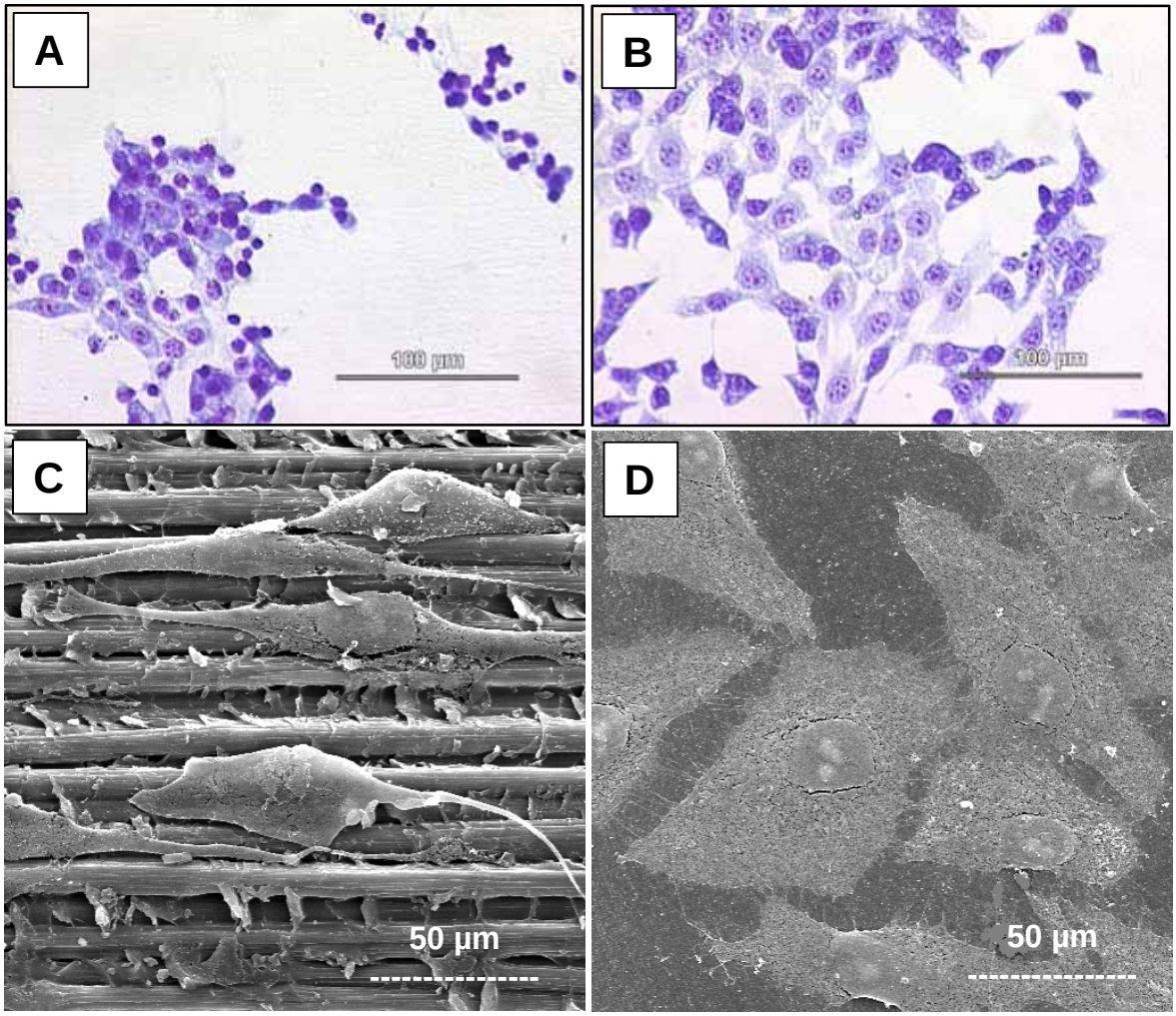


Fig. 1. Morphology of cells on physico-chemically modified artificial materials. **A**. Pristine unmodified polyethylene with less spread and often aggregated mouse embryonic fibroblasts of 3T3 line. **B**. Polyethylene implanted with O^+ ions (energy 15 keV, dose $3x10^{13}$ cm^{-2}) with well spread polygonal 3T3 cells distributed homogeneously throughout the whole polymer surface. **C.** Pristine unmodified carbon-based composite material with spindle-shaped human osteogenic MG 63 cells arranged in parallel to carbon fibres prominent on the surface. **D.** Well spread polygonal cells on the composite after polishing and coating with pyrolytic graphite. Stained with hematoxylin and eosin (**A, B**) or photographed in scanning electron microscope (**C, D**).

Also the surface roughness should be adjusted in some materials, especially those used for hard tissue engineering. For example, pristine unmodified carbon fibre-reinforced carbon composites are relatively rough due to the prominence of carbon fibres over the carbon matrix, so that the adsorption of collagen IV and other cell adhesion-mediating ECM proteins was relatively low and irregular. The cells adhering to these materials were not able to develop distinct focal adhesion plaques and bridge over the irregularities, and remained spindle-shaped and arranged longitudinally in the grooves between the fibres. If the surface was polished with colloidal SiO_2, metallographic paper or diamond paste, the number and spreading of initially adhered osteogenic and vascular cells, as well as their subsequent growth and expression of differentiation markers, significantly increased (Bačáková *et al.* 2001b,c, Starý *et al.* 2003) (Figs 1C and 1D). On the other hand, very smooth

surfaces cannot also ensure firm cell adhesion, formation of a sufficient number of bone-implant contacts and fixation of the implant inside the bone (for review see Bačáková *et al*. 2001b). Taken together, similarly to surface polarity and wettability, the parameters of the surface roughness also have their optimum range, and this may differ for each type of material (i.e. carbon-based composites, metals, ceramics, synthetic polymers etc.), and for each type of cells. In addition, not only the height and depth of the surface irregularities and distances between them are important, but also their shape, especially their sharpness, which may damage mechanically the cells.

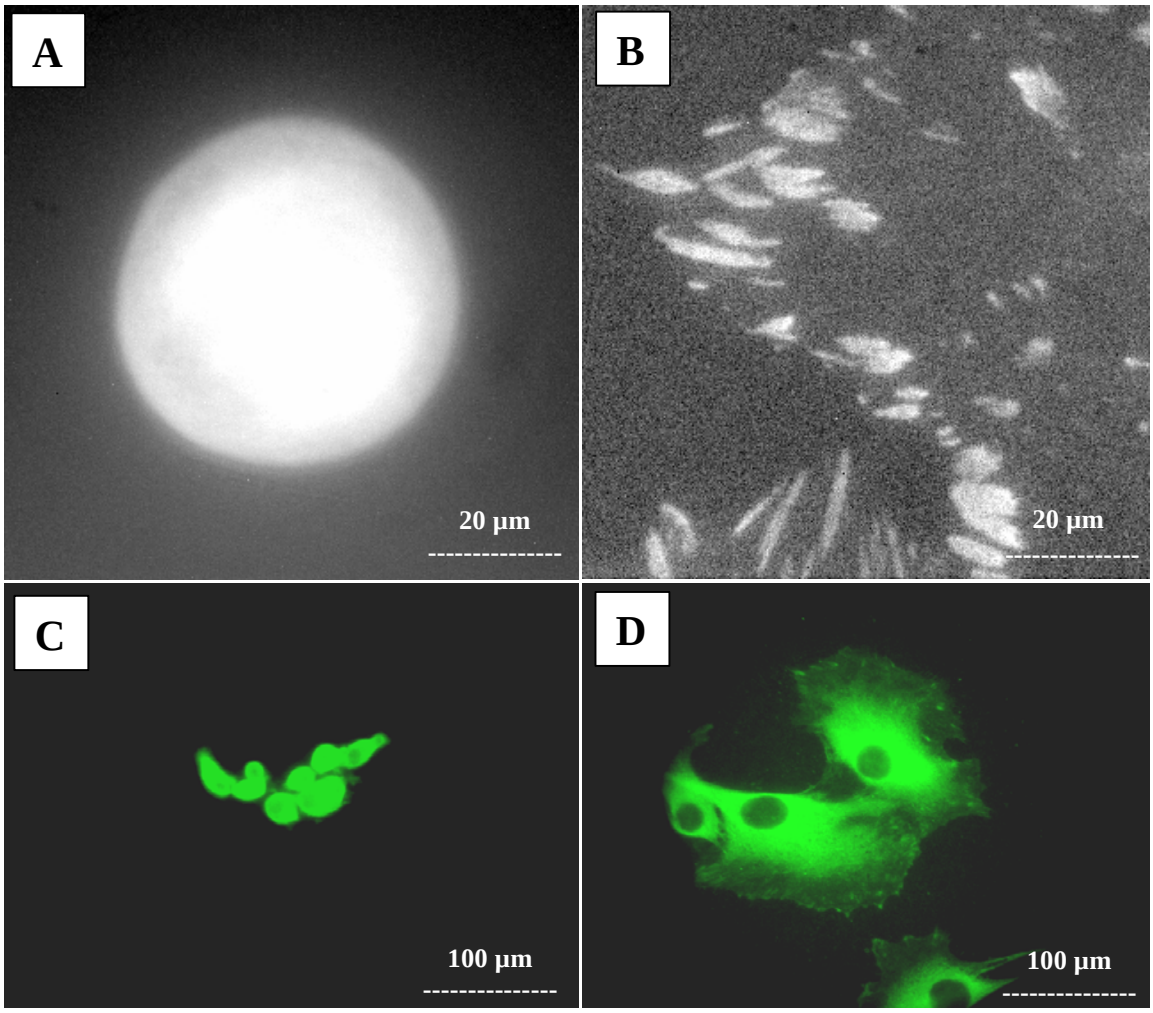


Fig. 2. Assembly of focal adhesion plaques in rat vascular smooth muscle cells on soft and hard (**A, B**) or inert and bioactive polymers (**C, D**). **A.** Round cell without focal adhesions on soft polyacrylamide gel (PAG) preadsorbed with collagen. **B**. Peripheral region of a cell with well-developed multiple streak-like focal adhesions on hard PAG preadsorbed with collagen. **C**. Non-spread round cells without focal adhesions on cell non-adhesive PDLLA-PEO copolymer. **D.** Spread cells forming dot-like focal adhesions on the copolymer functionalized with GRGDS peptides in 5 % concentration. Focal adhesions were visualized by transfection of cells with a gene construct encoding paxillin and green fluorescence protein (**A, B**) or immunofluorescence staining of vinculin (**C, D**).

Cells are able to detect very sensitively the mechanical properties of the adhesion substrate and regulate the integrin binding, assembly of focal adhesion plaques and cytoskeleton accordingly (Ingber *et al.* 1995, Huang *et al*. 1998, Wang *et al*. 2001). If the adhesion substrate is very firm, rigid and non-deformable, for example ECM molecules adsorbed on too hydrophobic surfaces, the cells are not able to reorganize these molecules in order to access the ligands for integrin receptors and recruit these receptors into focal adhesion plaques, which is a prerequisite for delivery of signals ensuring the viability of anchorage-dependent cells (Huang *et al.* 1998, Garcia *et al*. 1999, Groth *et al*. 1999). On the other hand, if the material is too elastic, compliant, flexible and irreversibly deformable, it does not allow the anchorage of cells, even if the ligands for

integrin receptors are present in satisfactory amounts and accessibility, and are bound by these receptors. Such type of substrate cannot resist the cell tractional forces generated by the assembling cytoskeleton. When collagen was adsorbed or covalently bound on glass or hard polyacrylamide gel (PAG), the vascular smooth muscle cells were normally spread with multiple streak-like focal adhesion sites and rich in actin cytoskeleton, and were viable. However, if soft PAG or pure collagen gel was used, the cells remained round (without signs of spreading and assembly of adhesion apparatus), and underwent apoptosis (Engler *et al*. 2004). As mentioned above, similar cell behavior was observed on extremely hydrophilic, i.e. very weak, fluid-like surfaces resembling water, e.g. hydrogels, PEG or PEO (Banerjee *et al*. 2000, Irvine *et al.* 2001, Liu *et al*. 2002, Kim and Kim 2002, Wang *et al.* 2002, Kubies *et al.* 2000, Rypáček *et al.* 2001, Bačáková *et al*. 2003a, Park *et al.* 2003, VandeVondele *et al.* 2003) (Figs 2A and 2B).

Incorporation of specific bioactive molecules

As mentioned above, in more advanced biomaterials, the relatively simple, short, chemically well-defined and usually synthetic oligopeptidic or carbohydrate ligands for integrin or proteoglycan-based receptors and their cooperating sequences can be bound against a cell non-adhesive background in defined species, amount and spatial distribution in order to promote specific cell responses in a controllable manner. These constructs, often called as "bioactive", "biospecific" or "biomimetic" surfaces or "artificial templates of ECM", provide the following advantages: 1) entire protein molecules, often adsorbed in less defined spectrum, amount and conformation, and promoting inflammatory responses, thrombosis or device-associated infections, can be avoided (Tang *et al.* 1998, VandeVondele *et al*. 2003, for review see Bačáková *et al.* 2000a, b), 2) a certain cell type could be preferentially seeded on a certain region of the biomaterial, e.g. endothelial cells on the luminal surface of a vascular prosthesis and smooth muscle cells in its intramural part, or drugs and genes could be delivered to a certain cell type in polymeric vesicles through receptor-mediated internalization pathway (Dee *et al.* 1998, Park *et al*. 1998, Bačáková *et al.* 2003a, Mann and West 2002, Suh *et al.* 2002, Heilshorn *et al*. 2003), and 3) further cell behavior, such as migration, proliferation, differentiation, appropriate functioning or long-term viability, could be regulated by the extent of initial cell adhesion. It is well known that the size and shape of cell spreading area, as well as the number, size, shape and distribution of focal adhesion plaques are decisive for further migratory, proliferative and differentiation behavior of anchorage-dependent cells. If this extent is very small (i.e. attachment of round cells without formation of focal contacts and spreading), the cells usually do not survive. At the intermediate adhesion strength, the cells are most active in migration and proliferation, and if the cell-material contact area is very large with multiple well-developed focal adhesion sites associated with a rich cytoskeleton, the cells tend to skip the proliferation phase and enter sooner the differentiation program. Construction of so-called "patterned surfaces" with adhesive microdomains of defined size and shape as well as grouping of ligands for integrin receptors into features mirroring focal adhesion sites on cells could provide an excellent physiological tool for attenuation of excessive proliferation of vascular smooth muscle cells on bioartificial vascular prostheses, often leading to restenosis and even full obliteration of these devices (Huang *et al.* 1998, Kim *et al.* 1999, Irvine *et al.* 2001, Mann and West 2002, Kubies *et al.* 2000, Rypáček *et al.* 2001; for review see Bačáková *et al.* 2000a, 2001a, 2002, 2003a,c, Filová *et al.* 2003, 2004a) (Table 1, Figs 2C and 2D).

Another possibility how to control the proliferation and other behavior of cells on advanced biospecific materials is incorporation and controlled release of functional parts of natural growth factors, hormones, enzymes or synthetic cell cycle regulators (Brooks *et al*. 1997, Vella *et al.* 1999, Lutolf *et al.* 2003; for review see also Bačáková *et al*. 2000b, 2003a). The latter are represented e.g. by inhibitors of cyclin-dependent kinases (CDK), especially the compound CVT-313 (Brooks *et al*. 1997). This purine analog inhibits the activity of CDK2, i.e. the phosphorylation of its substrates including histone H1 or Rb protein. In our experiments, a satisfactory restriction of cell proliferation by CVT-313 was observed in aortic smooth muscle cells derived from newborn rats, whereas the cells from adult animals were apparently less sensitive to this type of regulation (Filová *et al*. 2003, 2004a,b).

Conclusions

Artificial materials are increasingly used for construction of replacements of damaged tissues and organ. Cells can sense the physical properties and

chemical composition of these materials and regulate their behavior accordingly. There are at least three types of biomaterials: 1) inert materials not allowing adhesion of cells, including thrombocytes or cells of the immune system, 2) biomaterials allowing adsorption of entire extracellular matrix molecules which contain binding sites for adhesion receptors on cells, e.g. integrins or proteoglycan-based receptors, and 3) advanced biospecific and biomimetic materials consisting of a bioinert background endowed with ligands for adhesion receptors (usually short amino acid sequences, such as Arg-Gly-Asp, or carbohydrates) and/or functional parts of hormones, enzymes or growth factors. These bioactive molecules can be attached in defined spectrum, concentration and spatial distribution in order to control adhesion, growth, differentiation, functioning and viability of cells. In addition, advanced biomaterials could be used for site- or cell-specific drug or gene delivery.

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