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Tissue engineering and autologous transplant formation: practical approaches with resorbable biomaterials and new cell culture techniques

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The engineering of living tissues *in vivo* requires new concepts in cell culture technology. In contrast to conventional cell cultures, the development of tissues depends on a three-dimensional arrangement of cells and the formation or synthesis of an appropriate extracellular matrix. Special emphasis is given to the major role of the extracellular matrix and cell differentiation in an artificial tissue. New technical approaches of *in vitro* tissue engineering are compared to the natural development of tissues *in vivo*. Current methods using resorbable biomaterials, tissue encapsulation and perfusion culture are discussed. Major consideration is given to scaffold structures of biomaterials that define a three-dimensional shape of a tissue or guide matrix formation. The different goals of tissue engineering such as *in vitro* models and transplant production are taken into account in the described techniques. Practical concepts comprising cell multiplication and differentiation in subsequent steps for future clinical applications are outlined.

Keywords: Tissue engineering, cell transplantation, perfusion culture, extracellular matrix, cartilage

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In reconstructive surgery, there is an increasing demand for replacement material to fill defects especially in bone and cartilage. Usually, implants made of synthetic polymers, ceramics and metals, as well as transplants made of tissue derivatives such as collagen or fixed cartilage allografts, are used for tissue repair[1,2]. However, to date none of these materials can provide the quality of the original tissue. These artificial materials often fracture, induce immunological responses and are difficult to anchor[1–3]. In contrast to vital tissues they lack any process of regeneration. Thus, the challenging goal of tissue repair is the autologous growth or regeneration of the original tissue without remnants of foreign biomaterials. Recent investigations have opened new possibilities to construct vital autologous tissues *in vitro* using appropriate biomaterials to guide the tissue development[4–6]. An engineered autologous and vital transplant clearly would be the superior replacement material (*Figure 1*). Tiny biopsies from the patient would provide the initial source of cells and immune responses against a re-engineered tissue could be kept at a minimum when all steps are carried out in an autologous system. However, first the number of cells from a biopsy source has to be increased substantially and for making a tissue one has to conceive that a native tissue is far more complex than just a mass of cells glued together. The complexity of the extracellular matrix is striking and the signals that regulate its development *in vivo* are still only poorly understood[7].

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FORMATION AND IMPORTANCE OF EXTRACELLULAR MATRIX

Almost all of the cells in vertebrates are in contact with a very complex network of extracellular macromolecules which can be considered as the actual difference between isolated cells and a tissue (*Table 1*). This matrix is particularly plentiful in connective tissues where cells are only sparsely distributed within it. In cartilage for example, the matrix occupies up to 98% of the total volume of the tissue[8,9]. In epithelial tissues the extracellular matrix is represented by the basement

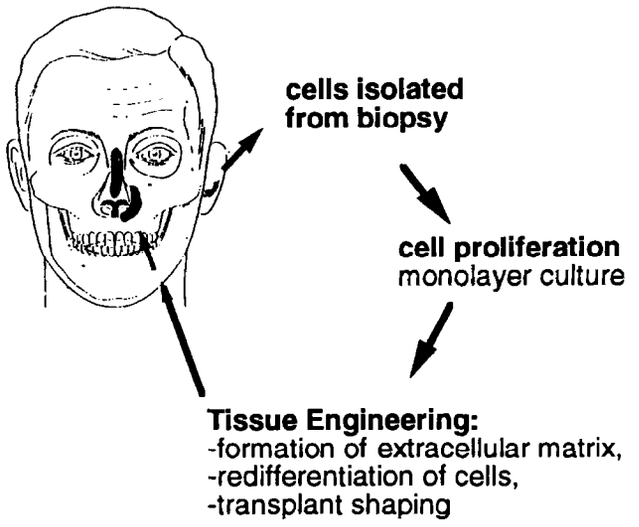

Figure 1 The concept of autologous transplant formation with tissue engineering. In the example depicted, tissue engineering is used to grow a piece of cartilage from cells of the ear to repair a defect of the nose. Isolated cells from the patient are loaded into an artificial resorbable matrix and cultured for maturation to a new tissue *in vitro* before they are transplanted into the same individual.

Table 1 Tissue engineering primarily requires the re-formation of an extracellular matrix. Various types of collagens and proteoglycans are the predominant components that have to assemble properly to form the matrix of cartilage

Cells $\xrightarrow{\text{extracellular matrix}}$ Tissue

Main components	Structure	Main function
Collagens	Mesh of fibrils	Tensile strength
Proteoglycans	'Ground-substance' hydrogel	Pressure resistance molecular sieve

membrane which underlies tightly connected sheets of cells. The main components of the extracellular matrix are collagens and proteoglycans. In connective tissues such as cartilage the collagens form fibrils which are embedded in a hydrogel consisting of extremely large aggregates of proteoglycans and hyaluronic acid with a molecular weight above 100×10^6 and the size of a bacterium (1 μm). Secreted collagens have a molecular weight of about 300×10^3. Proteoglycans, such as aggrecan in cartilage with a molecular weight of about 3×10^6, are secreted as whole large molecules consisting of a core protein and up to hundreds of glycosaminoglycans covalently bound to it[8]. Glycosaminoglycans are extremely hydrophilic and therefore can bind large amounts of water to create the turgor of a tissue. With respect to their molecular structure, proteoglycans are extremely complex and heterogenic and little is known about their possible functions. It is assumed that they can form gels with a varying pore size and charge density[9]. Recently, proteoglycans have been shown to be involved in even more active processes such as the modulation of growth factor activities[10]. For example, the glycosaminoglycan heparan sulfate is able to bind fibroblast growth factor (FGF) from where it can be released with a much higher affinity to its cell surface receptor[11]. As another example, the transforming growth factor TGF-β is inactivated by the proteoglycan decorin.

In contrast to proteoglycans, which can be imagined as the ground-substance, collagens primarily provide stress resistance to tissues which is particularly impressive in tendons (*Table 1*). Collagens are characterized by their typical triple-helical domains. After secretion from the cells, they lose their solubility by cleavage of extension peptides and self-assembly is driven by entropy[12] to form staggered fibrils which are strengthened by additional enzymatically formed interchain cross-links. About 90% of the human collagen is of type I found mainly in skin and bones, whereas collagen type II is the major constituent of cartilage. Collagen fibrils are usually heterotypic fibrils which means that they do not consist of only one type of collagen. In addition, non-fibrillar collagens, like type IX in cartilage, are associated to the surface of the fibrils where they are probably important in regulating the diameter of the fibrils[13].

As mentioned for collagen fibrils, self-assembly and enzymatic link processes are important in the extracellular space to form matrix structures. For fibril formation, however, the tissue cells even have the ability to form deep infoldings in the plasma membrane which partially compartmentalize the pericellular space[14].

Further understanding of the single components, structures and developmental control of the extracellular matrix will provide new insights and thereby novel tools to engineer this extracellular matrix between cells *in vitro*.

DEDIFFERENTIATION AND REDIFFERENTIATION OF CELLS

The isolation of mammalian cells and their culture in monolayer systems is a basic procedure for investigations in cell biology, intercellular molecular signals and the pathogenesis of many diseases. Furthermore, the effects of drugs and other substances on the human body are mainly evaluated by their influence on cultured cells. However, isolated cells can only simulate a small part of the body's tremendous complexity. A major problem of human cells in culture is the phenomenon of dedifferentiation. Normally, cells are isolated from their tissue-specific extracellular matrix. After suspension in growth medium they adhere at the bottom of cell culture plastic wares. Cells can grow and proliferate in these culture dishes to form a confluent cell layer. However, the cells frequently lose their morphology as well as their biochemical and functional properties[15,16]. Such dedifferentiated cells behave completely differently compared to the cells in their original tissue environment. A good example of this phenomenon is provided by cartilage cells (*Figure 2*)[17]. After a few days in monolayer culture, they begin to change their appearance to a fibroblast-like morphology[18]. The typical formation of chondrons and pericellular matrix is not observed in monolayer culture. Biochemical investigations reveal a switch of collagen synthesis.

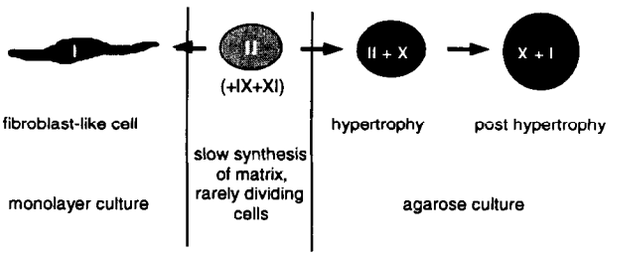

Figure 2 The phenotype of human cells is usually unstable in culture. Here, the changes of chondrocytes regarding cell shape and type of collagen synthesis are shown[18]. I, II, IX, X and XI refer to collagen types.

Thus, instead of the cartilage-typic collagen type II, cultured chondrocytes mainly synthesize collagen type I which is absent from normal cartilage[19]. The reason for the process of redifferentiation may be caused by cell adhesion to an unsuitable substrate, an unstable nutrient supply, the two-dimensional cell growth and most importantly the lack of an appropriate extracellular matrix.

If only a small biopsy is available as a donor source for tissue engineering, the number of cells needs to be increased to a great extent to generate tissues with sufficient sizes. So far, chondrocytes from adults can only be expanded effectively when cultured as a monolayer. However, they do not seem to divide before they alter their shape to fibroblast-like cells. Hence, during the phase of proliferation, the cells lose their overt phenotype and they later have to be redifferentiated for the artificial tissue formation. Especially for chondrocytes, a great number of investigations focussed on characterizing the so-called dedifferentiated cell. The dedifferentiated chondrocyte appears to be very similar to a fibroblast[20]. However, in contrast to a fibroblast it can still redifferentiate to a normal chondrocyte when cultured in an agarose gel[21]. Therefore, elongated chondrocytes in monolayer culture are probably still determined by a cell memory system to form cartilage in a favourable environment. It has not been investigated sufficiently whether this cell memory gradually ceases with the number of cell doublings in monolayer culture, but it is at least increasingly difficult to redifferentiate them in three-dimensional culture[22]. A deeper understanding of the relevant intercellular signals and the genetic regulation that controls cell differentiation and tissue development could provide new tools to induce and control redifferentiation processes for tissue engineering. One interesting example to mention here is the *myoD* gene. When its coding sequence is transfected into a skin fibroblast, this fibroblast is triggered to differentiate into a muscle cell[23]. In summary, tissue engineering and tissue maturation *in vitro* has to focus on conditions that control and induce the cells to appropriate phenotype expression.

THREE-DIMENSIONAL ARRANGEMENT OF CELLS *IN VITRO*

Tissue engineering has grown to a field of intense research in recent years. It concentrates on two major goals: (i) *in vitro* construction of transplantable vital tissues; and (ii) the development of *in vitro* models that are superior to conventional cell cultures mainly because normally connective tissue cell cultures lack an adequate extracellular matrix. Several investigators successfully used different types of hydrogel cultures to induce certain tissue-typic developments of cell cultures[24]. Hydrogels appear to be suitable tools for tissue engineering usually based on certain structural and physicochemical similarities to the natural extracellular matrix[25]. Natural matrix components were also active morphogenetically for tissue regeneration[26].

The engineering of tissues requires appropriate carriers that allow a three-dimensional distribution of cells. Suspending cells in gels like collagen or agarose are often applied procedures[21,27,28], useful to study phenotypical alterations. More recent approaches had the intention to construct transplantable tissue structures with cell carriers that degrade once the tissue has matured *in vivo*.

Chondrocytes were seeded on resorbable sutures[4]. Fibroblasts were seeded on resorbable meshes to create a skin replacement[5]. A porous matrix of poly(lactic acid) was used to reconstruct articular cartilage defects[29,30] and non-woven PLGA fleeces were used to form urothelial structures as well as cartilage tissues[6,31,32]. These more solid cell support alternatives to gel cultures provide improved mechanical characteristics with sufficient shape stability for the transplantation. There are several other factors that are important to an appropriate carrier structure (*Table 2*). One is the internal surface area per volume that allows loaded cells to anchor. High internal surface areas are given with porous structures as well as with non-woven fleeces of fibres; however, porous polymers tend to have more weight per volume, are less flexible and less elastic than structures of fibres. A low weight per volume has the advantage that only a small amount of biomaterial degradation products affects the tissue. Therefore, a fleece structure meets best the requirements for a cell carrier. However, a rather difficult problem with solid polymer scaffolds compared to gel cultures is to achieve a sufficient homogeneous distribution of cells. In the case of a non-woven textile carrier, a homogeneous distribution of its fibres is crucial[22]. In loose areas of a fleece hardly any cells might attach and too densely packed fibres get clotted with cell aggregates. Distances of about 50–100 μm between fibres are favourable. Cell distribution and attachment in three dimensions are mainly hampered by the force of gravity. The scaffolds have to be loaded from the top, and the cells have to be fished out of suspension

Table 2 Aspects favouring resorbable fleece structures for the development of transplantable tissues

Structural requirements for a suitable bioresorbable cell carrier:		
Stability of shape	→ high	→ fleece
Internal surface	→ high	
Amount of biomaterial	→ low	
Homogeneity of structure	→ high	

like molecules in a column of affinity chromatography. Thereafter, rapid attachment to the polymer surface is important. PLGA surfaces coated with poly-L-lysine were shown to be very effective to achieve a rapid attachment[31]. In addition, poly-L-lysine was recently found to stabilize the extracellular matrix in cartilage and promote chondrogenesis. It is assumed that the polycationic character of polylysine binds the polyanionic glycosaminoglycans of the matrix[33].

CONDITIONS AND TECHNIQUES FOR CULTURE OF ARTIFICIAL TISSUES

In native tissue, matrix molecules, like aggrecan in cartilage, are present in the tissue space and probably provide a natural scaffold for other newly synthesized structural components or enzymes of the extracellular matrix. In contrast, the initial secretion of matrix molecules into rather empty intercellular spaces as given with some three-dimensional cell arrangements is very different to the situation normally found *in vivo*. When cartilage cells develop from mesenchymal precursors, secreted matrix molecules can always immobilize into an already existing mesh of extracellular structures. If there are empty spaces between cells in culture, synthesized matrix molecules diffuse into the medium. However, large amounts of matrix components can be accumulated around cells when they are suspended in agarose or cultured as aggregates[29,34]. The gel retains the matrix molecules in the pericellular space. Therefore, the *in vitro* formation of a tissue from isolated cells requires two types of temporary scaffolds. One that defines the cell arrangement, mechanical stability and shape of tissue, and a second that helps to guide and anchor the components of a developing matrix. In a simple agarose culture both functional types of scaffolds are identical.

Compared to monolayer cultures, three-dimensional cultures in tissue engineering lead to rather high cell densities similar to the native tissue. Human articular cartilage normally contains about 15×10^6 cells ml^{-1} (Ref. 35). Such cell densities require very frequent changes of medium to maintain stable nutrient conditions. Using perfusion culture chambers as a convenient alternative provided a stable pH and constant concentrations of nutrients like glucose[22] (*Figure 3*). Culture handling was much easier and the risk of contamination was greatly reduced. This is especially important for cultures lasting for several weeks or months. These culture containers[36] are completely closed and a CO_2 incubator is not needed as long as the medium is buffered with HEPES (15 mM)[37].

The approach of tissue engineering mainly discussed here is based on a fusion of a cell suspension with a solid three-dimensional carrier such as a non-woven fleece structure made of poly(lactic-*co*-glycolic acid) and polydioxanon ('Ethisorb') (*Figure 4*). Such constructs of polymer fleeces and chondrocytes in perfusion culture were successfully applied to form tissues with cartilage-type characteristics[31]. Further, they were successfully implanted into nude mice

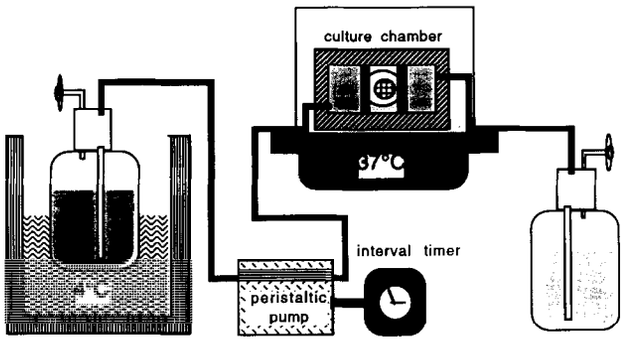

Figure 3 A constant flow of fresh culture medium provides stabilized culture conditions and reduces risks of contamination in long-term cultures.

where the resorbable carrier structure of PLGA eventually fully degraded within several weeks while the cartilage tissue remained stable[22].

Usually cultured cells like chondrocytes or fibroblasts synthesize extracellular matrix molecules into their environment. In regular culture flasks, most matrix components like collagens or proteoglycans diffuse into the culture medium and do not aggregate to an appropriate extracellular matrix[38]. Also a loose construct of cells seeded on a fleece could not sufficiently retain newly formed matrix components within the tissue[22]. However, encapsulation of the constructed tissues with semipermeable structures accumulates the matrix components within the tissue[31] (*Figure 5*). A simple material for that purpose is a solid agarose gel that cannot be penetrated by very large molecules like aggregan[39]. In principle, any biocompatible semipermeable material that allows sufficient diffusion of nutrients could be used to

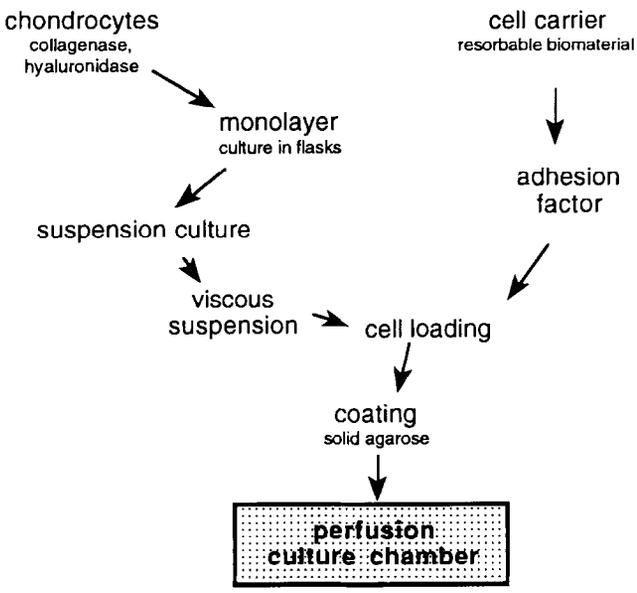

Figure 4 Preparation of cell–polymer tissues for perfusion culture. The adhesion factor is lyophilized onto the biomaterial. Then, the polymer fleeces are overlayed with slightly viscous cell suspensions. The cell–polymer construct is encapsulated in a pre-chilled solidifying gel (4% agarose). Constructs are cultured in perfusion culture (e.g. 2 weeks).

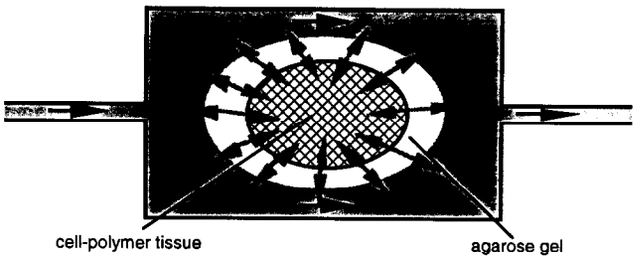

Figure 5 The engineered chondrocyte tissue is encapsulated by a semipermeable material like agarose to accumulate large extracellular molecules like aggrecan. Nutrients are only supplied via diffusion as is the case *in vivo*.

encapsulate a cellular tissue that is meant to develop an extracellular matrix *in vivo*.

TISSUE ENGINEERING AND TRANSPLANT PREPARATION: PERSPECTIVES FOR SURGERY

In recent years, novel structures of biomaterials offered alternative approaches for tissue engineering and *in vitro* transplant formation. Materials like resorbable polymers are expected to provide means to form pieces of tissues that can be conveniently handled by the surgeon and shaped as needed to accurately fill specific defects. These more mechanical aspects are important even though it has been recently shown that deep cartilage defects in joints can be repaired successfully when filled with a suspension of autologous chondrocytes and covered with a flap of periostium[40]. For corrections of the nose or the repair of large surface areas of skin or joint cartilage such suspensions of cells might not be sufficient; instead, ready-made shapeable tissue constructs seem more appropriate. For a successful application of such tissues, the development of biomaterials with suitable mechanical characteristics and degradation behaviour is very important. To minimize a loss of cells it might be of advantage to preform the biomaterial matrix to a needed shape.

The engineering of tissues from an autologous cell source requires sufficient amplification of the cell number. It has been shown that chondrocytes isolated from explants can be expanded easily in monolayer once the cells adhere to a polystyrene culture dish and stretch to fibroblast-like cells[22]. The time which is needed to prepare a sufficient amount of cells may vary from 2 weeks up to 2 months depending on the cell content of the biopsy, the size of the needed transplant and the proliferative capacity of the cell source. In addition to the time for cell expansion, the actual tissue engineering probably involves one or several weeks to condition the tissue during a period of redifferentiation *in vitro* possibly also supported by appropriate growth factors like bone morphogenetic proteins[41]. During months of *in vitro* tissue preparations the prevention of any bacterial or fungal contamination would be absolutely crucial. Besides sterile handling, only autologous serum from the patient should be used throughout the time of *in vitro* culture to exclude any viral infections or immune responses to foreign antigens.

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