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Tissue Engineering as a Tool in Reconstructive Surgery of Skeletal Tissues

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Tissue engineering is a relatively new branch of medicine which has been enthusiastically announced as a new tool in reconstructive surgery. Nine years ago it was being compared to genetic engineering, as an extremely promising area in medical treatment. Bone reconstruction was indicated as potentially one of the first major application of tissue engineering, as published in Science in 2000.
In the present review, tissue engineering is revealed as an alternative to currently used methods of skeletal tissue reconstruction. Issues, which are critical for preparation of tissue engineering products, are described. Among them, the potential sources and problems related to maintaining of cells which can be used in the reconstruction of the skeletal system are being discussed as well as the key expectations toward biomaterials which are the basis of scaffolds for cell and tissue transplantation.
The problem of the actual application of tissue engineering to healthcare, which seems not to meet expectations, is discussed. The perspectives for further development of tissue engineering both as a scientific field and as a medical treatment are considered.

Key words: *tissue engineering, scaffold, cell culture, bone reconstruction, cartilage, stem cells, growth factors*

1. Introduction

According to the most classical definition given by Langer and Vacanti, tissue engineering is defined as "... an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function...", [37]. It seems to be a promising and perspective tool in reconstructive surgery of tissues.

2. Tissue Engineering as an Alternative to Currently Used Methods in Skeletal Tissue Reconstruction

Tissue engineering is expected to be an alternative to autologous or allogenic transplantation or implantation of artificial, engineering materials. Each of this currently used methods of tissue/organs reconstruction has its advantages but also the important limitations.

Use of autografts is considered by surgeons as a gold standard, as the own biological material is the best basis for the regeneration of the tissue. However, the amount of the tissue which can be obtained for autotransplantation is strictly limited, the surgery is more invasive and it involves the injury of the donor site. This procedure is not recommended for the young patients, in the growth period. Also it cannot be applied if the quality of patient's own bone is not satisfactory, like in the case of osteoporosis. Besides, side effects may occur, e.g. donor-site morbidity due to infection and haematoma [65].

These problems can be omitted by using allografts. In the case of skeletal tissue, so called biostatic implants—nonviable allografts usually prepared in tissue banks are used. They are prepared by means of highly specialised procedures including preservation and sterilisation [11]. Also, the approval of each particular allograft is preceded by a verification whether the donor fulfils the precisely formulated serological criteria. Anyway, the risk of transmission of infection or disease by a transplantation of the allogenic material cannot be completely excluded. Especially for biostatic implants, where cadaver tissues are usually used, what eliminates the possibility of repetition of serological tests. Besides, although the amount of the available tissue may be higher than in the case of autologous transplantation, still it is not unlimited.

The solution that is free from such limitations is using of synthetic biomaterials. There is a very big and still growing offer of artificial implantable materials—metals, bioceramics, polymers and composites. On one hand, the fact that they are not biological is the advantage; on the other hand, it is the main drawback—artificial matter will not be recognised as the own material and will never be as excellent as the natural tissue, with its ingenious structure and abilities of rebuilding. Anyway, the continuous development can be observed in the field of biomaterials. Hench specified three generations of implantable materials [22]. First, it was thought, that the ideal implantable material should be inert in order to remain "unnoticed" by host tissues. However, in the case of materials which are intended to cooperate

with load-bearing bone such approach is not acceptable. Inert materials are usually not well anchored in tissues and unwanted implant movement and loosening appears. So, the next generation, the so called active biomaterials appeared, designed in order to achieve various forms of bonding with host tissues. The third, and the most sophisticated generation is based on the idea of providing an active tool for tissue self-regeneration rather than the end-point filler. Currently, so called biomimetic materials—as similar to the natural tissues as possible are of a great interest and establish the direction for the further development of implantable materials, [10, 42, 61, 86].

Taking together all advantages and limitations of currently used methods of skeletal tissue reconstruction discussed above, it can be realised that the concept of tissue engineering, which involves the use of the technologies of molecular and cell biology, combined with those of advanced materials science and processing, is a chance for gathering all advantages and, in the meantime avoiding the negative aspects of reconstructive surgery of skeletal tissue.

"*The scientist describes what is, the engineer creates what never was*" (Theodore von Kármán). This is remarkable, that the tissue engineering is being compared do the other medical field with the word "engineering" in its name, i.e. genetic engineering. "...Tissue engineering", Mc Carthy quoted Langer in 1996, "is at the stage that genetic engineering was at in 1981—no products approved, but some on the horizon. It is a potentially explosive area..." [46]. The concept of tissue engineering product (TEP) for skeletal tissue reconstruction is schematically represented in Fig. 1. Cells, isolated from small pieces of host bone are expanded and seeded on a 3-dimensional scaffold made of implantable natural or, more often artificial implantable material and such hybrid construction is implanted as a partly autologous graft to the host tissue.

Issues critical for manufacturing and application of TEP, are:

- scaffolds,
  - type of material—big offer, many questions,
  - design—looking for an optimal 3d structure,
  - delivery of biologically active molecules for implant/host tissue connection improvement (e.g. growth factors),
- cells,
  - source,
  - cell culture systems,

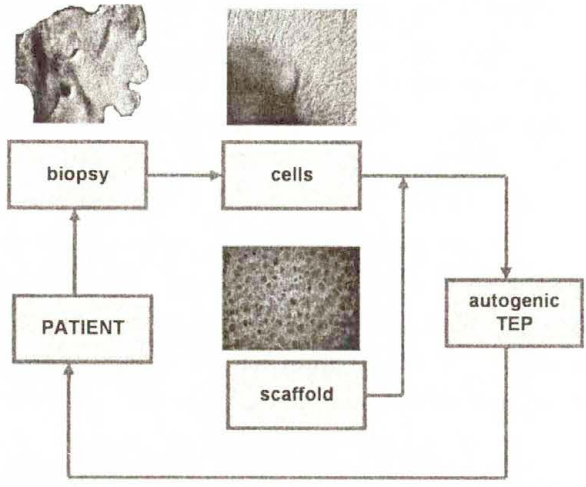

FIGURE 1. Schematic representation of the general concept of tissue engineering product (The pictures of cells originate from our laboratory, the picture of a scaffold originates from the Institute of Glass and Ceramics, Warsaw)

  - * tissue production in vitro,
  - * need for new methods of cell/tissue quantitative examination,
  - * necessity of avoiding factors of animal origin,
- cells and scaffolds put together,
  - problem of a "double" biocompatibility,
  - distribution of cells in scaffolds—bioreactors,
- way from laboratory towards the clinic,
  - regulations,
  - preclinical studies—lack of satisfactory experimental systems,
  - conservative clinicians vs application of very recent findings.

These problems will be now briefly discussed.

3. Scaffolds for Tissue Engineering Product

The importance of a scaffold in working on tissue engineering product can be reflected by a growing interest in this area in a basic research (illustrated in Fig. 2). The role of the scaffold may be expressed in the following three main points:

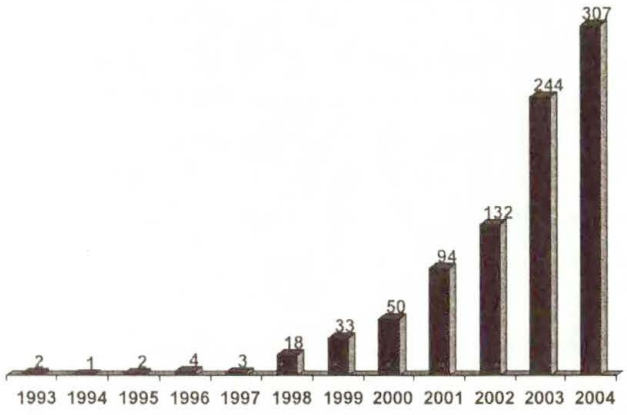

FIGURE 2. Numbers of articles found in the MEDLINE database under the entry: "scaffold" and "tissue engineering"

- provides support for cells to proliferate and differentiate/maintain their differentiated functions *in vitro*,
- plays the role of graft with the proper architecture after implantation *in vivo*,
- allows tissue remodelling *in vivo* (including the appropriate vascularisation).

In fact, almost all commonly used implantable materials can be taken into account as potential scaffolds for tissue engineering. Among them are metals: titanium and its alloys, Vitalium® and other cobalt and chromium-based alloys and stainless steel; bioceramics: inert—like alumina, and active or resorbable—like hydroxyapatite, and others ceramics based on calcium phosphates, including biphasic and multiphase ceramics, bioglasses of various chemical composition and manufacturing technology, glass-ionomers (mainly used in dental applications), carbon-based ceramics and others. However, the main materials of interest as candidate scaffolds are polymers. The title of the key-note lecture given by professor Feijen at the Sixth World Biomaterials Congress (Hawaii, 2000) was: "Was von Frankenstein a Polymer Chemist?" and it reflects very well the tendency in using synthetic materials in reconstructive surgery in humans. The biopolymeric offer is very wide. It includes nonresorbable polymers, like: silicone rubber, polytetrafluoroethylene (PTFE), polyethelene terephthalate (Dacron), polyurethanes (some polyurethanes may be resorbable), poly(hydroxyethyl methacrylate) (PHEMA),

polyvinyl alcohol (PVA Ivalon), ultra-high-molecular-weight polyethylene (UHMWP), polymethylmethacrylate (PMMA), hydrogels (e.g. PVP), and resorbable ones, eg.: polyglicolide (PGA), polylactide (PLA), poly(lactide-co-glycolide, PGLA), poly(L-lactide-co-caprolactone, L-PLCL), poly(glycolide-co-trimethylene carbonate) (PGTMC)—which are approved by FDA (Federal Drug Administration) [28]. Also, natural polymers are used, based on collagen sponge, hialuronic acid and other gel-like polymers of extracellular matrix origin. A special issue of *Biomaterials* related to polymeric scaffolds was edited by Mikos [48].

The most close to the ideal solution are bioresorbable materials which serve as a scaffold for cells/tissue in vitro and then gradually disappear, while being replaced by the host tissue, which is expected to regenerate on the basis of the implant. The perfect endpoint is thus the completely regenerated host tissue without any residuals of artificial material. This concept is illustrated in Fig. 3, and discussed in details by Hutmacher [27].

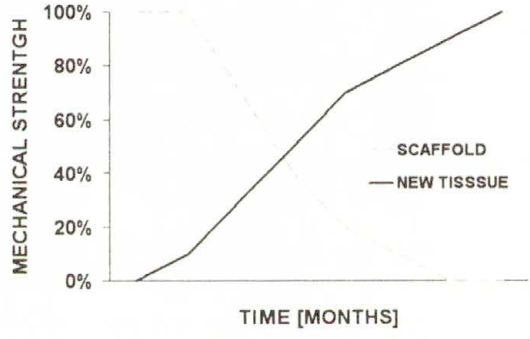

FIGURE 3. Schematic representation of the idea of gradual disappearing of the artificial scaffold while replacing by the host tissue which is expected to regenerate on the basis of the implant in vivo

Not only the chemical nature of a material which serves as a support for cell transplantation is crucial, but also a scaffold designing is of critical importance. In the case of bone substitutes, it is usually assumed that the optimal pore size which allows the proper bone organisation and remodelling after implantation in vivo, is between 200 μm and 500 μm. Thus, scaffold should have open porosity of 80–90% with appropriate pore size. Another important element of the structure is the interconnection of the pores, which is necessary for the organisation of blood vessel network in vivo. There are different technologies applied in order to achieve the proper scaffold architecture. The

most popular seems to be foaming, and/or polymerisation in the presence of salt crystals, which are added at the stage of polymer cross-linking and removed by washing at the end of the procedure [63]. Also, fiber bonding is used as well as sophisticated 3D printing [27, 47]. In the case of ceramics, one of the most popular methods is based on sintering of non-hardened ceramics embedded on an appropriate 3D structure made of polymer. Since the sintering temperature is much higher than the temperature of polymer evaporation, the final product consists solely of ceramics, and has the structure of the initial polymeric scaffold. Metals are usually used in a form of a network [6, 38, 50, 51, 68, 74]. Still basic reports on experiments looking for the most proper scaffold design are being published and both the description of the most appropriate scaffold architecture and technological possibilities to achieve it are under investigation [7, 23, 26, 64, 72, 78, 82]. Expectations toward scaffolds for chondrocytes are different, due to the completely different structure of cartilage—tissue with small number of cells nourished by diffusion where vascularisation should be avoided. The main role of the scaffold in this case is to keep cells in suspension, since chondrocytes spread on flat surfaces loose their phenotype. This dedifferentiation process is irreversible, so that the stage of culture when chondrocytes are spread is used for cell number expanding and this phase should be followed by moving cells to the 3D suspension in order to achieve their re-differentiation and thus make them ready to organise cartilage after implantation. Such sequence is applied in the procedure used in clinical reconstruction of joint cartilage when patient own cartilage is a source of cells which are therefore expanded in a culture and injected back to the joint. This procedure is approved and commercially available [2, 5, 57]. It seems however that seeding of cells in scaffold would better keep the sufficient number of cells at the implantation site, so works on optimal scaffolds for chondrocytes are going on in many laboratories and are followed by clinical trials [9, 17, 18].

Another expectation toward scaffolds for cells is related to a growth factors (GFs) delivery. The role of growth factors in cell differentiation is well documented [54, 58, 71]. Scaffold as a storage depot for growth factors at the in vitro stage of TEP creation may be interesting if stem cells are used (discussed in some more details in the further part of the present chapter). However, since growth factors may be added directly to the culture medium in vitro, this function of a scaffold is even more important after implantation, when growth factors entrapped on a surface of an implant may play an

essential role in the recruitment, differentiation and maturation of stem cells present at the implantation site [12, 33, 56]. Since the influence of the support on the activity of proteins is not fully recognised, it remains difficult to identify a suitable carrier for proteins. Some enthusiastic reports on successful osteoinduction achieved by bone morphogenetic proteins (BMPs) implanted on hydroxyapatite, tricalcium phosphate, or titanium have been published [1, 29, 31, 34, 49, 60, 76]. Also, some data on the role of implant geometry in efficiency of scaffold-donated BMPs in bone formation in vivo are available [32, 69]. However, effective systems for growth factor delivery, which maintain their biological activity, are still not specified.

In our own in vitro observations it was found that the rhBMP-2-induced[1)] osteogenic potential of human bone derived cells (HBDCs) was blocked by the presence of several biomaterials [40]. Particularly, it was found that rhBMP-2-related stimulation of HBDCs as measured by enhanced alkaline phosphatase (ALP) activity appeared in the culture on the tissue culture treated polystyrene, routinely used culture dish. On the contrary, inhibitory effect of BMP-2 on ALP activity appeared in the same experiment in the HBDCs culture on the surface of hydroxyapatite, alumina, and titanium (Fig. 4). There are several reasons which might explain this unexpected result. Firstly, oxides present on the surface of biomaterials may involve in some way active domains of rhBMP-2 and in consequence this protein cannot be recognised as a ligand by the cells in the culture. Secondly, membrane receptors for

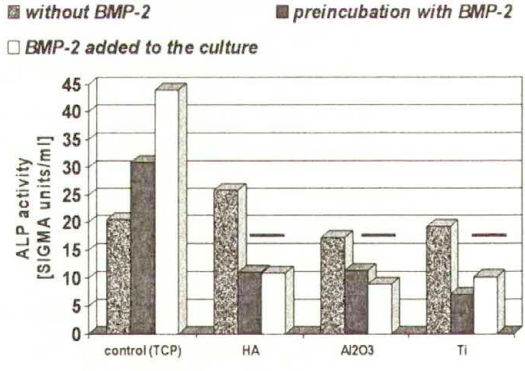

FIGURE 4. The influence of BMP-2 on the alkaline phosphatase activity in the culture of human bone derived cells on various supports, i.e.: hydroxyapatite, alumina, titanium and culture treated polystyrene (control). After [40].

[1)] rhBMP-2—recombinant human bone morphogenetic protein 2

rhBMP-2 present in the osteoblasts may become inactivated in contact with biomaterials via some unknown mechanism. The third option is connected with the possible activation of the BMP inhibitors, e.g. inorganic oxides might enhance the expression of noggin in cultured osteoblasts. Effectiveness of biomaterials in GFs-donor systems remains under intensive investigations. Another problem connected with using growth factors as biologically active agents in tissue engineering is that the efficacious (and cost-effective) cocktail for bone or cartilage induction and regeneration in clinical practice remains unknown. There are a lot of contributory results—the role of members of the TGF-β superfamily is well documented. Particularly, it is known that TGF-β and BMP-7 stimulate proteoglycan synthesis [75]. Bone morphogenetic proteins BMP-2 and BMP-7 are in clinical trials as bone healing promoters [8, 15, 21, 35, 55, 62, 79]. It seems that in the case of bone, demineralised bone matrix (DBM) is the attractive natural BMPs cocktail, optimal for individual when autogenic. Its osteogenic potential, known from the classic experiments of Marshall Urist was the key discovery of a bone inducing principle [70]. There are some new reports on the possibility of using DBM in tissue engineering [30, 45, 67, 87]. Our own studies confirmed the effectiveness of DBM as the stimulator of osteogenic capacity of HBDCs in culture [41]. In the applied experimental system DBM was added to the cell culture (Fig. 5) and it was found that the presence of autogenic DBM promotes HBDC prolifera-

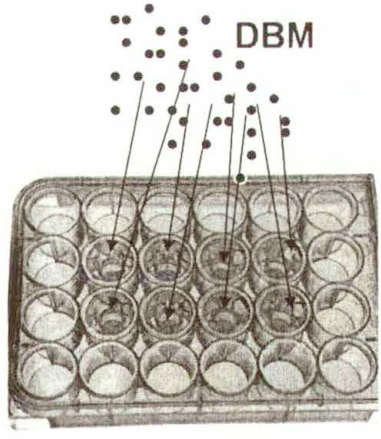

FIGURE 5. Experimental system in which demineralised bone matrix (DBM) particles are added to the culture of osteogenic cells in vitro; DBM particles are put on the milipore inserts and not directly on the cell layer

tion in culture while allogenic DBM is not effective as a proliferation-inducing agent in HBDC culture but it promotes HBDC maturation. Osteogenic capacity of DBM was still found after radiation sterilization (35 kGy) although not to the same extent as was observed without this sterilization procedure and this is a finding of a critical importance for practical purpose because including the allogenic DBM in the standard procedure of TEP preparation would require its sterilisation. It seems that if the DBM is available its use may be more effective and much cheaper than application of recombinant bone morphogenetic proteins. Anyway, looking for the optimal cocktail of recombinant growth factors is definitely up-to-date direction, interesting for both scientific and practical reasons.

4. Cells for Tissue Engineering

Potential sources of cells for transplantation in a tissue engineering system are gathered in Table 1. The advantages and disadvantages connected with using different types of cells are specified. There is no need to indicate one universal source of cells since in accordance with the main idea of tissue engineering, implants should be tailored individually—for each particular clinical case. As a consequence, tissue engineers should be able to obtain the adequate cell population from different sources depending on the biological material availability in each particular clinical case. For example, if arthroscopy is performed during diagnostic procedure, small pieces of tissue harvested during the procedure can be used. Otherwise, stem cells from different sources seem to be more promising material. In Table 1, only the most important sources of stem cells are listed. However our knowledge about the reservoirs of stem cells, including adult organisms is growing rapidly and is bringing revolutionary changes in our understanding of the regenerative potency of human tissues. Particularly, in the case of skeletal system, an extremely promising source of cells for transplantation seems to be adipose tissue. There are many reports confirming the potency of cells harvested from adipose tissue for differentiation toward osteoblasts, chondrocytes or myocytes [14, 19, 88]. The preliminary studies of our group confirmed that osteoblastic phenotype of stem cells isolated from adipose tissue can be achieved in the presence of biomaterials by relatively simple methods without the necessity of application of highly sophisticated and expensive procedures [36]. Our knowledge about the sources and the potency of stem cells is still limited, but there

TABLE 1. Potential sources of cells for transplantation in a tissue engineering system

cell source	advantages	disadvantages
cells isolated from the small pieces of tissue harvested from patient (usually at different stage of differentiation and maturation)	• source of native GFs in the optimal combination	• small proliferative potential • small amount of available tissue • not always satisfactory tissue quality
stem cells obtained from patient's own marrow or any other tissue of the body	• multipotential of cells • easier cell expansion (better proliferation as compared to mature cells)	• small amount of MSC in isolated population (only 1 MSC per 100 000 nucleated cells) • small amount of available tissue
stem cells obtained from the cord blood	• noninvasive harvesting	• doubts if non-haemopoietic stem cells are present in CB in sufficient quantity, • problem of CB ownership if used in allogenic system.
human embryonic stem cells	• great potential for proliferation and differentiation, • not evoking an immune response when implanted into patient	• ethical limitations (unacceptable for many people) • tumorogenecity (!) (undifferentiated ES cells must be completely removed before transplantation)

is no doubt, that using stem cells in tissue engineering is highly promising. Practical application is straight behind the basic research in this field. This concerns also other aspects of cell culture techniques. Cell culture is a commonly used tool in biological/medical scientific research. However, I agree with the opinion of T. Hardingham, who wrote: "... Cell and tissue culture until now has been a craft rather than a science and this will change..." [20]. Each step ahead in our understanding of cell behaviour in culture, especially

when cells are put in the direct contact with biomaterial—a candidate for scaffold, may become a milestone in TE.

Another factor associated with cell culture technique which should be mentioned here is a need to eliminate any substances of animal origin for the cells maintained in vitro, in order to avoid the risk of transmission of animal infections like, for example Kreutzfeld-Jacobs disease. Using specially prepared synthetic substitutes for substances of animal origin routinely used in culture makes cell culture procedure extremely expensive and this must change if TE is to become a widely used treatment.

5. Cells and Scaffolds Put Together

Each candidate for implantable material must be biocompatible. Biocompatibility is defined as "the ability of a material to perform with an appropriate host response in a specific application" [81]. In the case of TEP, biomaterial plays a double role, i.e. serves as a support for cells in a culture and works as an implant—tissue substitute after implantation in vivo. Thus, it must respond to the specific requirements connected with cell proliferation, differentiation and maturation in vitro, as well as provide the optimal condition for tissue ingrowth and rebuilding after implantation in vivo. The latter requirement is connected with the very difficult problem of revascularization. This is not the case in cartilage where cells are nourished via diffusion. But its the critical point in all other tissues, including bone. The prediction of events which appear after implantation in vivo, on the basis of in vitro observation is very difficult and doubtful. Experimental systems in animals are also not satisfactory due to differences in metabolism as well as tissue structure and dissimilar size relations. It should be also mentioned that, although some techniques of biocompatibility studies in vitro have been developed as an alternative to experimental implantation to animal tissues [39], the quantitative studies of cells cultured in 3D systems are very difficult and standard procedures do not give satisfactory results [52, 83]. There is still a lot to be done in the field of suitable experimental systems for reliable estimation of cell and tissue behaviour in sophisticated 3D systems for TEP research and development.

Another aspect of scaffold-cells relations is the problem of the effective cell distribution within 3D structure of the scaffold as well as the proper cell nutrition during the relatively long culture. This practically cannot be

achieved under static conditions. It was shown by many authors that applying of dynamic factors like fluid flow, perfusion, rotating of culture dish, etc. brings spectacular improvement in cell culture [3, 16, 24, 74, 85]. Better cell (HBDCs) expansion and distribution on alumina scaffolds with a porosity index 60 ppi (pore per inch) and with dimensions ($10 \times 10 \times 5$ mm) was confirmed also by our group [84]. The presence of cells, visualised by means of fluorescent staining of cell nuclei (Hoechst staining) deep inside the relatively big scaffold was confirmed when spinner flask technique was used (Fig. 6) and not under static conditions (not shown). Application of dynamic cell culture systems are especially useful when not only cells maintenance but also their expansion, maturation and finally, extracellular matrix (ECM) is wanted. The most convincing results which document the ability of tissue formation in vitro originate from experiments where the so-called *bioreactors* were applied. A very nice review of bioreactors design and function is given by L.E. Freed and G. Vunjak-Novakovic [12]. They use the term: engineering bioreactors, which is defined as "in vitro culture systems designed to perform at least one of the following four functions: establish spatially uniform cell distribution on 3D scaffolds, maintain desired concentrations of gases and nutrients in the culture medium, provide efficient mass transfer to the growing tissue, and expose developing tissues to physical stimuli". The growing interest in application of physical factors in cell culture systems can be observed and there are no doubts about the crucial role of bioreactors in the further development of TEPs.

FIGURE 6. Cells visualised by means of fluorescence technique (Hoechst staining) within dynamically seeded alumina scaffolds. After [84].

6. The Way from Laboratory toward the Clinic

The concept of tissue engineering has been enthusiastically accepted in early '90s [46]. In 2000 the title: "Tissue Engineers Build New Bone" appeared as news in Science [66]. However, the estimation of a tissue engineering products in USA discussed lately in the Journal Tissue Engineering seems rather disappointing [20, 43]. In 2002 there were only four TEPs approved by FDA (3 skin substitutes, 1 autogenic chondrocytes transplantation), the further ten products were in various stages of clinical trials and the next ten applications have been abandoned or failed to achieve product approval. The annual spending at the TEM market in USA raised from $246 million in 1995 to $610 million in 2000, but its value in 2002 decreased to $487 million. Also, the reduction in the capital valuation of publicly traded firms can be observed (it was $2.5 billion in 2000, and decreased to $0.3 billion in 2002; although it started from $1.9 billion in 1998) [43]. „It was the best of times, it was the worst of times"; so began Michael Lysaght's presentation at a recent TE conference with the quote from Charles Dickens' *Tale of Two Cities* [20]. Looking at the gap between expectations and the successful product portfolio in the field of tissue engineering product the statement can be postulated that the tissue engineering concept was ahead of the real possibilities in early '90s. Almost all elements of TEP creating are based on the newest scientific findings (Fig. 7):

- sophisticated cell culture systems with the lack of satisfactory methods of quantitative description,
- not fully recognised cell-biomaterial interactions,
- first trials with the application of growth factors when even their role is not definitely characterised and the donor systems have not been yet established.

In this respect, the delay in clinical application of TEPs is becoming understandable. What is more, one can be impressed by a very short distance between fundamental research and works on practical application of so many aspects which are involved in creation of TEPs. At the present stage it is certainly better reflected in experimental studies then in clinical application. However it should not be surprising if we take into account that not only very recent scientific findings are involved and expected to orchestrate together, but also that there is no validated regulations for approved using of tissue engineering product. Only recently in Europe the term: Tissue Engineering

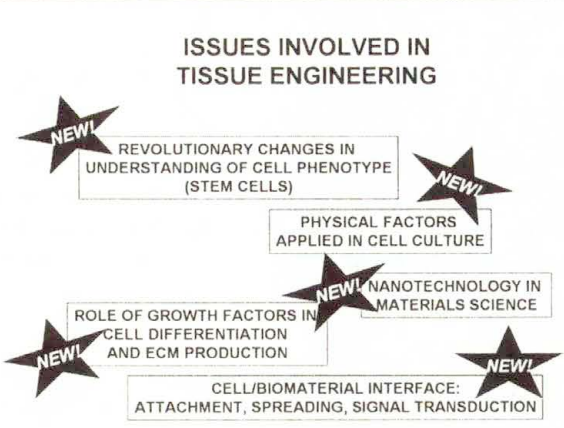

FIGURE 7. Chosen issues involved in tissue engineering, based on the newest and not fully recognized scientific findings

Product has been accepted and works on the appropriate regulations of the TEP preparation, maintaining and application are currently going on.

Going over the perspectives of tissue engineering, the very interesting article by David Williams can be recommended [80]. It addresses the underlying issues of benefit and risk in tissue engineering and the benefit-risk equation seems to confirm the promising perspective for this new concept for reconstructive surgery. It is reflected also in the M.J. Lysaght & A.L. Hazlehurst paper entitled: *Tissue Engineering: the End of the Beginning*, in which authors applied the quote from Winston Churchill: "This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

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