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Experimental Approach as Research Strategy for Cytocompatibility Assessment of New Materials for Bone Implants

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The study presents experimental design (direct and indirect experiments, assays, cell cultures) used by our group for the initial cytocompatibility assessment of new materials for bone implants. Some advantages and drawbacks of cell cultures (primary cultures and permanent cell lines; non-tumor and tumor cells) applied as model systems in the investigations are also discussed.

Key words: bone disease, bone implants, cytocompatibility, cell cultures

Introduction

The global incidence of bone diseases and conditions is rising steadily and is expected to double by 2020. The most affected are people over the age of 50 with reduced physical activity and obesity. The treatment of bone and joint degenerative and inflammatory processes, fractures, spinal pain, osteoporosis, scoliosis and other musculoskeletal problems requires the use of permanent, temporary or biodegradable materials/devices [4]. Suitable model systems and experimental strategies are needed for biocompatibility assessment of new materials for bone implants and estimation of their osteoinductive and osteoconductive properties [2, 9].

The aim of this study was to present the advantages and challenges of experimental strategies used by our group for cytocompatibility assessment of new materials for bone implants.

Materials and Methods

The cytocompatibility of 25 materials for bone implants from three different groups was investigated in our study: i) di- and tricalcium phosphate fine powders; ii) composite materials (Zn(13)-b-$Ca_3(PO)_4$ and hydrogels prepared from gelatin, xanthan gum and carrageenan; iii) cements. The cell cultures used as model systems are presented in **Table 1**.

Table 1. Cell cultures used as model systems in our investigations

Origin	Type (PCL / PCC; T /NT)	Established from (Name)
Rat	PCC, NT	Bone morrow
Mouse	PCC, NT	Bone marrow
	PCC, NT	Bone explants
	PCL, NT	Embryonal fibroblasts (BALB/c 3T3)
Bovine	PCL, NT	Kidney (MDBK)
Human	PCL, NT	LEP-3, MRC-5 – embryonic fibroblasts
	PCL, T	Osteosarcoma (Saos-2)
	PCL, T	Carcinoma of the uterine cervix (HeLa)
	PCL, T	Breast cancer (MCF-7)

PCL = permanent cell line; PCC = primary cell culture; T = tumor; NT = non-tumor

Primary cell cultures (PCC) were established as described earlier [5, 6]. Permanent cell lines (PCL) were obtained from the Cell Culture Collection of the Institute of Experimental Morphology, Pathology and Anthropology with Museum – Bulgarian Academy of Sciences. The cells were grown in Dulbecco's modified Eagle's medium (D-MEM) medium supplemented with 5-10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin and kept in a humidified incubator (Thermo Scientific, HEPA Class 100) at 37°C under 5% CO_2 in air. For routine passages the monolayer cell cultures were detached using a mixture of 0.05% trypsin and 0.02 % EDTA.

The effect of the compounds on cell viability and proliferation was studied in direct (the cells were seeded on the material) and indirect (the cells were grown in culture medium in which the material was pre-incubated) experiments using one or more of the following cytotoxicity assays - thiazolyl blue tetrazolium bromide (MTT) test, neutral red uptake assay, crystal violet staining (CVS), trypan blue dye exclusion technique (TBT). Double staining with acridine orange & propidium iodide (AO/PI) and/or hematoxylin & eosin (HE), single cell gel electrophoresis (Comet assay), light/fluorescent microscopy and scanned electron microscopy (SEM) were also performed [5, 6, 7].

Results and Discussion

The investigations were performed by:

Indirect experiments (IDE) – the "material extracts" were prepared by incubating the materials in culture medium (D-MEM) for various periods of time (for example 1h, 4h, 8h, 24h, 3 days, 6 days, 10 days). The parent "extracts" (100%) were then diluted in D-MEM to obtain a series of dilutions with concentrations of 75%, 50%, 25%, 10% and 1% which were

used further for cell viability/proliferation tests. IDE can be carried out by a wide range of cytotoxicity assay, cytological, (immuno) cytochemical and other methods.

Direct experiments (DE) – the cells were cultivated directly on the material's surface. DE provide valuable information about the interactions between the material and the cells, including the ability of the material to allow and support cell viability, adhesion, proliferation and differentiation. However, the application of some cytotoxicity assays in this approach may require additional modification depending on the physicochemical properties of the material examined.

Various cell cultures were used as model systems for cytocompatibility assessment of the materials. Our decision to include bone marrow cells (BMCs) in the experiments was not occasional because of at least three reasons: 1) the possible contact of BMCs with the material in the body; ii) BMCs are characterized by low or absent expression of P-glycoprotein which makes them extremely sensitive to the toxic effects of xenobiotics; iii) BMCs are mixture of various stem / progenitor cells including mesenchymal stem cells (MSCs) that are suitable for application in bone tissue engineering [1, 3, 13].

Primary cultures from bone explants of laboratory animals are attractive model systems because represent at least partially the regenerative process of damaged bone in laboratory conditions.

MCF-7 and HeLa cells were used in our investigations because they are established from two of the most common cancers in women – breast cancer and carcinoma of the uterine cervix. Both cancers are known to induce bone metastasis that may cause major morbidity including fractures [8, 11]. In addition, endocrine treatment of hormone-dependent breast cancer increases the risk for osteoporosis and fractures [10]. That is why data on the potential effect of bone substitutes on the proliferation of cancer cells are needed.

Some advantages and drawbacks of cell cultures used as model systems in our experiments are presented in **Table 2.**

One of the most important questions is what kind of cell cultures to be used as model systems in biocompatibility asssessment of new materials for bone implants. On one hand, osteoblasts, that are known to produce calcified bone matrix and contribute to bone formation and remodeling [14] as well as their progenitors seem to be the most appropriate model systems for this purpose. On the hand, human body is a highly coordinated complex system, where different organs/tissues have overlapped and interconnected functions. One can suggest that the local bone implant will more or less interact with the whole organism (through body fluids that "wash" the material, by switching on or off various signaling pathways).

In this relation various cell cultures must be taken into account when examining the potential cytotoxicity of bone implants including liver and kidney cells. The challenge is that we need information coming from normal cells. Most of the available human kidney and liver cell lines were obtained from cancer tissues and as a result exhibit different biology/behavior as compared to normal cells. Even permanent cell lines established from healthy liver or kidney can possess genetic/epigenetic alterations obtained during prolonged cultivation in laboratory conditions. Most of the available non-tumor kidney and liver PCL are of non-human origin.

Theoretically, a better option to solve this problem is to use primary cultures from healthy tissues. In reality, there is some difficulty in doing this at least because healthy human tissue is not easily accessible and the establishment of primary cell cultures has a low successful rate.

Primary cell cultures obtained from the same type "starting material" and following the same protocol can differ in some of their characteristics.

The mesencymal stem cells (MSCs) are among the most appropriate model systems for biocompatibility assessment of new materials for bone implants and bone tissue

Table 2. Advantages and drawbacks of cell cultures used as model systems for cytocompatibility assessment of new materials for bone implants

Cell culture	Advantages	Drawbacks
Fibroblasts	Important for bone tissue growth and regeneration Well known and readily accessible permanent cell lines are available	Terminally differentiated cells (their conversion into functional osteoblasts for the needs of bone tissue engineering may require special strategies)
Bone marrow cells	(Possible) contact with material in the body The stem/progenitor nature of these cells	Heterogeneous population The cells grow (with some exceptions such as MSCs) in suspension that requires adequate cytotoxicity assays (For example MTS test instead of MTT test)
Primary cultures from animal (rat, mouse) bone explants	*In vitr*o model of an *in vivo* bone regeneration	Heterogeneous population? Each cell culture has its individual own characteristics (low repeatability) The type/nature of the cells has to be determined
Osteosarcoma cells	Their (osteoblast/osteocyte) origin	The biology and behavior of cancer cells differ from those of normal osteoblasts/osteocytes.

engineering. The main advantages of MSCs include their high ability to proliferate, self-renew and differentiate into osteoblasts and chondroblasts; the possibility to be isolated from various embryonic and adult tissues; immunomodulating properties and the lack of teratogenic potential.

Co-cultures, including fibroblasts, endothelial cells and osteoblasts can be helpful for the evaluation of bone implant materials [13].

In conclusion, the successful development of improved new biomaterials for bone implants requires adequate experimental strategies and cell culture models to test their biocompatibility, osteoinductive and osteoconductive properties and to provide valuable predictive information.

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