ACTA FAC. MED. NAISS. UDK 619:616.71:615

Original article

ACTA FAC. MED. NAISS. 2005; 22 (3): 135-138

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CHARACTERISTICS OF GROWTH OF MICE BONE MARROW CULTURES IN PRESENCE OF BIOMATERIALS BASED ON HYDROXYAPATITE AND POLY-L-LACTIDE

SUMMARY

Biodegradable hard polymers have wide medical application, especially as amend bone defects in the form of implants, bone cement, or matrices for tissue engineering. Examination of biocompatibility includes the evaluation of effects of physiologic environment on material and material on biologic ambience. Therefore, it is very important to assess possible cytotoxicity of biomaterials, their influence on cell morphology, growth division in direct or indirect contact with the material.

The purpose of this study was to examine in vitro effects of different biomaterials on the growth and bone marrow cells of mice. Biomaterials HAp/PLLA with poly-L-lactide 50000 D(HP3) and 430000 D (HP2), titanium plates covered with HP2, and PLLA 200 000 D were examined. Bone marrow cell culture were maintened in MEM medium, 14 days on 33°C and 5% CO_2, and after that stained with Giemsa. The highest cell density was found in cell culture without biomaterial, and the lowest in HP2. The cell phenotype is much more diverse in control culture than we found in the presence of biomaterials. Adherent phenotype was seen with many phylopodic, lamelipodic and pseudopodic extensions. Also, there was a cell change as vacuolization, azurofilic granules and exocentric placed nucleus. Prominent phagocytosis of biomaterial HAp/PLLA which is comparable with the same process in nature bone demonstrates its good biocompatibility.

Key words: **biomaterials, cell growth, cell culture, bone marow**

INTRODUCTION

Biodegradable hard polymers have wide medical application, especially as amend bone defects in the form of implants, bone cement, or matrices for tissue engineering. Biofunctionality and biocompatibility stand for two basic characteristics which the material must satisfy. Examination of biocompatibility includes the evaluation of effects of physiologic environment on material and material on biologic ambience. Therefore, it is very important to assess possible cytotoxicity of biomaterials, their influence on cell morphology, growth division in direct or indirect contact with the material (1-4).

Because calcium hydroxyapatite (HAp) is a constituent of the vertebrate bone, artificially synthesized HAp is used for development of phosphate ceramics as potential material for implantation into the bone. Experimental and clinical studies have shown that HAp granules and powder can be successfully applied for reconstruction of the bone defects. It has been shown that HAp possesses excellent biocompatible properties, high osteoconductive activity, slow degradation and osteoinductive potential. Therewith, HAp possesses neither antigenicity nor cytotoxicity (5). Improvement of properties and

expanding of area application of HAp is achieved by synthesis of composite material based on HAp and poly-L-lactide (PLLA). Desired porosity, microstructure, compression strength, as well as bioresorbility of HAp/PLLA can be achieved by using PLLA with different molar weight ranging from 50 000 - 430 000g/mol, which ca be obtained by fluctuation of synthesis parameters (5). In this manner, it is possible to obtain nonporous and porous blocks for the reconstruction of bone defects without limitations of dimensions and forms. Molar weight of poly-L-lactide directly conditions the polymer bioresorbility lifetime, while directly influences osteogenesis time and vascular tissue proliferation. In our previous experiments, biocomposite HAp/PLLA is successfully used for filling bone defects (unpublished data). In histological assessment, we did not register any cellular reaction of the surrounding tissue, which accompanies the foreign body presence. The examination of biocomposite Hap/PLLA material conducted on mice showed a negative inflammatory reaction of peritoneum as well as proliferation of new bone tissue (5).

The purpose of this study was to examine *in vitro* effects of different biomaterials on growth and cell morphology of the bone marrow in mice.

MATERIALS AND METHODS

Mice: Because of necessary sterility, mice were washed in 70 vol% ethanol, and posterior extremities were cut off. The further complete procedure was performed in a sterile box. After cleaning the bones from musculature and conective tissue, the bone marrow was obtained by exhausting femur and tibia. Previously, the bones were opened at their ends and by a syringe filled with cold medium (MEM), the bone marrow was injected into the test-tube.

Cells: Bone marrow cells were washed three times for 10 minutes in a centriphuge (Hetich) on 150g and 4°C. In the end, the pelet was resuspended in 1 ml medium and cells density was counted in chemocytometer chamber. By adding of medium, the desired density of cells was obtained.

Culture: Two milion viable bone marrow cells were put in 2 ml of MEM medium (HIMEDIA) for culturing. Culturing medium contained 10% bovine fetal serum, 300 mg/l L-glutamine, 10-6 M dexametasone and 200 U/l penistrepto. Cultivation was performed in 24-well tissue culture plate (Sarstedt) on 33oC and 5% CO_2. Exchanging of medium was performed twice a week.

Biomaterials: *In vitro* analysis of bone marrow cell growth and morphology with presence of specimens (granules, scales, or plates, respectively) of examined biomaterials was performed in triplicate. We examined materials HP2 (HAp with 20mass% PLLA of 430000 D), HP3 (HAp with 20mass% PLLA of 50000 D), titanium plate coated with material HP2 and PLLA of 200000 D.

Analyses: After one and two weeks of cultivation the analysis of cell culture was done. Cell morphology was analyzed on phase-contrast inverted microscope (IM Olympus) at 150 time magnification after one week of cultivation. Density of cultivating cells near around biomaterials, after two weeks, served as a parameter for evaluation of cell growth *in vitro*. In this purpose, cultivation was disrupted and cells were fixed and stained (May-Grünwald-Giemsa). Density was expressed by average cell number in five fields on 40x10 magnifications. Cultures without biomaterials served as a control.

RESULTS

Among examined cultures the greatest cell density was found in the control culture. Material signed as HP3, titanium plates coated with same material and PLLA show the effect of slight suppression onto cell growth, while HP2 material reduces cell density more than 50%. (Table 1).

Table 1. Cell density in presence of tested biomaterial

	titanium plate	HP2	HP3	PLLA	control
Avv. ± SD	31.3± 11.3	16.4± 6.1	28.1± 9.8	23.7± 8.2	35.5± 13.6

Cell phenotype changes accompany changes in cell density. As a rule, the cultures growing in higher density are characterized by pleomorphic, adherent cell phenotype with numerous extensions of phyllopod, lamellipodia and pseudopod type.

Greater PLLA molecular mass within composite, or separately, had an inhibitory effect on the cell growth compared to PLLA with minor molecular mass. The same goes for the composite coating titanium plates.

DISCUSSION

Biodegradable materials are widely examined for biomedical, as well as common use. Understanding of degradation characteristics have crucial importance for the use of all types (6-11). Lactide polymers degrade very slowly by unusual hydrolytic mechanism (12). Namely, degradation inside of

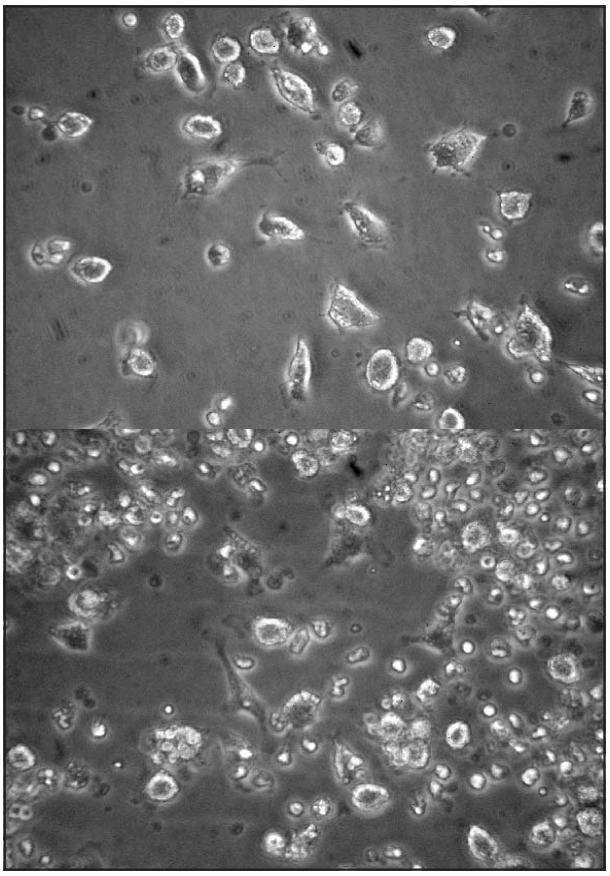

Figure 1. Bone marrow cells after two days of cultivation (Phase contrast on inverted microsope, mag. 400X)

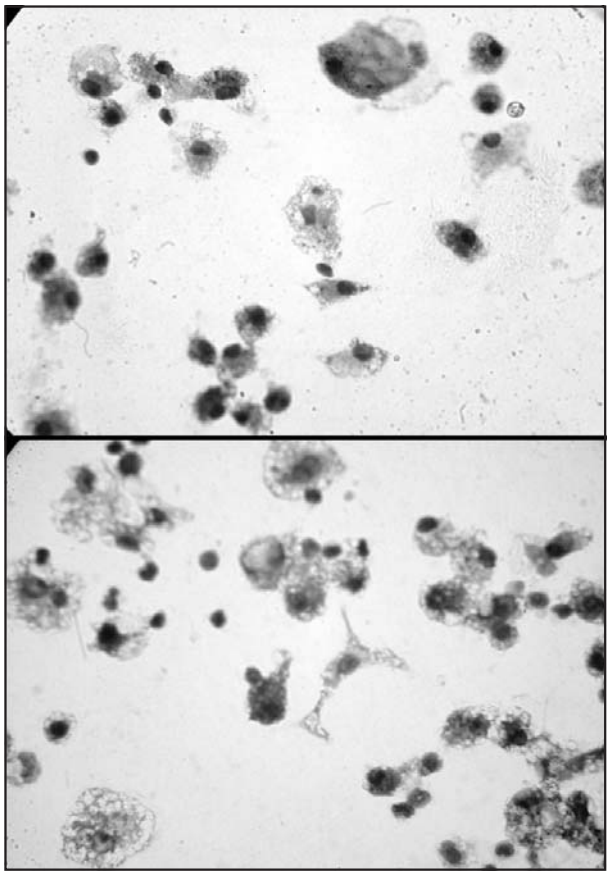

Figure 2. Bone marrow cells after two week of cultivation (MGG staining, mag. 400X)

specimen is faster than on the surface. It is caused by participation of mechanisms: lighter diffusion of soluble oligomers from the surface into the medium than from the inside and neutralization of carboxyl groups on the surface of PLLA. Both phenomena reduce surface acidity, while the inside is characterized by increased degradation rate due to autocatalytic activity of terminal carboxyl groups. Also, it was shown that polylactide decomposition mainly depend on environmental conditions (12).

Our previous experiments have shown that phagocytosis is included in resorption of examined HAp/PLLA biocomposites (5). The other researches have shown that macrophages during phagocytosis of biomaterials release pro-inflammatory mediators as interleukin-1, interleukin-6, tumor necrosis factor- and prostaglandin E2. Besides, macrophages release metalloproteins, chemokines and growth factors into extracellular matrix (6-11; 13-14). Also, reactive oxygen intermediaries are produced and released during phagocytosis, and they stand for very aggressive chemical agents. Many of the aforementioned macrophage products act osteolytic in direct or indirect manner.

Comparison of different materials in whose presence the bone marrow cultures were growing suggests that there is a possibility that the difference in biomaterial phagocytosis has probably caused the difference in the culture growing. More intensive phagocytosis might have been the cause of greater suppression of culture growing. Our previous findings show that phagocytosis of H2 material is long-term and become more intense in time (5). Their medical use requires their not being detrimental to health.

CONCLUSION

All the examined biomaterials show suppressive effect to growth of mice bone marrow culture. Greater PLLA molecular mass had stronger inhibitory effect on the cell growth compared to PLLA with minor molecular mass. Phagocytosis of biomaterial could produce factors with negative effects on the cell growth. Cultures growing in higher density are characterized by pleomorphic, and more frequently adherent cell phenotype with numerous extensions of phyllopod, lamellipodia and pseudopod type.

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KARAKTERISTIKE RASTA KULTURA KOSTNE SRŽI MIŠA U PRISUSTVU BIOMATERIJALA NA BAZI HIDROKSI APATITA I POLI-L-LAKTIDA

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SAŽETAK

Bodegradabilni čvrsti polimeri, imaju široku primenu u medicini, a naročito kod nadoknade koštanih defekata u vidu implanta, koštanog cementa i matrice za tkivni inženjering. Procena biokompatibilnosti uključuje sagledavanje efekta fiziološke okoline na materijal i efekat materijala na okolinu. Zato je vrlo važno ispitati moguću citoksičnost biomaterijala, njegov uticaj na ćelijsku morfologiju, rast i deobu ćelija u direktnom ili indirektnom kontaktu sa materijalom. Cilj našeg ispitivanja je bio da se u *in vitro* uslovima ispita efekat različitih biomaterijala na rast i morfologiju ćelija kostne srži miša. Ispitivani su materijali HAp/PLLA sa polilaktidom od 50000 D (HP3) i 430000 D (HP2), titanijumske pločice presvučene materijalom HP2, kao i sam PLLA od 200000 D. Kulture koštane srži su postavljene u 2 ml hranjive podloge (MEM) u kultivacione ploče sa 24 komore i ostavljene da rastu 14 dana na 33°C i 5% CO_2, posle čega su fiksirane i obojene Giemsaom. Najveća gustina ćelija nađena je u kulturi bez biomaterijala, a najmanja u prisustvu HP2. Ćelijski fenotipovi su raznovrsniji u kontrolnoj kulturi nego u kulturama raslim sa biomaterijalima. Adherentni fenotip analiziranih ćelija odlikuju filopodije, lamelipodije i pseudopodije. U prisustvu biomaterijala u ćelijama se češće sreće vakuolizacija, prisustvo azurofilnih granula i ekscentričnih jedara. Izražena fagocitoza biomaterijala HAp/PLLA koja je uporediva sa istim procesom u prirodnoj kosti pokazuje njegovu dobru biokompatibilnost.

Ključne reči: **biomaterijali, ćelijski rast, ćelijska kultura, kostna srž**