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Changes of gene expression of osteoblast cells stimulated by hydroxyapatite and hydroxyapatite composite by protein microarray – preliminary study

Zmiany w ekspresji genów komórek osteoblastów stymulowane hydroksyapatytem i kompozytem hydroksyapatytu przy wykorzystaniu techniki mikromacierzy białkowych – badania wstępne

INTRODUCTION

Nowadays, the problem of skeletal surgery requiring partial bone substitutes increases rapidly among hospitalized patients. Therefore, scientists are interested in developing fully accepted biomedical materials. Hydroxyapatite (Hap) ceramic $Ca_{10}(PO_4)_6(OH)_2$ demonstrates an excellent biocompatibility and mechanical properties similar to natural bone tissue [4]. The main disadvantage of Hap in skeletal tissue engineering is a lack of cohesive strength and surgical handiness. In order to overcome this problem, HAp has been combined with many organic agents such as collagen fiber [7, 10] or gelatin [9]. The new designed hydroxyapatite / plant derived polymer composite demonstrates lack of brittleness and it fits excellently in the implantation site [1]. On the other hand, the new biocomposites with better physiochemical properties should be carefully examined with regard to their biocompability. *In vivo* application of HAp composites may cause multi gene response of host organism. A parallel study of all genes and majority of proteins in a quick and efficient way is carried out with the use of the microarray technology [8]. This microarray technology is represented on two main expression levels: RNA and protein. The gene level technology permits only measurement of expression based on the mRNA quantity. The problem of the lack of correlation between mRNA level and corresponding protein has been solved by the development of protein microarray technology [3, 6]. The use of antibody-protein reaction provides global and direct information on functional state of examined cells [5]. The data obtained from protein microarray analysis allows to studies cell signaling pathways with the use of specific software. Molecular studies of necrosis, apoptosis and carcinogenesis are important subjects of intensive investigations of material biocompability.

The aim of this study was to identify the differences of gene expression on protein level in osteoblast cells stimulated by hydroxyapatite and hydroxyapatite / plant-derived polymer composite and changes in P53 cell-signaling pathways.

MATERIAL AND METHODS

Cell culture. An experiment was carried out using hFOB 1.19 cell line (human fetal osteoblasts) obtained from ATCC (American Type Culture Collection, Menassas, VA). The cells were cultured in a 1:1 mixture of DMEM/Ham F12 medium without phenol red supplemented with 10% FBS, 2,5 mM L-glutamine, 300 μg/ml G418, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and maintained at 34˚C in a humidified atmosphere of 5% CO_2 and 95% air. All reagents were obtained from Sigma (St. Louis, USA).

The extracts of Hap and Hap composite were obtained by immersing the test materials in a complete culture medium supplemented with 2% FBS under standard conditions: 72h, at 37˚C without agitation, according to standard procedures (ISO 10993-5). The ratio between the sample weight and the volume of the extraction vehicle was 0.1 g/ml beyond the absorptive capacity of the material.

The cultures were grown to near confluence in 100 mm diameter Petri dishes. Then the medium that nourished the cells through 48 hours was replaced by pure HAp or Hap composite extracts. After 24h of incubation at 34˚C, the extracts were removed and the cells were prepared for further analysis.

The cytotoxicity level deriving from the HAp and the HAp composite was evaluated indirectly by means of fluid extracts with the use of MTT and NRU tests.

Antibody microarrays analysis. The research was done according to the manufacturer's protocol of Panorama Ab Microarray Cell signaling Kit (Sigma, St. Louis, USA). 224 antibodies were spotted in a duplicate in 4 x 8 grids. Protein extraction from cell line stimulated by Hap and Hap composite extracts was performed with the use of extraction buffer. Equal amounts of protein samples induced by HAp and HAp composite extracts were labeled with Cy3 and Cy5 (Amersham, UK), respectively. Free non-incorporated dyes were removed by SigmaSpin post-reaction clean up columns. To provide DNA degradation and to eliminate small particles, Benzonase was added. The incubation of sample arrays lasted for approximately 30 min. During the air-drying, the slides were protected from light to avoid any additional effect of the fluorescent dies. The scanning of the antibody array was performed with the use of Tecan high-resolution microarray scanner. The experiment was normalized by reference (housekeeping) proteins. The whole experiment was repeated at least twice.

The analysis of the microarray cell signaling slides was performed with the use of the Ingeunity software.

RESULTS

In accordance with the aim of our study, the Panorama Ab Microarray Cell signaling Kit was chosen as a useful tool for tracking the changes in biological pathways including apoptosis, cell cycle

and signaling transduction. The results obtained in our study are illustrated in Fig.1. which shows the same slide scanned at the two fluorescence emission wavelengths for Cy3 and Cy5.

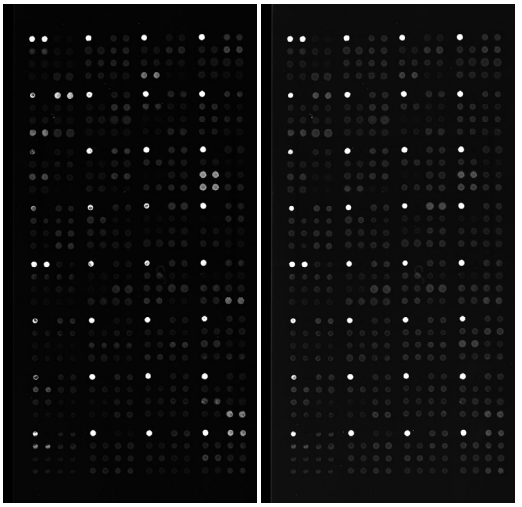

Fig. 1. Expression of protein in human osteoblast cells hFOB 1.19 induced by HAp (left) and HAp composite (right). The proteins were labeled with Cy3 and Cy5, respectively. Equal amounts of Cy3 and Cy5 labeled protein were incubated on the slide

The intensity of the signal obtained from each single spot during scan procedure was recorded as the numerical value. The proteins showing a ≥ 2-fold difference in expression between HAp and HAp – composite induced samples were assumed as functionally significant. Interestingly, almost 90% of examined proteins were found not to be significantly up or down regulated (see Fig.2). The cutoff level used to determine the functional significance of gene expression allowed to identify unchanged protein concentration engaged in p53-dependent pathways such as: MDM2, CHK1, CHK2, p21, CDK4/6, Cyclin B, CASP8, P53AIP, CSP9, CASP3, GD-AiF, p53R2, PTEN. These results are summarized in Fig. 3.

DISCUSSION

A new molecular technology represented by protein microarrays is distinguished by examining a broad overview of the state of a signaling-pathway target after HAp or HAp composite inducing cell extracts. This method stands out for a unique ability to analyze signaling pathways using small sample volume. Molecular profiling using protein microarray presents a necessary approach to research new biomaterials used in skeletal engineering. Various biochemical tests have been used so far to analyze cytotoxity of new bioactive ceramics. One of them estimates the integrity of the cell membranes by measuring the activity of lactic and glutamate dehydrogenase or liver tissue specific enzymes (ASPAT, ALAT). Moreover, the Neutral Red Uptake (NRU) Phototoxicity Assay as well

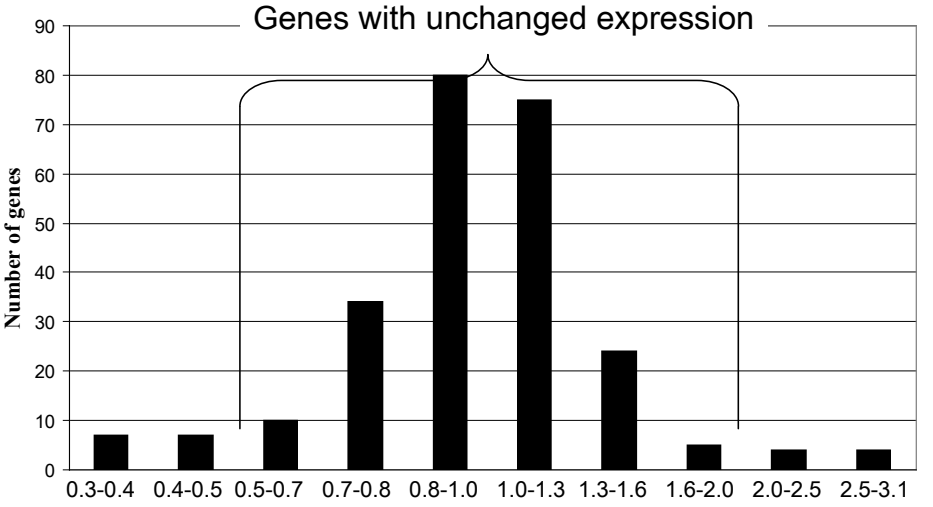

Fig. 2. Detection of signaling molecules. The distribution of protein concentration changes. The proteins showing a ≥ 2-fold difference in expression were deemed significant. The numbers on axis of abscissa show Cy3/Cy5 absorbance intensity ratio

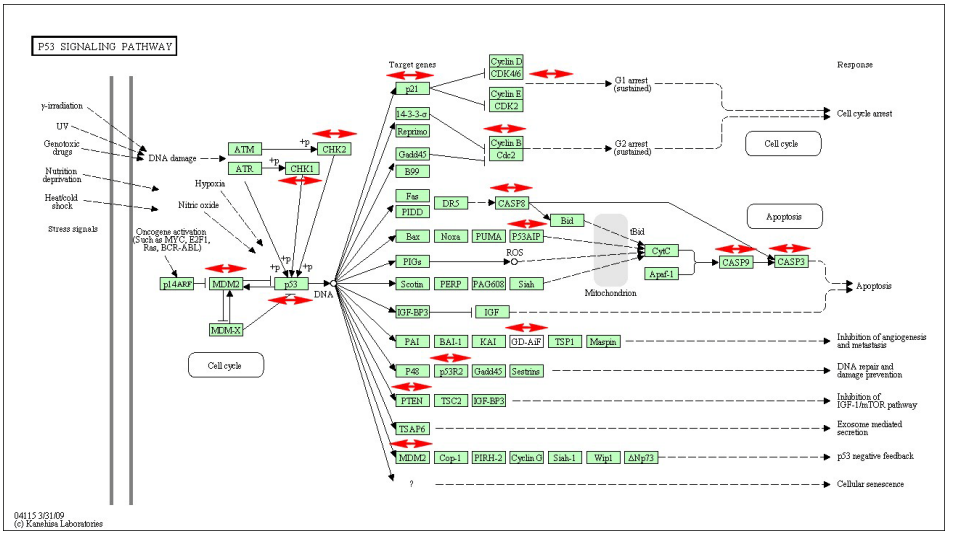

Fig. 3. Localization of examined genes with unchanged expression on p53 signaling pathways marked with double-arrow (↔)

as the MTT colometric assay are used to estimate the viability and growth of the cell. Considering the fact of Hap composite clinical implantation, it is important not only to measure its cytotoxity level, but also the influence on any biochemical process. To achieve complexity, in our research we decided to use The Signaling Pathway Protein Microarray, covering almost all proteins involved in regulation of cell reactions. The measurement of the cell proteome excludes a disadvantage of gene microarray, where mRNA level, not fully corresponding to its protein, is calculated. The instant results of present cell signaling protein concentrations create a unique ability to track changes in biochemical pathways. Traditional methods of protein research like western blot or ELISA are useful to drill down any potential changes only in particular protein reactions. The chosen ≥ 2-fold change in expression cutoff level between the examined samples was used according to protein microarray analysis standard [2, 11].

It is worth noticing that the control points in pathway responsible for arresting the cell cycle in G1 or G2 phase are not disturbed by adding the HAp composite extract, because of unchanged p21, CDK4, CDK6 or Cyclin B protein level, respectively. The apoptosis pathway controlled by p53 and expressed by a stable level of Caspase 8, p53AIP, Caspase 9 and Caspase 3 protein seem not to be disrupted, too. The metabolic pathways responsible for inhibition of angiogenesis and metastasis or DNA repair and damage prevention are also unchanged.

The results achieved so far, by using protein microarray tool, confirm an excellent biocompability of new HAp composite on molecular level.

CONCLUSIONS

The protein microarray tool is useful for screening changes in biochemical pathways created in response to the introduction of HAp and new HAp composite to osteoblast cell culture. We found that over 90% of signal-regulated proteins concentration stayed unchanged after addition of new HAp composite extract and, most importantly – the p53 controlled signaling pathway remain unchanged. However, finding the changes in expression of almost 10% genes does not allow to exclude the influence of HAp composite on the other cellular signaling pathways. Continuing our research on biocompability of HAp composite we are going to make use of gene microarrays, protein microarrays, Western-blotting or RNAi – techniques as well as specialized computer programs designed for cell-signaling pathways research.

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SUMMARY

The development of biomaterials for medical applications is an evolving subject in the area of human health care. Osteoconductive properties and biocompatibility of calcium phosphate ceramic materials such as hydroxyapatite (HAp) allow their use in skeletal tissue engineering. In order to overcome their limitations, such as brittleness, various composites have been developed so far. Combining different materials in composites generates better physiochemical properties of biomaterial. However, such a new mixture needs special attention in terms of its biocompatibility. Biomaterials used in bone repair, by nature of their *in vivo* applications, inevitably cause multi gene response of host organism. Gene microarray technology has been used successfully in bioengineering to characterize genes expression on mRNA level. The poor correlation between mRNA level and corresponding protein expression leads to the development of protein microarray. Direct screening of the proteins level changes in cells responding to different biomaterials stimulation, permits evaluation of their biocompatibility. Our study has been conducted with osteoblast cell line stimulated by hydroxyapatite and hydroxyapatite/plant-derived polymer composite. The gene expression changes have been analyzed on the protein level with the use of The Panorama Ab Microarray Cell Signaling kit. The microarray tool for cellular-signaling protein has been chosen for noting the development of changes in signaling pathways with the use of pathway-finder software. In our studies, cells stimulated by HAp and HAp/plant-derived polymer composite showed no differences in the level of protein expression in almost 90%. Interestingly, the p53-controlled signaling pathways have not been changed by hydroxyapatite composite. Further analysis should be performed to identify the role of up- or down-regulated proteins in apoptosis and cell cycle pathways crucial for bioengineering applications.

Key words: protein microarrays, hydroxyapatite, p53, cell signaling pathways

STRESZCZENIE

Hydroksyapatyt (HAp) odznacza się uznanym i wysokim stopniem biokompatybilności, jednakże jego zastosowanie limitowane jest jego kruchością i łamliwością. Czynniki organiczne, jak włókna kolagenowe, żelatyna czy chitosan, w połączeniu z hydroksyapatytem prowadzą do powstania materiału o lepszych mechanicznych właściwościach. Badany przez nas nowy kompozyt hydroksyapatytu z polimerem organicznym wykazuje zdolność do częściowego dopasowania się do kształtu i wymiarów miejsca implantacyjnego, a tym samym zapewnia lepszą poręczność chirurgiczną. Jednakże zastosowanie nowych biokompozytów *in vivo* musi być poprzedzone skrupulatnymi i kompleksowymi badaniami. Ważne jest określenie potencjalnych zmian, jakie może wywołać nowy biokompozyt w ekspresji genów, a co za tym idzie proteomie komórek organizmu biorcy. Szczególny nacisk należy położyć na określenie zmian w szlakach karcynogenezy, apoptozy i nekrozy. Wykorzystując technikę mikromacierzy białkowych, celem naszej pracy było porównanie wpływu HAp i nowego biokompozytu na zmiany zachodzące w proteomie osteoblastów, ze szczególnym uwzględnieniem różnic w szlakach przekazywania sygnałów kontrolowanych przez białko p53. Po uzyskaniu monowarstwy linii prawidłowych ludzkich osteoblastów podłoże zastąpiono ekstraktami z HAp i testowanego biokompozytu, zgodnie ze standardami (ISO 10993-5). Wyizolowane białka posłużyły do testów na mikromacierzy Panorama Ab Microarray Cell Signaling. Uzyskane białka były inkubowane z mikromacierzami. Wynik odczytano przy użyciu skanera o wysokiej rozdzielczości. Stwierdzono, że dla ok. 90% genów, których ekspresję mierzono na poziomie proteomu, ekspresja nie uległa zmianie po zastosowaniu nowego kompozytu w porównaniu do samego hydroksyapatytu. Nie uległy zmianie także szlaki sygnałowe kontrolowane przez białko p53, takie jak: szlak apoptozy, angiogenezy oraz mechanizm zatrzymania cyklu komórkowego w fazie G1 i G2. W świetle uzyskanych wyników stwierdzono, że biokompatybilność badanego kompozytu jest porównywalna z biokompatybilnością HAp. Mimo braku widocznego wpływu na szlaki sygnałowe, w których regulację zaangażowane jest białko p53, znaleziono zmianę ekspresji nielicznych genów, która będzie celem dalszych badań. Na obecnym etapie nie możemy jeszcze wykluczyć w pełni wpływu kompozytu hydroksyapatytu na pozostałe szlaki sygnałowe w komórce.

Słowa kluczowe: mikromacierze białkowe, hydroksyapatyt, p53, szlaki sygnałowe