Collagen-based hydrogel functionalized with rhBMP-2

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Abstract. The study showed the cytocompatibility, matrix, and osteoinductive properties of collagen-fibronectin hydrogel impregnated with rhBMP-2. After 7 days of MSCs incubated with osteoplastic material the cell viability was 93.4 ± 3.6%. Expression of osteoblast-related genes after 14 days was increased by 1.4 - 4.8 times. Furthermore alkaline phosphatase activity and calcium ion quantity in cell lysates were increased by 2 and 2.7 times. It was accompanied by extracellular matrix mineralization. The results indicate that rhBMP-2 fully retains its activity inside the collagen-based material. The use of rhBMP-2 at a concentration of 1 μg/ml was ineffective and unable to induce osteogenic differentiation of MSCs. An effective osteoinductive concentration of rhBMP-2 was determined as 10 μg/ml.

1. Introduction

Provide organotypic bone tissue regeneration is an important problem that still does not have an effective solution. Success in this field can be obtained by using new biotechnological approaches based on the application of thermosetting hydrogels from natural polymers. They are capable of retain and prolonged release osteoinductors while maintaining their functional activity and can completely fill the bone defects providing blood vessel ingrowth, cell migration, and having an osteoinductive effect on mesenchymal stem cells. These properties can be used in bioengineering materials for bone tissue regeneration. The use of collagen for this case is justified by its biocompatibility, abilities to gel formation, and involvement its metabolic products in bone formation. One of the generally known and most effective osteoinductors is BMP-2: its low doses can induce osteogenic differentiation in vitro and in vivo [1, 2]. Compared to other BMPs, the BMP-2 gene knockout leads to absolute fetal mortality, which emphasizes the critical importance of this factor [2]. Use of BMP-2 at concentrations higher than 150 μg/ml can cause such complications as cyst-like bone formations [3], tumors, inflammation, soft-tissue swellings, and hyperostosis [1]. In this case, the important task is to search an approach for reducing side effects of BMP-2, for example due to reducing dosage with prolonging its action. As we have shown earlier, the incorporation of fibronectin in collagen enhanced the gel formation ability and
prolonged release of BMP-2 [4]. The aim of this study is to evaluate the osteoinductive properties of rhBMP-2 in the composition of a collagen-fibronectin hydrogel.

2. Materials and methods

2.1. Obtaining collagen-fibronectin hydrogel impregnated with rhBMP-2
A sterile 10% porcine collagen type I neutral solution “ViscollTM, PA100” (“Imtek Company Ltd”, Russia) was mixed with a sterile human fibronectin solution H Fne-C (“Imtek Company Ltd”, Russia) in a volume ratio of 1:4. To impart osteoinductive properties to the material, rhBMP-2 (“Akron Biotech”, USA) to a concentration of 10 μg/ml was added to the collagen-fibronectin mixture. The resulting mixture was incubated at 4 °C for 24 hours.

2.2. Cell cultivation and differentiation
Human adipose-tissue-derived MSCs (ADSCs) were cultured on passage 2-3 in the basal medium consisting of DMEM (“Paneko”, Russia) with 10% FBS (“PAA Laboratories”, USA), 4 mM L-glutamine (“Paneko”, Russia) and 100 mg/l amikacin (“Syntez OJSC”, Russia) at 37 °C and 5% CO2. The medium was changed every 3 days. For osteogenic differentiation, MSCs were incubated in basal medium supplemented with 1 or 10 μg/ml rhBMP-2 (“Akron Biotech”, USA) or in the presence of the collagen-fibronectin hydrogel impregnated with rhBMP-2. Cultured in the basal medium MSCs were used as a negative control.

2.3. Assessment of osteoblast-related genes expression
Total RNA was isolated from cells using the “RNeasy Plus Mini Kit” (“Qiagen”, Germany); the synthesis of the first strand of total cDNA on the RNA matrix was performed using the “RevertAid kit” (“Thermo Scientific”, Germany) according to the manufacturer’s recommendations. Quantitative real-time PCR analysis was carried out on the “CFX96 Touch ™” amplifier (“Bio-Rad”, USA), using a “SYBR Green I” intercalating dye (“Eurogen”, Russia) and gene-specific primers for detection of osteoblast-related genes: ALPL, Runx2, BMP-2 (“Eurogen”, Russia) (table 1). The analyzed genes expression levels were normalized by average expression values of the identified reference genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (beta-actin).

| Primers       | Sequence (5'-3')                        |
|---------------|-----------------------------------------|
| ACTB forward  | CCTGGAACCAAGCACAATA                     |
| ACTB reverse  | GGGCCGAGACTAGTCTAC                     |
| ALPL forward  | CTCGGAAGACACTCTGACGCT                  |
| ALPL reverse  | TAGTCCACCATGGAGACATTTCTCT              |
| BMP-2 forward | ACTACCAGAAGCGGGAGGAA                    |
| BMP-2 reverse | GCATCTGGTCTCGGAAACCT                   |
| GAPDH forward | GAAGTGAAGGTCGGAATGCT                  |
| GAPDH reverse | TTCACACCCATGACGACAT                    |
| Runx2 forward | AGTGGAGACAGTCTGACTT                   |
| Runx2 reverse | GGTGAATGCCTAGACTGTG                    |

2.4. Evaluation of alkaline phosphatase activity and extracellular matrix mineralization
The determination of alkaline phosphatase activity (ALPL) and the calcium ion quantification in cell lysates on the 14th and the 21st days of osteogenic differentiation was carried out using the “Alkaline Phosphatase-Novo” (“Vector-Best”, Russia) and “Calcium Colorimetric Assay Kit” (“Sigma-Aldrich”, USA) reagent kits, respectively, according to the manufacturer’s instructions on the “EnSpire Multimode Plate Reader” (“PerkinElmer”, USA). To detect extracellular matrix mineralization (ECM),
fixed MSCs were stained by a 2% aqueous solution of alizarin red (‘‘Sigma-Aldrich’, USA) at pH = 4.1 for 10 minutes.

2.5. Biocompatibility assessment

To evaluate the cytotoxic effect, MSCs were incubated in basal medium with collagen-fibronectin hydrogel impregnated with rhBMP-2 in a volume ratio of 3:1 for 1, 4 and 7 days. On each of these terms the MTT assay was performed according to the standard method. The formazan absorption was evaluated by determining the optical density of the eluate at a wavelength of 570 nm and subtracting the background value at 620 nm on the “EnSpire Multimode Plate Reader” (“PerkinElmer”, USA). To visualize cells on the surface of materials, it’s were pre-stained using a “PKH26” dye (red fluorescent cell linker kit, “Sigma-Aldrich’, USA) according to the manufacturer’s recommendations. To count the number of living and dead cells, cultures on the surface of the material were stained with “Calcein AM” dye (“Biotium”, USA) for 40 minutes and “DAPT” dye (“Sigma-Aldrich’, USA) for 10 minutes at 1 μg/ml. Images were obtained and processed using an “AG Axio Observer D1” microscope with an “AxioCam HRc” camera and “ZEN” software (“Carl Zeiss”, Germany).

2.6. Statistical processing

Statistical processing and graphing of the results were performed in “GraphPad Prism 8.00” (USA). Intergroup differences were determined using the Holm-Sidak test when comparing 3 or more groups and Student’s t-test when comparing 2 groups. Differences at p ≤ 0.05 were considered statistically significant. Statistical differences at p ≤ 0.05 is indicated by “*”, p < 0.01 – “**” and p < 0.001 – “***”.

3. Results and discussion

3.1. Effective concentration of rhBMP-2

Incubation of MSCs cultures in a basal medium supplemented with rhBMP-2 at a 10 μg/ml concentration promoted increased expression of osteogenic markers, ALPL activity and ECM mineralization compared to control groups. On the 7th day of the experiment, the Runx2 gene expression levels increased by 1.3 ± 0.4 times (p = 0.05), on the 21st day BMP-2 by 2.9 ± 0.4 times (p < 0.001). After 14 days, ALPL activity levels increased by 2.5 ± 0.2 times (p = 0.009) and after 21 days – by 2 ± 0.3 times (p = 0.01) (figure 1). Alizarin red staining revealed extensive ECM mineralization on the 21st day of MSCs cultivation in a medium supplemented with rhBMP-2 at a 10 μg/ml concentration (figure 2). Its volume exceeded that in the group using rhBMP-2 at a lower concentration of 1 μg/ml. The use of rhBMP-2 at a 1 μg/ml concentration did not lead to changes in ALPL activity on the 14th and 21st days of the experiment and a statistically significant increase in the osteogenic markers expression levels.

In this study, the minimum effective concentration of rhBMP-2 in vitro was determined as 10 μg/ml. This dose induces the osteogenic differentiation of MSCs and is an order of significantly less than the doses causing undesirable side effects.

**Figure 1.** Expression of osteoblast-related genes Runx2 after 7 days, BMP-2 after 21 days and ALPL activity after 14 and 21 days of incubation with rhBMP-2 relative to the initial in MSCs cultures.
Control

200 μm

1 μg/ml rhBMP-2

200 μm

10 μg/ml rhBMP-2

200 μm

Figure 2. ECM mineralization in MSCs cultures after 21 days of incubation with rhBMP-2 at a 1 μg/ml and 10 μg/ml concentrations. Alizarin red staining.

3.2. Biocompatibility of collagen-fibronectin hydrogel impregnated with rhBMP-2
The collagen-fibronectin hydrogel did not exert a cytotoxic effect and supported the adhesion of MSCs. Cell viability in the presence of the material was not significantly different from the values of the control group and amounted to 94.4 ± 7% on the 1st day, 92.8 ± 4.4% on the 4th day and 93.4 ± 3.6% on the 7th day of the experiment (figure 3). Most cells adhered to the material remained viable after 24 hours (figure 4).

In several studies have shown that collagen and fibronectin promoted MSCs proliferation, cell survival under stress, high cell adhesion on the surface of such materials, as well as osteogenic differentiation [5]. Xenogenic collagen materials have high biocompatibility, in contrast to materials based on other ECM components, such as hyaluronic acid, chondroitin sulfates, elastin [6]. These results confirm the preservation of high biocompatible properties of the collagen hydrogel in the composition with fibronectin.

Figure 3. MSCs viability after 1, 4 and 7 days incubation with collagen-fibronectin hydrogel impregnated with rhBMP-2 relative to control group. MTT assay.

3.3. Osteoinductive properties of collagen-fibronectin hydrogel impregnated with rhBMP-2
Incubation of MSCs cultures in a basal medium with a collagen-fibronectin hydrogel impregnated with rhBMP-2 for 14 days contributed to the elevated expression of osteogenic markers, ALPL activity and calcium ion quantity, as well as extensive ECM mineralization compared to control groups. BMP-2 gene expression levels increased by 4.8 ± 0.6 times (p < 0.001), Runx2 by 1.8 ± 0.1 times (p = 0.002) and ALPL by 1.4 times ± 0.1 (p = 0.001). The ALPL activity and calcium ion quantity in cell lysates increased by 2 ± 0.2 times (p = 0.007) and by 2.7 ± 0.3 times (p < 0.001), respectively (figure 5). In addition, alizarin red staining shows enhanced ECM mineralization focus in contrast to the control group (figure 6). Thus, the osteoinductive effect of a collagen-fibronectin hydrogel impregnated with rhBMP-2 at a 10 μg/ml concentration was demonstrated. This indicates the complete preservation of the functional activity of the protein in the composition of the osteoplastic material.
Figure 4. MSCs adhesion on the surface of the collagen-fibronectin hydrogel impregnated with rhBMP-2 – a, b, c, and in the control group – d, e, f, after 1 day incubation; a, d – PKH26 staining; b, e – Calcein AM staining (viable cells), c, f – DAPI staining (non-viable cells). Fluorescence microscopy.

Figure 5. Expression of osteoblast-related genes Runx2, BMP-2, ALPL, ALPL activity and calcium ion deposition (μg Ca/μg protein) after 14 days of incubation with collagen-fibronectin hydrogel impregnated with rhBMP-2 relative to the initial in MSCs cultures.

Figure 6. ECM mineralization in MSCs cultures after 21 days of incubation with collagen-fibronectin hydrogel impregnated with rhBMP-2. Alizarin red staining.
4. Conclusion
This study showed that the use of a hydrogel-based on the collagen and fibronectin composition can fully preserve the functional activity of impregnated rhBMP-2, while ensuring its prolonged release, which was shown earlier [4]. The developed material does not exert a cytotoxic effect, is able to provide cell adhesion of MSCs cultures and promotes osteogenic differentiation of these cultures \textit{in vitro}. The results obtained in the study allow us to recommend the material for bone tissue regeneration, including usage as a scaffold for cells in the development of bone tissue engineering.

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