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Selection of the Fragments of the BMP-2 Protein. Components of Materials for Bone Tissue Regeneration

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Introduction

Bone morphogenetic protein 2 (BMP-2 Figure 1) belongs to the transforming growth factor β (TGF-β) superfamily that plays essential roles in many processes, including cardiogenesis, neurogenesis, and osteogenesis. BMP-2 by associating with type I receptor BMPR1A and type II receptor BMPR2 initiates the canonical signalling cascade reactions leading to bone and cartilage tissue regeneration. It has been identified and characterized by over twenty BMPs in mammals, but the multifaceted action of BMP-2 in tissue regeneration attracts the most attention of scientists [1,2]. This biomolecule consists of 396 amino acids, from which 19 create signaling sequence and its secondary structure is highly ordered, constituted mainly from β-sheet and α-helix chains [3]. BMP-2 is an FDA-approved osteoinductive growth factor applied as a bone graft substitute. Nowadays physicians use INFUSE® Bone Graft, which contains BMP-2 adsorbed to a collagen sponge, during spinal cord injuries treatment [4]. However, after surgery, some undesirable effects such as infections, severe swelling, heterotopic ossification or urogenital problems have been detected in patients [5]. This led to the hypothesis that these adverse events might be eliminated by reducing the delivered growth factor amount to a suitable yet clinically relevant dose. Therefore, the development of a delivery system that provides a sustained release of the protein at sufficient concentrations is a challenge of high priority. On the other hand, it has been searching for new modifications of BMP-2 with lower immunoactivity. One of the approaches postulates finding shorter BMP-2 fragments with high osteoinductive character and selectively interacting with bone morphogenetic protein receptors on mesenchymal stem cells but with less effect on the human immune system [6,7].

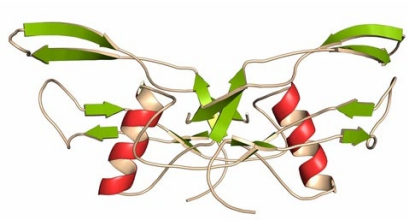

Fig. 1. BMP2 structure [3].

This paper will present studies leading to the implementation of the last hypothesis in medical practice. In this aim it has been performed: 1) synthesis of the library of immobilized BMP-2 fragments on cellulose according to SPOT methodology using a triazine coupling reagent and a linker based on 1,3,5-triazine derivatives; 2) selection of BMP-2 fragments reproducing the external sphere of the protein, capable of interacting with specific anti-BMP-2 polyclonal antibodies, in the dot-blot test; 3) synthesis of selected peptides on the 2-chlorotrityl chloride resin, according to Fmoc/tBu methodology, applying triazine coupling reagent (DMT/NMM/TosO$^-$); 4) checking the influence and cytotoxicity of the synthesized peptides on cells line; 5) checking the usefulness of BMP-2 fragments useful in regenerative medicine on cell viability.

Results and Discussion

Our studies started from the initial preparation of cellulose matrix, namely its functionalization using 1,3,5-triazine derivative (DCMT) (Figure 2a) [8]. In the next step immobilized on cellulose matrix DCMT was transformed into a triazine-based coupling reagent using *N*-methylmorpholine (NMM) and next treated with Fmoc-glycine to obtain a stable linker for peptide synthesis. At elevated temperature, O → N rearrangement was performed to the stable form of the isocyanurate derivative [9]. Finally, the functionalized cellulose matrix could have been successfully used for SPOT peptide synthesis according to the Fmoc/tBu protection strategy. We synthesized 39 element library of

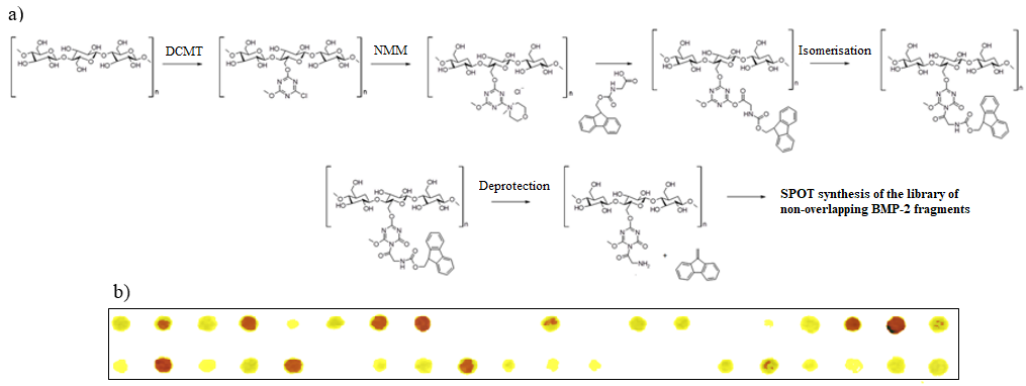

Fig. 2. a) Cellulose functionalization and immobilization BMP-2 fragments library according to SPOT methodology using a linker based on 1,3,5-triazine derivatives (DCMT) and a triazine coupling reagent; b) selection of BMP-2 fragments reproducing the external sphere of the protein in the dot-blot test.

decapeptides and one hexapeptide of BMP-2 which in the next step were treated with polyclonal *anti*-BMP-2 antibodies.

The strength of possible interactions peptide-antibody was determined according to the standard procedure, by measuring the coloration of the spot after treatment with antihuman secondary antibodies labeled with horseradish peroxidase. In the next stage of the research, modelling of the selected nine fragments strongly interacting with polyclonal antibodies was performed. Molecular modeling and optimization of the structure of the BMP-2 protein required the use of an optimized model of the structure of the whole protein (Figure 3). For this purpose, the structure available in the AlphaFold database was used. The model AlphaFold Model AF-P12643-F1 was used. The model of matching the protein structure is very high (pLDDT> 90) or it is characterized by high certainty (90> pLDDT> 70). The use of an optimized protein structure enabled imaging in the Swiss-Pdb Viewer 4.1.0 program [10,11].

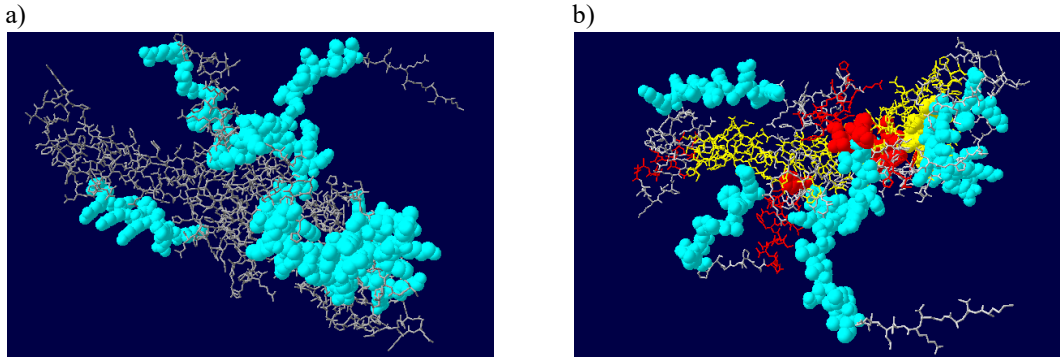

Fig. 3. a) Location of nine strongly interacting fragments with polyclonal antibodies on the structure of BMP-2; b) location of nine fragments strongly interacting with polyclonal antibodies on the structure of BMP-2, taking into account the secondary structure, fragments with β-sheet conformation are marked in yellow, fragments with helical conformation are marked in red.

Based on the structure imaging in the Swiss-Pdb Viewer program, the following secondary structure models were distinguished in the BMP-2: fragments of the alpha helix structure: 47(Asp)-61(Phe), 77(Pro)-87(Ser), 97(His)-103(Ala), 210(Thr)-218(Ala), 254(Glu)-258(Ser), 306(Ser)-308(Phe), 341

(Asn)-352(Val) and the fragment with the β-sheet structure: 107(Asn)-112(Phe), 128(Thr)-132(Phe), 144(Phe)-154(Arg), 167(Phe)-177(Ile), 188(Val)-198(Asn), 203(Arg)-209(Val), 225(Gly)-231(Ala), 263(Leu)-269(His), 297(Lys)-304(Phe), 313(Trp)-315(Val), 318(Pro)-326(His), 360(Cys)-367(Ser), 369(Ile)-375(Asp), 389(Val)-396(Arg).

In the fragments selected in the dot-blot test, helical fragments are compacted: 51-61, 77-80, 101-103, 211-218, 341-352 and β-sheet fragments: 107-110, 128-132, 167-176, 188-198, 225-231, 263-269, 297-300, 360-677, 369-370, 389-396.

The applied model of the protein structure confirmed the assumptions that the external sphere of the protein is largely responsible for interactions with polyclonal antibodies. A large area of the protein structure is characterized by a high value of the fitness parameter (pLDDT > 70).

Based on the obtained results we selected 9 decapeptides from the external sphere of BMP-2 which were able to have strong interactions with polyclonal antibodies (Figure 2b). Subsequently, these peptides (^{11}LLLPQVLLGG^{20}, ^{31}RKFAAASSGR^{40}, ^{61}FGLKQRPTPS^{70}, ^{71}RDAVVPPYML^{80}, ^{171}INIYEIIKPA^{180}, ^{181}TANSKFPVTR^{190}, ^{211}PAVMRWTAQG^{220}, ^{241}KRHVRISRSL^{250}, ^{281}KRQAKHKQRK^{290}) were synthesized on 2-chlorotrityl chloride resin under SPPS conditions using a triazine coupling agent (DMT/NMM/TosO$^-$) [12]. The final monoisotopic masses and purity of products were assessed using high-performance liquid chromatography coupled with electrospray mass spectrometry. It has been found that the purity of the crude products was in the range of 80-98%. The purified BMP-2 fragments were tested for their cytotoxicity against human osteoblasts-femoral (HO-f) and human osteosarcoma (Saos-2) cell lines (Figure 4).

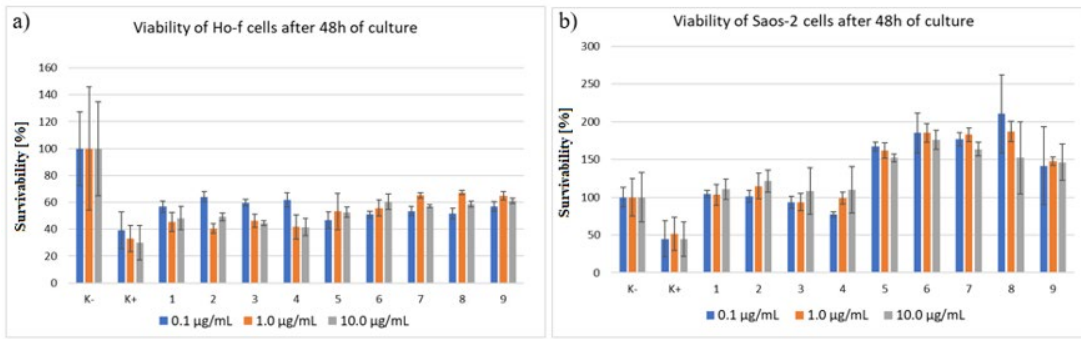

Fig. 4. The survivability diagrams of Ho-f (a) and Saos-2 (b) cells in the presence of BMP-2 fragments.

Cells cultured in a medium without the addition of any substances or harmful agents were used as the Control$^-$ (K^-), while cells treated with DMSO on the second day of incubation were used as the Control$^+$ (K^+). A colorimetric MTT assay was used to estimate the cytotoxicity of peptides. It has been found that the survivability of Ho-f cells in presence of each analyzed peptide was higher in comparison to a positive control (K^+) (Figure 4a). This was the first important clue that reflects the possible potential of BMP-2 fragments in regenerative medicine. Moreover, the concentration of the peptides had a crucial influence on cell proliferation – its increase not in all cases favoured cell growth. The very interesting results were obtained for the more resistant cell line Saos-2 (Figure 4b). Namely, also in this case, it was observed a remarkable increase in cell survivability, even more than it was noticed for cells cultured in a medium without the addition of any substances (K^-). Furthermore, for peptides 5-9 cells proliferation was the most effective, which meant that different decapeptides might have differential osteogenic activity.

Based on the results shown in this paper it can be concluded that the triazine coupling reagent was effective both for immobilization BMP-2 fragments on cellulose as well as in SPPS synthesis. Moreover, the selected fragments of the external sphere of BMP-2 can be successfully used as scaffolds in regenerative medicine. On the other hand, the osteogenic activity of the selected peptides might be regulated by their concentration within the damaged tissue. The synthesis of covalent polysaccharide-peptide conjugates as well as biological research are still continuing.

Acknowledgments

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