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Selection of Fragments of Collagen II Useful in Regenerative Medicine

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Introduction

At present, much emphasis is placed on the research of collagen-based scaffolds. Collagen, due to its properties and functions, and its widespread presence in the composition of almost every tissue, is one of the main research objects in modern regenerative medicine. From the point of view of the development of materials mimicking the natural matrix, the most important are collagens I, II, and III, able to form macrostructures in the form of fibers that are able to imitate ECM. It has been shown that collagen can also be used to obtain multidimensional porous structures [1]. Literature data show that scaffolds based on native collagen has the ability to initiate and support the tissue regeneration process. In the form of a hydrogel, the collagen materials even initiate the chondrogenesis process [2]. However, materials made of native, unmodified collagens are characterized by unsatisfactory mechanical properties and low structural stability [1]. For this reason, work was undertaken to modify scaffolds made of native collagen or to obtain composite materials in which the non-collagen fraction responsible for improving the strength properties will not eliminate the desired biocompatibility and bioresorbability. Strategies for cross-linking the native collagen backbone allow for enhancement of its mechanical and structural properties. Additionally, this process allows for extending the biodegradation time [3]. The cross-linking of collagen fibers adversely affects the activity of cells embedded in the modified material [4]. However, the negative influence of modified collagen on the cellular response *in vivo* has led to attempts to use mixtures of natural or synthetic polymers with collagen. Composites of collagen and synthetic polymers have found wide applications in tissue engineering [5,6]. Natural polymers are widely used in tissue engineering due to their similarity to native ECM [7]. Moreover, there are many literature reports that indicate that it is possible to use short peptides as scaffolds and their positive influence on the life processes of cells. The self-organizing peptides RADA16-I and RADA16-II form materials that favor the proliferation and differentiation of neuronal stem cells [8]. Another example of a self-organizing peptide that positively affects the proliferation processes and the ability to take up vital functions by cells is the RAD peptide [9]. KLD-12 peptide is used for the regeneration of cartilage tissue, e.g. for repairing intervertebral discs [10]. In order to obtain a material providing a comprehensive ECM substitute, work has been undertaken to combine raw materials with fiber morphology and fragments responsible for the interaction of the natural extracellular matrix with the surrounding environment. The primary method was to directly link a functional cell-interacting fragment to the *C*-terminus of the self-assembled peptide. Typically, a linker of two glycine residues is inserted between the self-assembled peptide and the functional fragment to improve the display of the active fragment. Research on this type of conjugate resulted in obtaining material consisting of the RADA16-I peptide and two RGD peptides, which showed the ability to stimulate proliferation in tests performed with fibroblasts. The use of the PRG motif derived from the laminin sequence made it possible to obtain derivatives significantly improving the activity of periodontal ligament fibroblasts [11].

The aim of our research was to select fragments of human collagen II useful for tissue regeneration. It was assumed that the key is to select the exposed fragments of collagen II, which should guarantee their biological activity. This assumption is due to the fact that the protein-protein / protein-peptide interaction takes place only at the junction of the outer domains of proteins. Assuming this assumption, the use of polyclonal antibodies that recognize structurally diverse and exposed fragments of a given protein should allow for the reconstruction of the external structure of collagen II. Despite the fact that the selected fragments form immune complexes with antibodies (i.e. they are immunologically active), due to their "length" they are too weak antigens to trigger the activation of the immune system, and in addition, they are fragments of human proteins, which further reduces the possibility of triggering activation the immune system in humans.

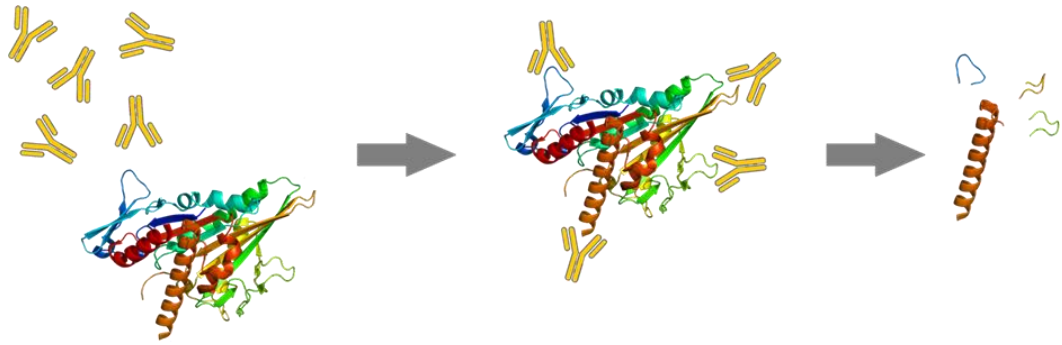

Fig. 1. Schematic diagram of the search for biologically active fragments of collagen II.

Results and Discussion

Results As part of the research, attempts were made to select biologically active fragments of collagen II, expecting that they would have the function of a native protein. The implementation of the aim of the work required dividing the research part into several stages: 1) synthesis of a collagen II fragment library comprising whole proteins, 2) selecting biologically active fragments, 3) checking the biological activity of selected fragments.

The automatic SPOT method with the use of cellulose modified with 1,3,5-triazine derivative [12] was used to synthesize the library of collagen II fragments immobilized on cellulose. The 1,3,5-triazine derivative acts as a linker separating the peptide from the cellulose matrix and ensuring its appropriate exposure to interaction with antibodies [13].

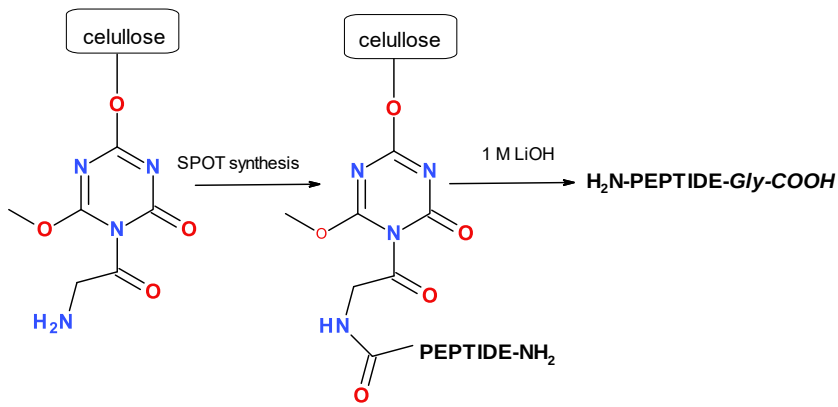

Fig. 2. Cellulose matrix with an immobilized 1,3,5-triazine derivative and a glycine residue (H_2N-Gly-iso-methoxy-1,3,5-triazine linker), to which subsequent amino acids of the synthesized peptides are attached.

4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium p-toluenosulphonate (DMT/NMM/$TosO^-$) was used as a coupling reagent in the synthesis of peptides by the SPOT method [14]. Collagen II contains 1487 amino acid residues, so the library of cellulose-immobilized non-overlapping collagen II fragments comprised 148 decapeptides and one heptapeptide. Transformation to a 256-point greyscale of the dot-blot-stained cellulose matrix allowed for the quantification of the binding capacity of the antibodies to collagen II fragments. This approach made it possible to select the fragments that make up the outer sphere of the protein.

From the 149-component library of collagen II fragments, 26 fragments were selected for further study. Out of 26 selected decapeptides, 4 formed complexes with polyclonal antibodies and were classified as very strong (++), 19 as strong (+), for the three decapeptides marked as +/- the results were not

identical and it was difficult to assign them to the group + or inactive fragments, therefore it has been assumed that they will be included in the pool of tested fragments. The next stage of research was to find the right fragments recognized by polyclonal antibodies, which should be included within 26 fragments selected in the first screening. These studies were necessary because at the first screening stage, collagen division was based on an arbitrary division of the protein into non-overlapping decapeptide fragments. It was assumed that epitope mapping of the selected 26 fragments would be performed, shifting the reading frame by five amino acid residues towards both the *N*- and *C*-terminus. The synthesis of cellulose-immobilized peptides followed the synthetic protocol described above. Finally, for the selected 26 fragments of collagen II, a 296-component library of immobilized decapeptides was obtained.

Table 1. Collagen II fragments able to interact with polyclonal anti-collagen II antibodies. Results of the dot blot test with polyclonal antibodies with a library of non-overlapping collagen fragments (1st screening) and a library resulting from epitope mapping of selected fragments (2nd screening).

Entry	*1st screening*		*2nd screening*	
	Fragment	*Strength of interaction*	*Fragment*	*Strength of interaction*
1	11-20	++	10-23	++
2	31-40	+/-	25-41	+
3	41-50	+	42-55	++
4	241-250	+	236-248	+
5	271-280	+	273-287	++
6	281-290	+	283-295	+
7	321-330	+	316-331	++
8	431-440	+	426-441	+
9	511-520	+	511-520	+
10	541-550	+/-	536-552	+
11	551-560	+/-	554-565	+
12	621-630	+	620-635	+
13	701-710	+	696-711	++
14	711-720	+	714-725	++
15	931-940	+	927-945	+
16	971-980	+	971-987	+
17	981-990	+	984-995	+
18	1131-1140	+	1130-1144	++
19	1181-1190	+	1181-1194	++
20	1331-1340	++	1327-1338	++
21	1341-1350	+	1344-1355	+
22	1371-1380	++	1367-1384	++
23	1391-1400	+	1385-1396	+
24	1431-1440	+	1427-1440	+
25	1441-1450	+	1443-1454	+
26	1451-1460	++	1449-1461	++

The possibility of cleavage of the peptide from the modified cellulose matrix in the presence of a diluted LiOH solution allowed for the cleavage of the collagen II fragments selected in the first screening and the use of an equimolar mixture of 26 selected collagen II fragments: 11-20G, 31-40G, 41-50G, 241-250G, 271-280G, 281-290G, 321-330G, 431-440G, 511-520G, 541-550G, 551-560G, 621-630G, 701-710G, 711-720G, 931-940G, 971-980G, 981- 990G, 1131-1140G, 1181-1190G, 1331-1340G, 1341-1350G, 1371-1380G, 1391-1400G, 1431-1440G, 1441-1450G, 1451-1460G in biological studies (in each cleaved peptide at the C-terminus present there is a Gly residue derived from the isocyanurate linker). An assessment of biological activity was performed on human Hs 680 fibroblasts. One of the tests was the PrestoBlue viability and proliferation test, which allows determining the viability of cells and their ability to proliferate. Another test used was ToxiLight™ Bioassay allowing the measurement of cytotoxicity. Damage to *in vitro* cultured cells and loss of cell membrane integrity results in leakage of many compounds into the surrounding medium. The ToxiLight test is based on the quantitative measurement of adenylate kinase (AK) released from damaged cells, which allows for an accurate and sensitive determination of cytotoxicity and cytolysis.

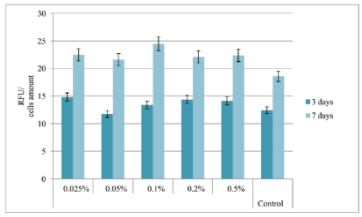

Fig. 3. Viability (proliferation) of Hs 680 cells cultured with the addition of an equimolar mixture of increasing concentration of collagen II fragments as a percentage of the total cell count in the culture. PrestoBlue assay. RFU- Relative Fluorescence Units.

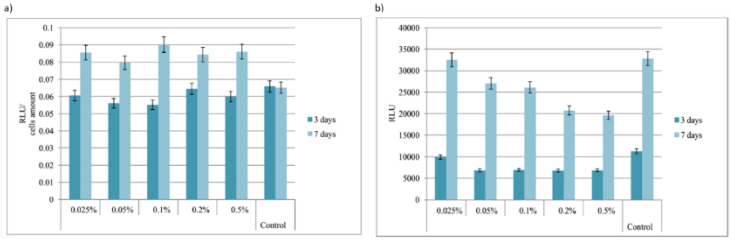

Fig. 4. Cytotoxicity of Hs 680 cell with the addition of an equimolar mixture of collagen II fragments, a) ToxiLight assay, b) ToxiLight 100 Lysis Control assay. RLU - Relative Light Units.

The amount of ATP is determined as a result of the conversion of luciferin in the presence of luciferase. As a result, the intensity of the emitted light is linearly dependent on the concentration of AK.

Preliminary results of the research indicate that selected fragments of collagen II can be used to obtain materials useful in regenerative medicine.

Acknowledgments

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