Summary of the article

Collagen-binding bone morphogenetic protein-2 designed for use in bone tissue engineering

(骨の組織工学に用いるコラーゲン結合性骨形成因子-2 の設計)

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Introduction

Bone tissue engineering is a promising approach to restore oral and maxillofacial bone defects caused by tumor resection or traumatic injury. In most cases, biodegradable porous scaffolds have been utilized to provide substrates for cells to adhere, proliferate, and differentiate, and simultaneously create spaces for bone tissue formation. With recent advances in technologies related to stem/progenitor cells, increasing attention has been paid to the regulation of cellular behavior. Various attempts have been made to incorporate proteins, such as growth factors, into biodegradable scaffolds, allowing them to actively engage the surrounding cells and promote tissue formation.

Collagen-based scaffolds containing bone morphogenetic protein-2 (BMP2) have been studied for bone formation. However, controlling the initial bursting and sustained release of BMP2 is one of the most crucial issues in optimizing therapeutic effects.

In this study, attempts were made to design functional chimeric proteins consisting of bone morphogenetic protein-2 (BMP2) and a collagen-binding domain (CBD), specifically the A3 domain of von Willebrand factor (vWF), which has an affinity for type I collagen. When incorporated into collagen-based scaffolds, the chimeric protein is expected to provide osteogenic microenvironments for a long period of time. First, computer-based structural prediction was employed to gain insight into the three-dimensional (3D) structure of the chimeric proteins consisting of BMP2 and CBD. Predictions were performed for chimeric proteins consisting of BMP2 and CBD with different domain orders (BMP2-CBD and CBD-BMP2), with or without the linker peptide. Based on the results of the *in silico* prediction, we prepared a recombinant chimeric protein consisting of BMP2 and CBD and evaluated its collagen-binding capacity of the CBD domain and osteogenic activity of the BMP2 domain to confirm consistency with the results of the structural prediction.

Materials and Methods

Two types of proteins were designed in this study: Human vWF A3 domain was fused to the N- or C-terminus of BMP2. Two glycines were added as linkers in between. A hexahistidine sequence was added to the N-terminus for protein purification. In addition, GSS and SSGLVPRGSHM were added upstream and downstream of hexahistidine, respectively.

The 3D structures of the chimeric and non-chimeric control proteins were computationally predicted using the AlphaFold2 program. The integrity of the predicted 3D structure was assessed by comparison with a reference structure obtained from the Protein Data Bank. The root-mean-square deviation was calculated from the distance between the respective backbone atoms in the target and reference proteins.

According to the results of the structural prediction, CBD-BMP2, as well as CBD and

BMP2 control proteins, was prepared using a bacterial expression system. First, a chimeric gene was prepared by overlap extension polymerase chain reaction and cloned in pET-15b vector. The chimeric and control proteins were expressed in and extracted from *E. coli*, and purified by metal chelate affinity chromatography. The purified proteins were subjected to refolding by stepwise dialysis. The concentrations of the chimeric and control proteins were determined using a Micro BCA Protein Assay Kit. The purity and molecular size of the proteins were analyzed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). To evaluate the function of the CBD domain, binding assays of chimeric and control proteins were performed on collagen-coated surfaces. On the other hand, the function of the BMP2 domain was evaluated by osteogenic differentiation assays in which human bone marrow-derived mesenchymal stem cells (hBMSCs) were cultured on the CBD-BMP2-bound surface. Promotion of osteogenesis was further confirmed by gene expression analysis of bone-related genes including ALP, Col-I, OCN, OPN, BSP, and Runx2 by qPCR. The results of alizarin red staining and gene expression analyses were statistically analyzed by Tukey's multiple comparison test

Results

Computer-based structural prediction revealed that the CBD domains in both BMP2-CBD and CBD-BMP2 refolded at a similar level of integrity. BMP2 domain in CBD-BMP2 have a similar 3D structure to those of the respective wild types. In contrast, BMP2 domain in BMP2-CBD exhibited a slightly altered structure in the C-terminal region of the BMP2 domain compared to the wild-type. From these findings, CBD-BMP2, instead of BMP2-CBD, was prepared and evaluated for its functions.

It was found from SDS-PAGE analysis that the chimeric protein CBD-BMP2 was successfully prepared. In the collagen-binding assay, the surface density of CBD-BMP2 increased with an increase in the concentration in solution with a dissociation constant of 4.8×10^{-7} M. This result demonstrated that the CBD domain in CBD-BMP2 chimera was able to exert their functions. CBD-BMP2 chimeric protein appeared to have greater binding capacity for collagen binding than control CBD. Control BMP2 was non-specifically adsorbed onto the collagen surface.

In the osteogenic differentiation assay, BMP2 domain in CBD-BMP2 as well as in control BMP2 promoted osteogenic differentiation of hBMSCs. The effect was stronger on CBD-BMP2. The expression of bone-related genes in hBMSCs and calcium deposition were enhanced on the surface with CBD-BMP2 than control surfaces.

Discussion

Chimeric proteins are attractive building blocks for constructing bioactive tissue engineering scaffolds. However, predicting the structure and function of chimeric proteins is difficult and their properties can only be determined through actual preparation and testing. Computer-based structural prediction may serve to avoid such time-consuming and labor-intensive processes in trial and error and accelerate the speed of development. In this study, we demonstrated that 3D structural prediction using AlphaFold2 provides valuable information on the structure of chimeric proteins consisting of BMP2 and CBD. The findings obtained by the prediction were consistent with the functional evaluation of this chimeric protein, CBD-BMP2: CBD-BMP2 bound to the collagen surface and promoted the osteogenic differentiation of hBMSCs and the formation of calcium-containing bone-like tissue. Accordingly, the advantage of using CBD-BMP2 has been well demonstrated in hBMSC culture experiments, although our study was limited to *in vitro* evaluation of the model surface.

In this study, to maintain the local concentration of BMP2, we engineered BMP2 fused with polypeptide domain A3 contained in vWF which have an affinity for collagen. The chimeric protein temporally binds to collagen scaffolds and is gradually released upon dissociation from collagen or the degradation of collagen substrates.

CBD-BMP2 chimeric protein appeared to have an affinity for collagen with a binding capacity greater than that of the control CBD. This suggests that the CBD domain of CBD-BMP2 can correctly fold and exert its function. This finding was consistent with the structural prediction results for the CBD domain of the chimeric protein. The results of the collagen-binding assay further suggested that collagen binding was not hindered by the BMP2 domain fused to the C-terminus of the CBD domain with the GG dipeptide linker.

The *in vitro* cell culture assay demonstrated that the osteogenic differentiation of hBMSCs and calcium deposition were promoted more notably on the surface with CBD-BMP2 than on the other surfaces. This result suggests that the BMP2 domain in CBD-BMP2, as well as in the control BMP2 protein, refolds into a functional 3D structure. A promotive effect was also observed on the surface treated with BMP2; however, this effect was weaker than that of CBD-BMP2. This was probably due to the higher availability of the BMP2 domain on the surface with CBD-BMP2 compared to that with control BMP2.

Promotion of osteogenesis by CBD-BMP2 was further confirmed by gene expression analysis. The highest levels of Col-I, OCN, OPN, BSP, and Runx2 expression were observed on the surface treated with CBD-BMP2 on day 14, indicating that osteogenesis was most strongly promoted on this surface among four surfaces studied. In the case of ALP, the highest expression was observed on the surface with control BMP2 at this time point. We speculate that the peak of the early-stage maker ALP expression might have occurred slightly earlier on the surface with CBD-BMP2.

Conclusion

Structural prediction using AlphaFold2 facilitates the efficient design of chimeric proteins consisting of CBD and BMP2. The CBD-BMP2 chimeric protein serves to promote the osteogenic differentiation of hBMSCs and the deposition of calcium when attached to collagen substrates through CBD−collagen interactions. The observed functions, including collagen binding by the CBD domain and promotion of osteogenesis by the BMP2 domain, appear to be essentially dependent on the structural integrity of these domains.