論　文　内　容　要　旨

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.

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 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.

Results

Computer-based structural prediction revealed that the CBD or BMP2 domain in CBD-BMP2 have a similar 3D structure to those of the respective wild types. In contrast, 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. In the osteogenic differentiation assay, the expression of bone-related genes in hBMSCs and calcium deposition were enhanced on the surface with CBP-BMP2 than control surfaces.

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.