論 文 の 内 容 の 要 旨

論文題目

Application of low molecular weight compounds to osteogenic differentiation in vitro and bone regeneration in vivo with biomaterials

(骨形成細胞分化及び骨再生への低分子化合物含有バイオマテリアルの応用)

氏　　名　　趙 基源

Introduction:

Although bone is able to heal innately, repair of large bone defects is still a big challenge. The need for osteogenic bone substitutes has led to the development of many bone tissue engineering strategies. By combining different components of the tissue-engineering paradigm (biomaterial scaffolds, cells, and bioactive molecules), the conditions of normal tissue development *in vivo* may be mimicked to recreate functional and structural tissues *in vitro.*

In my thesis, my research is mainly focused on bone regeneration using tissue engineering strategy. To achieve this purpose, two aspects of research have been carried out: 1. *in vitro* mechanism study on new potential bone anabolic compounds; 2. *in vivo* bone regeneration with several different scaffolds.

I start with the calcium enriched medium which is able to enhance mineralization *in vitro*. However, high concentration of calcium alone can't induce osteogenic differentiation and bone regeneration. Then I focus on a new bone anabolic drug-icarrin and find that its osteogenic effect was dependent on BMP- and Runx2- signaling pathway. Further *in vivo* study using a calcium phosphate cement scaffold showed enhanced bone regeneration together with icariin. Meanwhile we have evaluated the new drug's effect on bone remodeling using mouse senescence model. Icariin might be more promising in osteoporosis disease study if the positive data can be achieved. Furthermore, a modified collagen gel-vitrigel was applied for BMP-2 delivery. The system showed a sustained release pattern *in vitro* and significant new bone formation was observed *in vivo*.

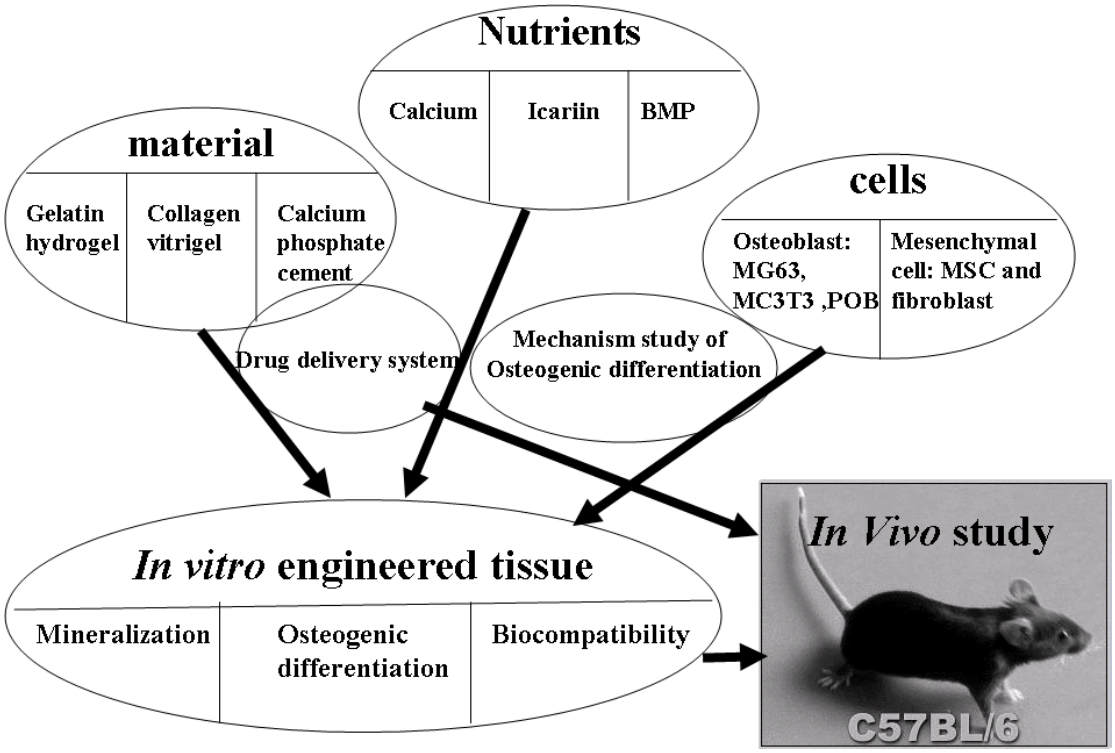

Fig.1. Research Scheme of this thesis.

Results and Discussion:

1. **Calcium enriched medium enhances mineralization *in vitro* but lacks the ability to induce osteogenic differentiation *in vitro* and bone regeneration *in vivo***

   Previous research in our lab has found that calcium enriched medium is able to induce greatly enhanced mineralization in osteosarcoma cell line (Takagishi et. al. Tissue engineering 2006). Other cells including mouse osteoblastic cell line and mouse primary fibroblasts have been tested here. High concentration of calcium enhances both mineralization and osteopontin mRNA expression in these cells. Enhanced calcium depositions also were observed on gelatin hydrogel and collagen sponge scaffolds with cells cultured in calcium enriched medium. But calcium alone can't induce upregulation of ALP and osteocalcin which is the marker of early and later osteogenic differentiation respectively. Further *in vivo* study using these scaffolds failed to induce bone regeneration in critical defect. The key transcription factor runx2 has been studied and its expression in MC3T3-E1 cells became upregulated under extremely high calcium condition. But it failed to induce late stage differentiation; we suggest that it is due to inhibition effect of runx2 on late stage osteogenic differentiation.

2. **Icariin induce osteogenic differentiation in a BMP- and Runx2- dependent manner *in***

To effectively treat bone diseases using bone regenerative medicine, there is an urgent need to develop safe and cheap drugs that can potently induce bone formation. Here, we demonstrate the osteogenic effect of icariin, the main active compound of Epimedium pubescens. Icariin induced osteogenic differentiation in pre-osteoblastic MC3T3-E1 cells and mouse primary osteoblasts. Icariin upregulated the mRNA expression levels of the osteoblast marker genes runt-related transcription factor 2 (Runx2) and inhibitor of DNA-binding 1 (Id-1). The osteogenic effect was inhibited by the introduction of Smad6 or dominant-negative Runx2, as well as Noggin treatment. Furthermore, icariin induced mRNA expression of bone morphogenetic protein (BMP)-4. These data suggest that icariin exerts its potent osteogenic effect through induction of Runx2 expression, production of BMP-4 and activation of BMP signaling. Furthermore, combination of icariin and TH synergistically induce osteogenic differentiation to a similar extent as BMP-2 does in MC3T3-E1 cells. Icariin also enhance BMP's osteogenic ability in fibroblast cell line. The anabolic effect of icariin was also confirmed in mouse calvarial defect model. Eight-week-old male C57BL/6N mice were transplanted with icariin-calcium phosphate cement (CPC) tablets or CPC only (n=5) and bone regeneration was evaluated at 4w, 6w and 8w after the operation. Significant new bone formation was observed in icarrin-CPC group at 4w and thickness of new bone increased at 6w and 8w. Obvious blood vessel formation also occurred in icariin-induced new bone. We are now evaluating the effect of icariin on age-related osteoporosis. After 6 weeks IP administration, thickness of trabicular bone of SAMP6 mice were significantly increased in icariin treated group.

3. Application of Type I collagen vitrigel to BMP-2 local delivery

Bone morphogenetic protein-2 is a very promising candidate for the treatment of bone diseases and defects, but more effective therapeutic methods are required due to its instability *in vivo*. A controlled and localized delivery system of bone morphogenetic protein-2 would be appropriate for effective bone regeneration. Here, we developed a novel delivery system of bone morphogenetic protein-2 using vitrigel (a novel stable collagen gel membrane prepared from vitrified type I collagen) for *in vivo* bone regeneration. Scanning electron microscopy revealed that the collagen vitrigel formed a tightly woven network with average pore sizes of about 1-2 μm. The vitrigel scaffold delivery system exhibited sustained release of bone morphogenetic protein-2 and

more than 80% of the total bone morphogenetic protein-2 was still retained in the vitrigel after 15 days in phosphate-buffered saline *in vitro*. Bone morphogenetic protein-2-containing vitrigel was transplanted into mouse calvarial defects. The enhanced mechanical strength of the vitrigel made it easier to implant into defects without damage. Obvious bone regeneration was observed in the defects of mice treated with as little as 0.19 μg of bone morphogenetic protein-2 at 4 weeks after the transplantation. The local and sustained delivery system for Bone morphogenetic protein-2 developed in the present study may represent a powerful modality for bone regeneration.

Conclusion

This thesis was focused on small molecular compounds to induce osteogenic differentiation and mineralization. Calcium enriched medium and icariin were studied with different kinds of cells and *in vivo* experiments were carried out with some biomaterials.

Calcium enriched medium was able to induce greatly enhanced mineralization in osteosarcoma cell line and other cells including mouse osteoblastic cell line and mouse primary fibroblasts. High concentration of calcium enhances both mineralization and osteopontin mRNA expression in these cells. Enhanced calcium depositions also were observed on gelatin hydrogel and collagen sponge scaffolds with cells cultured in calcium enriched medium. But calcium alone can't induce upregulation of ALP and osteocalcin which is the marker of early and later osteogenic differentiation respectively. Further *in vivo* study using these scaffolds failed to induce bone regeneration in critical defect. The key transcription factor runx2 has been studied and its expression in MC3T3-E1 cells was upregulated under extremely high calcium condition. The highly expressed runx2 inhibited the late stage differentiation and bone regeneration.

A compound isolated from traditional Chinese medicine, icariin was applied to bone tissue engineering. Icariin is a safe and strong bone anabolic agent that may exert its osteogenic effects through induction of Runx2 expression, production of BMP-4 and activation of BMP signaling. Icariin activates BMP signaling at least partly through the induction of BMP-4 expression. However, further studies are required to clarify the molecular mechanism underlying the induction of Runx2 expression by icariin. Combination of icariin and TH induces osteogenic differentiation of MC3T3-E1 cells to the similar extent as BMP-2 does. Icariin also enhances BMP's ability to induce osteogenic differentiation in fibroblast cell line. Two animal models were applied here to

study icariin's effect on bone regeneration *in vivo*. In mouse calvaria defect model, calcium phosphate cement scaffold was used as the drug delivery system which showed a sustained release pattern of icariin *in vitro*. Icariin plus calcium phosphate cement accelerates bone regeneration at week 4 and week 6 after transplantation. Obvious new blood vessels also appear in the new formed bone. The senescence osteoporosis model using SAMP1 and SAMP6 mice also showed icariin has the ability to enhance bone formation *in vivo*. Furthermore, the extremely low cost of icariin and its high abundance in nature make it appealing for bone regenerative medicine.

Finally, a novel drug delivery system was established with vitrigel. A delivery system based on collagen type I vitrigel scaffolds released BMP-2 in a sustained manner for at least 15 days. The BMP-2 released from the delivery system retained its biological activity. The extended BMP-2 delivery by the vitrigel scaffolds induced much more effective new bone formation in a calvarial defect model, indicating that the sustained and controlled delivery of BMP-2 increases the bone regenerative efficacy of BMP-2. The local and sustained delivery system for BMP-2 developed in the present study may represent a powerful modality for bone regeneration.