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Ectopic Bone Formation in vivo Induced by a Novel Synthetic Peptide Derived from BMP-2 Using Porous Collagen Scaffolds

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Abstract: To investigate the osteoinductive and ectopicly osteogenic effects of a novel peptide P24 derived from bone morphogenetic protein 2 (BMP2), biodegradable collagen scaffolds (CS) were used to load BMP-2-derived peptide solutions with different concentrations (0.4 mg peptide/CS, 0.1 mg peptide/CS and pure CS, respectively), and the implants were implanted into muscular pockets on the back of Wistar rats. Radiographs and histological analysis were performed to evaluate the ectopic bone effects. Active ectopic bone formation was seen in both groups containing the peptide at different concentration (0.4 mg and 0.1 mg), whereas no bone formation and only fibrous tissue was seen in the pure CS group. The new bone formation induced by the peptide P24 displayed a dose-dependent and time-dependent efficiency. The new bone formation in the 0.4 mg peptide/CS group significantly increased than that of the 0.1 mg peptide/CS group. This novel BMP-2-derived peptide had excellent osteoinductive and ectopicly osteogenic properties which were similar to those of BMP2.

Key words: bone tissue engineering; biomimetic material; bone morphogenetic protein 2; osteoinduction; peptide

1 Introduction

The BMP family is currently assumed as the only group that can induce bone formation in both ectopic and orthotopic sites in vivo. Currently, more than 15 different kinds of BMPs have been identified. It has been widely accepted that BMP-2 is one of the most important growth factors among BMPs. BMP-2 appeared to have the highest potent osteoinductive property to induce mesenchymal cells to differentiate into osteoblasts and chondroblasts compared with other BMPs[1]. However, the use of BMP requires large amounts for its short half-life and it can not maintain marked osteoinductive effect in vivo. Furthermore, it is difficult to produce BMP-2 in mass and then put them into broad application in medicine. So the above problems become focuses in the field of bone tissue engineering now[2,3].

At present, BMP-2 has been produced mainly by gene engineering, but it can not be produced in a great quantity for the complex production units and technologies of preparation, long cycle length, low yields and expensive prices. Moreover, the problem of security in the relative productions of gene engineering is in debate currently[4,5].

In this study, a novel peptide derived from BMP2 was designed and synthesized, then loaded it in porous collagen scaffolds and implanted them into the muscular pockets on the back of 24 Wistar rats to evaluate whether this novel peptide could be used as a high-efficient and economic substitute for BMP-2.

2 Experimental

2.1 Materials

The BMP-2-derived peptide, P24, was synthesized by FMOC/tBu solid-phase peptide synthesis (SPSS). The purity of the peptide was 96.8% determined by high performance liquid chromatography (HPLC). HPLC and mass spectrometer (MS) were used to evaluate synthesis of the peptide.

Porous collagen scaffold (CS) was fabricated by a solvent casting, particulate leaching process. The scaffolds were square with a volume of 125 mm^3,

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and their pore size ranges were controlled by using NaCl particles with a diameter of 150-250 μm in the processing.

2.2 Preparation of implants

The peptide P24 was dissolved with a buffer (PBS (0.01 M, pH 7.4)) at a concentration of 1 mg/mL, and the buffer solution was filter-sterilized. For the 0.4 mg or 0.1 mg peptide/ CS implant, a sterile CS was mixed with each of the peptide solutions which containing 0.4 mg or 0.1 mg of the peptide. Pure CS was used as the control implant.

2.3 Surgical procedure

Male Wistar rats weighed about 150±20 g were used for the study. The rats were anesthetized by intraperitoneal injection of ketamine(KT). The hairs around the surgical wound areas were shaved and the areas were prepared in a sterile fashion. 1-cm longitudinal linear incisions were made in the rats' backs bilaterally to expose the musculus sacrospinalis. The animals were randomly assigned to either the 0.4 mg peptide/CS composite implant (n=12) or the 0.1 mg peptide/ CS composite implant (n=12). The experimental material was placed into one side of musculus sacrospinalis of each rat, while the pure CS implant was put into the other side of the muscles, and the wounds were closed with 5-0 nylon interrupted sutures.

2.4 X- ray evaluation

At the postoperative 3rd, 6th and 8th week, the rats were examined by X-ray to observe whether new bone formation was formed in vivo or not.

2.5 Histologic evaluation

After the X-ray examination, four rats in each group were sacrificed. The implanted region, together with the surrounding muscles, was removed. Then each specimen was rinsed in sterile saline and fixed in 10% neutral formalin, sectioned at 5 μm thick, stained with hematoxylin/eosin(HE) stain and examined using a light microscope. In short, formalin-fixed and paraffinized sections were hydrated by successive immersion in xylene, ethanol, and PBS (0.01 M, pH 7.4). Histologic findings were compared between groups.

2.6 Image analysis

Photographs from each section under 4-fold object lens including graft materials were captured and analyzed using Scion Image v4.0.2 (Scion Corp., Frederick, MD). The percentage of the area of new bone formation in every field of vision was calculated and compared.

2.7 Statistical analysis

All data were expressed as arithmetic mean standard deviation ($\bar{x}\pm s$). F test was used for comparison of significant variance among several groups. The significance of difference between groups was checked by q check of analysis of variance. A value of P<0.05 was considered significant.

Table 1 Results

Peak No.	Peak ID	Ret time	Height	Area	Conc.
1		11.755	19784.504	274955.719	1.8660
2		12.238	505505.781	14257374.000	96.7562
3		13.967	12102.517	202024.625	1.3779
Total			537389.802	14735364.344	100.0000

3 Results

3.1 Characterization of the peptide

High performance liquid chromatogram and mass spectra spectrum show that the peptide P24 was successfully synthetized(Figs.1-2) and the purity of the peptide P24 is 96.8%(Table1).

3.2 X- ray evaluation

In the 0.4 mg peptide/ CS group, high-density tissue consistent with new bone was observed in muscles at the postoperative 3rd week, and the imbedded materials were not absorbed obviously and combined tightly with the surrounding osseous tissue (Fig.3). In the observation period, the imbedded materials were absorbed gradually. At the postoperative 8th week, the bone density of the new bone was similar to that of the autogenous bone. In the control side, there

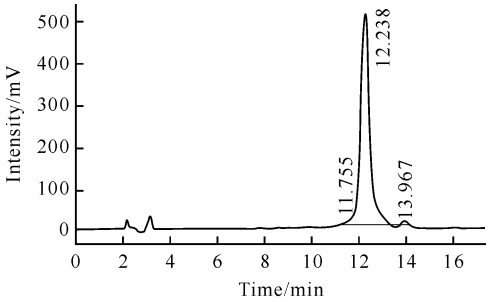

Fig.1 High performance liquid chromatogram

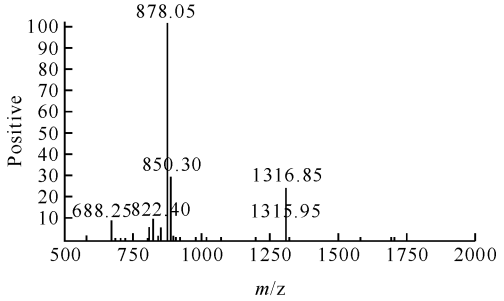

Fig.2 Mass spectra spectrum

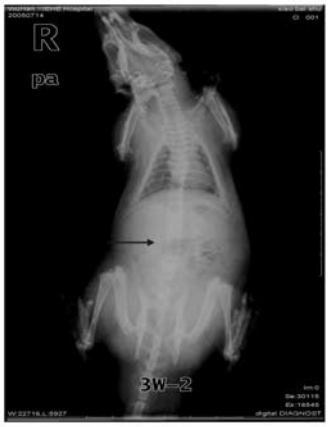

Fig.3 X-ray of the 0.4mg peptide/ CS group 3 weeks postoperatively:high-density tissue consistent with new bone was formed(arrow)

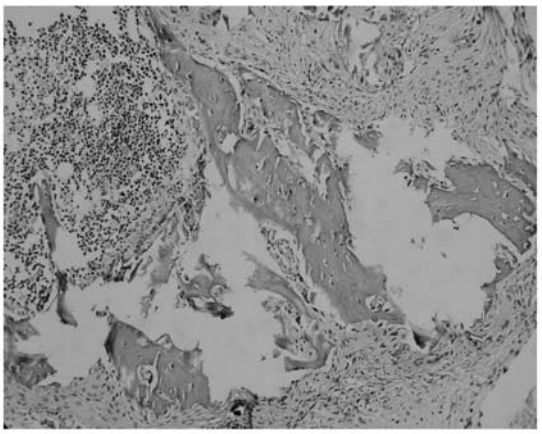

Fig. 4 The 0.4mg peptide/ CS group 8 weeks postoperatively: thick trabecular bones has formed and serried osteoblasts could be observed around the trabecular bones, × 200

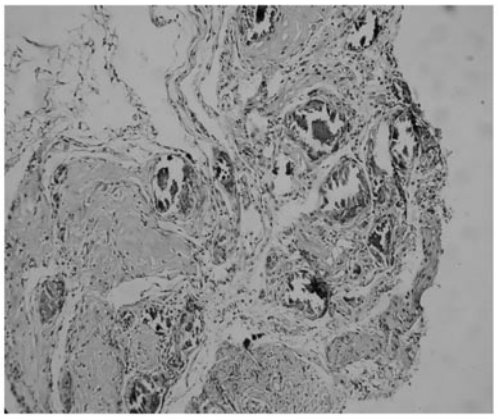

Fig.5 The 0.4mg peptide/ CS group 8 weeks postoperatively: a little new bone with small volumes diffused distribution has formed, × 100

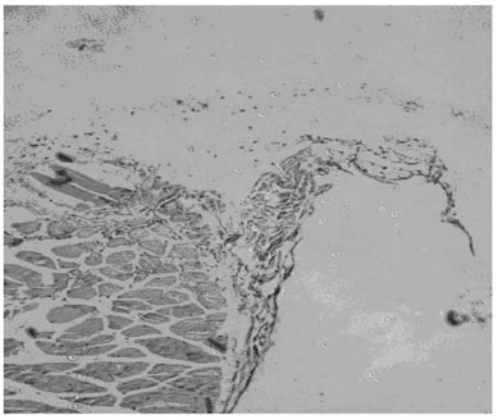

Fig.6 The control group 8 weeks postoperatively: no new bone but a little fibrous connective tissue has formed, × 100

Table 2 The ratio of the area of new bone in each group at 3, 6, 8 weeks($\bar{x} \pm s$)

Groups	Ratios of new bone areas/%		
	3 weeks（n=4）	6 weeks（n=4）	8 weeks（n=4）
0.4mg peptide/CS group	14.7329 ± 5.2356*	18.4987 ± 5.6321*$^{\triangle}$	21.5784 ± 4.4875*$^{\triangle\#}$
0.1mg peptide/CS group	5.1249 ± 2.6892	8.4586 ± 2.8540	8.9412 ± 2.9740

* P<0.05, as compared with 0.1mg group; Δ P<0.05, as compared with 3w 0.4mg group; # P<0.05, as compared with 6w 0.4mg group

was not new bone formation at all.

In the 0.1 mg peptide/CS group, no evident bone formation can be observed in the entire observation period. In the control side, there was not new bone formation neither.

3.3 Histologic evaluation

Histological examination showed that, in the 0.4 mg peptide/CS composite group, immature, diffused bone-like tissues were observed in muscles at 3 weeks, and a lot of bone marrow derived stroma cells (BMSCs), bone lacuna and osteoblasts with large amounts of particles of materials could be seen there. At 6 weeks, woven bones were in lamellar distribution and they were thick and close-up. At 8 weeks, trabecular bones became more thick and wide than before and serried osteoblasts could be observed around the trabecular bones, while there were not many inflammatory cells could be seen (Fig.4).

In the 0.1 mg peptide/ CS composite group, a little woven bone with small volumes and diffused distribution could be observed at 3 weeks. At 6 weeks, the bone mass had increased in contrast with that of 3 weeks, while there was not obvious increasing of the bone mass at 8 weeks(Fig.5).

In the pure CS control group, there was no new bone formation in each observation period, and only a little fibrous connective tissue has formed. With the time increased, the mass of the materials gradually

degraded. The materials degraded and were absorbed completely at 8 weeks (Fig. 6).

3.4 Image analysis

With the time increasing, the 0.4 mg P24/CS group demonstrated an increase in the ratio of the area of new bone formation. The ratio of the new bone area in 0.4 mg P24/CS group revealed a significant difference in vivo over time(P<0.05), while that of 0.1 mg P24/CS group did not show a significant difference over time(P>0.05) (Table 2).

4 Discussion

Replacement of natural bone tissue by graft materials and products of tissue engineering having composition, structure, and biological features that mimic natural tissue is a goal to be pursued. Natural bone is a complex biomineralized system with an intricate hierarchical structure. It is a typical example of an "organic matrix-mediated" self-assembling and biomineralization process which constituted of nanosize plate-like crystals of carbonated hydroxyapatite(HA) orderly grown in intimate contact with collagen fibers[6,7]. Meanwhile, investigations showed that this biomineralization process was affected by the content and space distribution of anionic functional groups. Mineralization during vertebrate bone growth is a classic example. The best biomineralization effects could be attained only when the density of anionic functional groups on the surface of the material is ample and suitable[8-14].

It has been widely recognized that it is the phosphoprotein that promoted nucleation and self-assembling mineralization of the apatite. Previous research indicated that Asp (aspartic acid) and phosphorylated Ser (serine) were abundant in the phosphoprotein. Anionic group can make this group of protein more adhesive to calcium ion and phosphate ion. It also contains functional groups that can allow further modification to combine with cell-specific recognition factors, which induce cells to adhere, differentiate and proliferate on the surface of the materials[8-14].

In the present study, we designed and synthesized the peptide P24, which was derived from knuckle epitope of the amino acids sequence of BMP-2 that can induce bone formation[15-18]. The peptide P24 included Asp and phosphorylated Ser, which could form acidic surrounding to promote deposition of calcium and phosphate ions and accelerate nucleation and mineralization, so it can mimic the function of organizing and accumulating mineralization of natural bone. At the same time, peptide with short chain can thoroughly expose active sites that can be combined with the specific cell surface receptor efficiently through mechanisms of intercellular communication and signal transduction system.

The radiological evaluation of the rats showed that the peptide P24 can induce ectopic bone at 3 weeks and this result identified the successful design of the peptide P24 sequence. The result of histologic evaluation showed that both groups which contained the peptide P24/CS can show new bone formation. The 0.4 mg peptide P24/CS group demonstrated evident new bone formation in contrast with moderate new bone formation in the 0.1 mg peptide P24/CS group. Meanwhile, only some fibrous tissue can be seen in the group of pure CS without any bone formation. It can be concluded that the BMP-2-derived peptide P24 had excellent osteoinductive property and the ability of ectopic bone formation similar to the that of BMP2.

We chose porous collagen scaffold as a carrier for the peptide P24 for several reasons: collagen has excellent biocompatibility, hydrophilicity, and suitable biodegradation which may sufficiently absorb large amounts of peptide and release it for a long time. Natural materials such as collagen are advantageous in that they contain biological information that provides a good interface for the adhesion, migration, proliferation and differentiation of cells. Three-dimensional interconnected porous collagen scaffolds also provide a good support and sufficient nutrition exchange for cells than other kind of biomaterials such as amorphous hydrogels[19-22]. So, after loaded with peptide, the mesenchymal stem cells from the surrounding muscle or connective tissue will be recruited by the peptide and result in active ectopic bone formation. Porous collagen scaffolds provide biomimetic micro-environment cue for the osteogenesis differention of the stem cells.

5 Conclusion

In Conclusion, this novel BMP-2-derived peptide had excellent osteoinductive and ectopicly osteogenic properties which were similar to those of BMP2. Collagen scaffold was a good local delivery system for the peptide. This study represent the first successful demonstration of ectopic osteogenesis using BMP2-derived peptide and collagen porous scaffolds, which may be a high-efficient and economic substitute for the

expensive BMP2 and the insecure BMP2 gene. Further studies directed at characterizing the time course and the dose-response relationship of the effect of this peptide on bone formation with more suitable materials are under way.

References

[1] Cheng HW, Jiang W, Phillips FM, *et al*. Osteogenic Activity of the Fourteen Type of Human Bone Morphogenic Proteins(BMPs)[J]. *The Journal of Bone and Joint Surgery* (*American volume*), 2004, 86- A (1): 141-147

[2] Boden SD. The ABCs of BMPs[J]. *Orthopaedic Nursing*, 2005, 24(1): 49-52; quiz 53-4

[3] Malafaya PB, Silva GA, Baran ET, *et al*. Drug Delivery Therapies II: Strategies for Delivering Bone Regenerating Factors[J]. *Current Opinion in Solid State and Materials Science*, 2002, 6(4): 297-312

[4] Gugala Z, Olmsted D. Osteoinductive by ex vivo Adenovirus-mediated BMP-2 Delivery is Independent of Cell Type[J]. *Gene Therapy*, 2003, 10(16): 1289-1295

[5] Mountain A. Gene Therapy: the First Decade[J]. *Trends in Biotechnology*, 2000, 18(3): 119-128

[6] Guo XD, Du JY, Zheng QX, *et al*. Molecular Tissue Engineering: Concepts, Status and Challenges[J]. *Journal of Wuhan University of Technology - Mater. Sci. Ed.*, 2002, 17(3): 30-34

[7] Tana J, Saltzmana W. Biomaterials with Hierarchically Defined micro-and Nanoscale Structure[J]. *Biomaterials*, 2004, 25: 3593-3601

[8] Hubbell JA. Materials as Morphogenetic Guides in Tissue Engineering[J]. *Current Opinion in Biotechnology*, 2003, 14(5): 551-558

[9] Hartgerink JD, Beniash E, Stupp SL. Self-assembly and Mineralization of Peptide-amphiphile Nanofibers[J]. *Science*, 2001, 294: 1684-1688

[10] Jadlowiec JA, Celil AB, Hollinger JO. Bone Tissue Engineering: Recent Advances and Promising Therapeutic Agents[J]. *Expert Opinion on Biological Therapy*, 2003, 3(3): 409-423

[11] Riminucci M, Bianco P. Building Bone Tissue: Matrices and Scaffolds in Physiology and Biotechnology[J]. *Brazilian Journal of Medical and Biological Research*, 2003, 36(8): 1027-1036

[12] Shin H, Jo S, Mikos AG. Biomimetic Materials for Tissue Engineering[J]. *Biomaterials*, 2003, 24(24): 4353-4364

[13] Cui DX, Gao HJ. Advance and Prospect of Bionanomaterials[J]. *Biotechnology Progress*, 2003, 19(3): 683-92

[14] Murphy WL, Mooney DJ. Bioinspired Growth of Crystalline Carbonate Apatite on Biodegradable Polymer Substrata[J]. *Journal of the American Chemical Society*, 2002, 124(9): 1910-1917

[15] Saito A, Suzukib Y, Ogata SI. Activation of Osteo-progenitor Cells by a Novel Synthetic Peptide Derived from the Bone Morphogenetic Protein-2 Knuckle Epitope[J]. *Biochimica et Biophysica Acta*, 2003, 1651: 60- 67

[16] Kirsch T, Sebald W, Dreye MK. Crystal Structure of the BMP-2-BRIA Ectodo Main Complex[J]. *Nature Structural & Molecular Biology*, 2000, 7: 492-496

[17] Kirsch T, Nickel J, Sebald W. BMP-2 Antagonists Emerge from Alterations in the Low-affinity Binding Epitope for Receptor BMPRII[J]. *The EMBO Journal*, 2000, 19: 3314-3324

[18] Knaus P, Sebald W. Cooperativity of Binding Epitopes and Receptor Chains in the BMP/TGFbeta Superfamily[J]. *Biological Chemistry*, 2001, 382: 1189-1195

[19] Wozney J, Seeherman H. Protein-based Tissue Engineering in Bone and Cartilage Repair[J]. *Current Opinion in Biotechnology*, 2004, 15(5): 392-398

[20] Saito N, Takaoka K. New Synthetic Biodegradable Polymers as BMP Carriers for Bone Tissue Engineering[J]. *Biomaterials*, 2003, 24(13): 2287-2293

[21] Geiger M, Li R H, Friess W. Collagen Sponges for Bone Regeneration with rhBMP-2[J]. *Advanced Drug Delivery Reviews*, 2003, 55(12): 1613-1629

[22] Winn SR, Uludag H, Hollinger JO. Carrier Systems for Bone Morphogenetic Proteins[J]. *Clinical Orthopaedics and Related Research*, 1999, 367(Suppl): S95-106