Simple and Efficient Gene Therapy for Bone Regeneration

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Introduction

Therapy using recombinat human bone morphogenetic protein-2 (BMP-2) is expected to promote bone healing, and regeneration. The protein is soluble and disperses soon after implantation, so that many researchers have sought the delivery system to retain the protein at the target site. Previous studies using BMP-2 protein or BMP-2 gene expressing viral vectors had required a suitable carrier matrix, expensive bone morphogenetic protein to purify, or antigenic viral vectors. On the other hand, non viral delivery systems do not require an expensive BMP protein, a carrier matrix, or treatment of immunosuppression for general or local. In particular, electroporation is an efficient and simple method. Under these conditions, the combination of plasmid vectors and electroporation has been used for tissue regeneration, considering clinical applications. But, the value of this technique for local tissue regeneration has not yet been fully defined. The aim of our first study was to determine whether gene transfer by transcutaneous electroporation could induce ectopic bone formation in the muscles of rats.

It has been generally accepted that BMP-2 can induce osteogenesis in skeletal muscles via endochondral ossification. However, it is not clear how the ossification process occurs after the BMP-2 gene transfer to skeletal muscles in rats using *in vivo* electroporation. In our second study, we evaluated the ossification process by BMP-2 gene transfer using *in vivo* electroporation.

In this model, the volume of the injected plasmid is restricted to 50 ml, given the surface area the electrodes can cover. Furthermore, any relationship between the plasmid dose and the electrical parameters will affect the efficiency of the gene transfer. As the third study, our trial was designed to improve the method to enhance bone formation at the intrinsic level of the osteoinductive activity. Here, to enhance bone regeneration or repair by BMP gene transfer, we examined the effects on osteoinduction of the direct and simultaneous gene transfer of BMP-2 and BMP-7 expression plasmid vectors into skeletal muscles by electroporation.

Materials and Methods

Plasmid vector

We constructed the pCAGGS-BMP-2 and pCAGGS-BMP-7. PCAGGS vector has the CAG promoter that shows a high potential of gene expression.

Direct injection and trascutaneous in vivo electroporation

At first, we nipped the muscle with plate type of electrodes via skin without a skin incision. Next, each dose of plasmid in PBS (total volume 50ul) was injected into the center of the target areas between the electrodes. And electroporation was started immediately after the injection by applying eight electrical pulses (on condition with 100V, 50msec).

X-gal staining

To confirm the gene expression that was transferred after transcutaneous electroporation, we performed X-gal staining one day after the injectetion of lacZ expression plasmid with or without electroporation.

RT-PCR

To identify the expression of the BMPs and osteoblastic marker gene in the skeletal muscles after the treatment, we performed RT-PCR.

Western blot

BMPs productions were identified by western blot analysis in the skeletal muscles,

Histologic, Immunohistochemical and Radiographic analyses

We employed soft X-ray, HE staining, von Kossa staining and immunohistochemistry for detection of BMP-2 in the time course studies.

Biochemical Tests

For quantitative analyses of bone formation by gene transfer with pCAGGS-BMP-2 or pCAGGS-BMP-7 using transcutaneous *in vivo* electroporation, ALP activity (IU/mg protein) and the calcium (Ca) content (mg/mg tissue) of the tissues were measured.

Results

Transcutaneous in vivo electroporation is successful for repeated gene transfer to the skeletal muscles without surgical operation.

A single electroporation with 25ug of pCAGGS-lacZ, showed much wider distribution of X-gal stain product among muscle fibers than that of after 5 injections of plasmid without electroporation. Human BMP-2 mRNA was detected in all muscles after the transfection with pCAGGSBMP-2 by transcutaneous electroporation, but not in muscles with pCAGGS. After 1, 3, or 5 sessions of electroporation with 25ug of pCAGGS-BMP-2, we detected the production of BMP-2 in the target muscles. On day 7 after 5 repetition of electroporation with 25ug of pCAGGS-BMP-2, histologic examination revealed prominent mononuclear cell infiltration into the target muscles, and the degradation of muscle fibers, although it was not severe. On the other hand, the damage of muscles subjected to a single electroporation was mild. It was revealed that BMP-2 was highly expressed in the muscle fibers around blood vessels.

Transcutaneous electroporation with pCAGGS-BMP-2 induces ectopic bone formation

X-ray radiographs revealed opacities which had clear margins in the target muscles of all groups of electroporation with pCAGGS-BMP-2. Histologic examination revealed ectopic bone in the target muscle with bone marrow, and the bone showed to be well-mineralized trabecular bone, including active osteoblasts, osteoclasts and osteocytes in the matrix. ALP activity and Ca

content had become significantly high after *in vivo* electroporation with pCAGGS-BMP-2 as compared with treatment using pCAGGS.

Human BMP-2 gene transfer using transcutaneous in vivo electroporation induced both intramembranous and endochondral ossification

Regarding ossification patterns in our studies, both endochondral ossification and intramembranous ossification were identified. Cartilaginous tissues were identified by histology and immunohistochemistry of type II collagen, and these cartilages underwent calcified cartilage, leading to endochondral ossification was also found. Another ossification pattern, the bone that had undergone intramembranous ossification, that is, the bone matrix was intensively stained for type I collagen, but negative for type II collagen by immunohistochemistry, and light and electron microscopic observations revealed characteristic appearances for bone, was identified also at 10 days after treatment.

BMP-2 and BMP-7 gene transfer by in vivo electroporation induces rapid bone formation and BMP-4 expression

Ten days after treatment, direct and simultaneous transfer of the BMP-2 and BMP-7 genes induced higher ALP activity, more bone formation, and more intense calcification than did the transfer of either gene alone. Moreover, the calcified areas observed 10 days after the gene transfer with pCAGGS-BMP-2 were mostly cartilaginous tissue with very little bone. The muscles transfected with pCAGGS-BMP-7 electroporation did not form cartilaginous tissue, bone, or calcified areas during the observation period. BMP-4 mRNA was also expressed, and its level increased for 3 days in a time-dependent manner in the double gene transfer group.

Conclusions

Transcutaneous *in vivo* electroporation is successful for repeated gene transfer to the skeletal muscles without surgical operation

Transcutaneous electroporation with pCAGGS-BMP-2 induces ectopic bone formation

Human BMP-2 gene transfer using transcutaneous in vivo electroporation Induced both intramembranous and endochondral ossification

BMP-2 and BMP-7 gene transfer by *in vivo* electroporation induces rapid bone formation and BMP-4 expression

Human BMPs gene transfer using transcutaneous *in vivo* electroporation is expected to be an useful technique for clinical application.