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Biodegradable hydrogels for bone regeneration through growth factor release

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Abstract: For successful tissue regeneration, growth factors should be released over a long period of time at the site of action, but their *in vivo* half-life time is very short. The sustained release of growth factors could be achieved by taking advantage of biodegradable hydrogels prepared from acidic gelatin with an isoelectric point (IEP) of 5.0. When mixed with this negatively charged gelatin, positively charged growth factors ionically interacted at the neutral pH to form a polyion complex. Gelatin hydrogels were enzymatically degraded in the body with time and the time profile of growth factor release was in good accordance with that of in vivo hydrogel degradation. This indicates that the growth factor complexed with the acidic gelatin constituting hydrogels was released as a result of their biodegradation. This article briefly overviews the *in vivo* release of basic fibroblast growth factor (bFGF) and transforming growth factor β1 (TGF-β1) from gelatin hydrogels.

INTRODUCTION

Cell growth factors are known to greatly contribute to tissue regeneration at different stages of cell proliferation and differentiation (ref. 1). However, tissue regeneration by use of growth factors has not been always successful because of several reasons. One of them is too short half-life periods of growth factors in the body. A possible means to circumvent this problem is to incorporate a growth factor into an appropriate polymer matrix for achieving its sustained release at the site of action. It is very likely that the growth factor in the matrix is protected from proteolysis and antibody neutralization, resulting in prolonged retention of the biological activity in vivo. Many researchers have attempted to release growth factors from various polymer matrices over a long time period (ref. 2-20). The largest problem associated with this release technology is the loss of biological activity of growth factors during the growth factor-polymer formulation process due to denaturation and deactivation of growth factors. Protein is generally denatured and loses its biological activity when exposed to harsh environments, such as heating, sonication, and organic solutions (ref. 21-23). Therefore, it is required to exploit a new formulation method using polymer carriers for proteins under mild conditions to minimize protein denaturation. From this point of view, polymer hydrogels seem to be preferable as release matrix candidate of growth factors because of their biosafety and high inertness toward protein drugs (ref. 24). However, sustained release of growth factors over a long time period could not be expected from hydrogels, since the release is generally diffusion controlled through aqueous channels in the hydrogels. Thus, to achieve the sustained release of growth factors, it will be a key strategy to immobilize growth factors to polymer carrier molecules constituting the hydrogel through molecular interactions. As one trial for sustained release of growth factors from polymer hydrogels, we have been attempting to take advantage of polyion complexation which takes place between the growth factor and polymer molecules in hydrogels.

Figure 1 schematically shows the concept of growth factor release from a biodegradable polymer carrier on the basis of polyion complexation. A positively charged growth factor will be electrostatically complexed with negatively charged polymer chains constituting the carrier matrix. It seems unlikely that all of the ionic interactions between two polyelectrolytes are dissociated at the same time, in contrast to low-molecular-weight electrolytes. However, the complexed growth factor will be released from the growth factor-carrier complex, if a significant environmental change, such as increased ionic strength, takes place. Even if such an environmental change does not occur, degradation of the polymer carrier in the body will also lead to growth factor release. The latter is more likely to happen in vivo than the former for the polyion complex. Thus, the release carrier is preferred to be prepared from biodegradable polymers. In this case, the release of growth factors is regulated by controlling the carrier biodegradation.

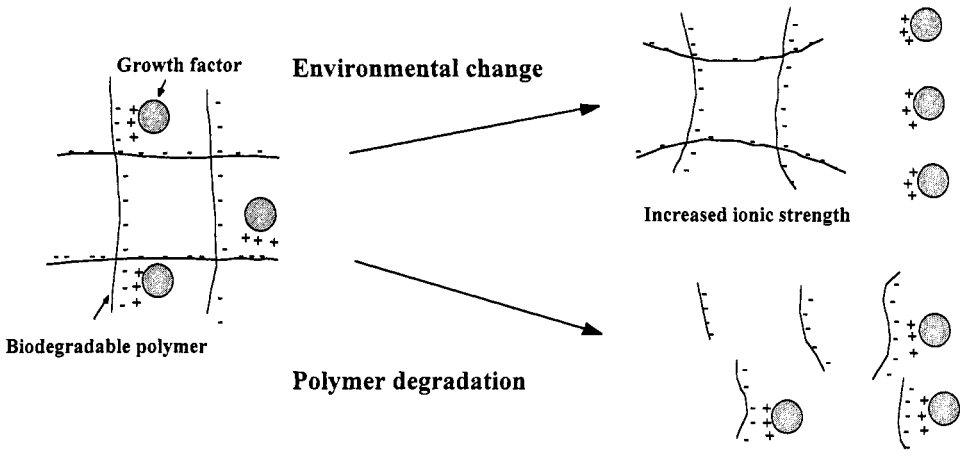

Fig. 1 Conceptive scheme of growth factor release from biodegradable polymer carrier on the basis of polyion complexation.

To make use of polyion complexation for the sustained release of a growth factor, it is absolutely necessary to employ a highly bio-safe polyelectrolyte as their carrier matrix. In addition, if biodegradability is required for the carrier, the material to be used is limited to natural polymers with charged groups, such as proteins and polysaccharides, because of poor availability of synthetic polymers having similar properties. Therefore, as the carrier polymer, we have selected biodegradable gelatin, because the biosafety of gelatin has been proved through its long clinical usage as a plasma expander, surgical biomaterials, and drug ingredients. Another unique advantage of gelatin as drug carrier is the electrical nature of gelatin which varies by the collagen processing method (ref. 25). For example, the alkaline process through hydrolysis of amide groups of collagen yields gelatin having a high density of carboxyl groups, which make the gelatin molecule negatively charged and lower the IEP of gelatin. If the growth factor to be released is basic, the acidic gelatin with an IEP of 5.0 is preferable as the carrier material.

GELATIN HYDROGELS AS RELEASE MATRIX

The acidic gelatin is chemically crosslinked with water-soluble carbodiimide or glutaraldehyde to prepare gelatin hydrogels (ref. 18, 20). As a measure to evaluate the crosslinking extent of gelatin hydrogels, one can use their water content, which is determined from the hydrogel weight before and after swelling in phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C and expressed as the weight ratio of water in hydrogel to the whole wet hydrogel (ref. 18). The water content of gelatin hydrogels decreases with an increase in the crosslinking agent and gelatin concentration and the reaction time, ranging from 99 to 85 wt%. Gelatin hydrogels are degradable in the body, but not in vitro, where there is no enzymes. The hydrogel loses its weight with implantation time and finally the mass disappears from the implantation site. The degradation rate depends on the water content of hydrogel; the higher the water content of the hydrogel, the faster the degradation. A similar dependence of in vivo hydrogel degradation on the water content was observed in a biodegradation study conducted using ^{125}I-labeled gelatin hydrogels (ref. 26). Variation of the degradation period of hydrogels from 5 days to 5 weeks was possible. Hydrogel preparation in the presence of growth factors leads to their activity loss probably because of concurrent chemical crosslinking of growth factors (ref. 18). On the contrary, our method, in which aqueous solution of growth factor is dropped onto freeze-dried gelatin hydrogels, followed by leaving them under various conditions to allow the growth factor to sorb into the gelatin hydrogels, will prevent the protein molecules from chemical deactivation. This method is also effective in quantitatively incorporating growth factors into gelatin hydrogels with high reproducibility, irrespective of their water content, because the volume of growth factor solution is much less than that theoretically required to impregnate the growth factor into the hydrogels. For example, a bFGF-incorporating gelatin hydrogel can be prepared by dropping scores of microliter of bFGF onto several milligram of a freeze-dried hydrogel of acidic gelatin with an IEP of 5.0,

followed by standing overnight at 4 °C. bFGF was released from the bFGF-incorporating gelatin hydrogel in PBS at 37 °C within 1 day up to about 30 % of the initial loading (ref. 18). This demonstrates that bFGF cannot be released from acidic gelatin hydrogels under the in vitro non-degradation condition if basic bFGF molecules are complexed with acidic gelatin. Addition of NaCl into the PBS suppressed bFGF sorption to the hydrogel in a dose-dependent manner (ref. 26). These findings indicate that an electrostatic interaction between bFGF and the acidic gelatin contributes to the bFGF sorption.

To assess the in vivo profile of growth factor release from gelatin hydrogels, an acidic gelatin hydrogel incorporating ^{125}I-labeled bFGF was implanted subcutaneously into the back of mice and the residual radioactivity was measured at different time intervals. The ^{125}I-labeled bFGF-incorporating gelatin hydrogels showed a decrease in residual radioactivity with implantation time. The decrement pattern of radioactivity depended on the hydrogel degradability in such a manner that the radioactivity retained for a longer time with the lower water content of hydrogel. Irrespective of the hydrogel water content, the decrement pattern of bFGF radioactivity in the hydrogel was in good accordance with that of the gelatin radioactivity evaluated by use of ^{125}I-labeled gelatin hydrogels incorporating bFGF (Fig. 2).

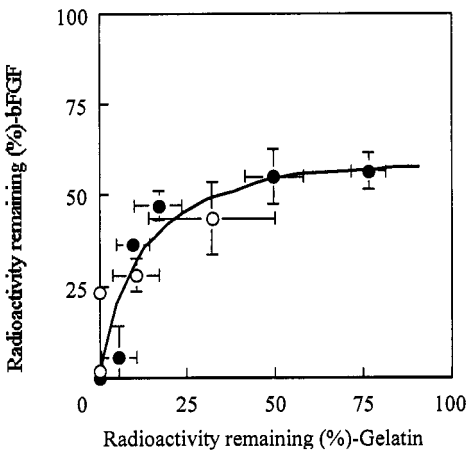

Fig. 2. Relationship between the radioactivity of bFGF and gelatin remaining after subcutaneous implantation of ^{125}I-labeled bFGF-incorporating hydrogel prepared from ^{125}I-labeled acidic gelatin. The water contents of the hydrogels are (□) 98.8 and (●) 96.9 wt%.

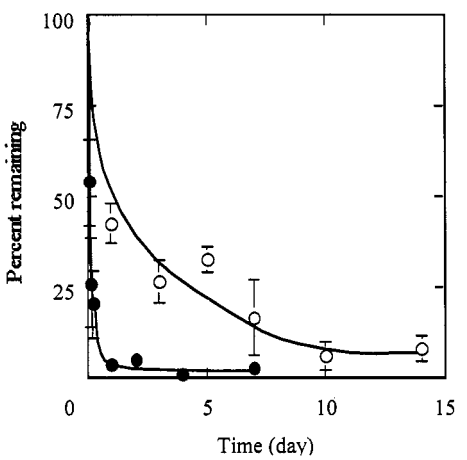

Fig. 3. In vivo decrement patterns of radioactivity in the back subcutis of mice after subcutaneous implantation of ^{125}I-labeled TGF-β1-incorporating acidic gelatin hydrogels (□) and subcutaneous injection of ^{125}I-labeled TGF-β1 aqueous solution (●).

In addition, the half-life period of bFGF retention in gelatin hydrogels with different water contents was found to be linearly related to that of the amount of hydrogels remaining. It is apparent from these findings that bFGF is released from the gelatin hydrogel as a result of hydrogel degradation in the body, probably together with degraded gelatin fragments. Figure 3 shows the time profile of TGF-β1 retention in the back subcutis of mice after subcutaneous implantation of TGF-β1-incorporating acidic gelatin hydrogels. Obviously, incorporation of TGF-β1 into the acidic gelatin hydrogel enhanced the in vivo retention, whereas free TGF-β1 was rapidly cleared from the injected site, similar to that obtained for bFGF (ref. 26).

Bone regeneration through growth factor release

As mentioned earlier, the most important issue regarding the delivery of growth factors is whether or not the growth factor released into the body still retains its biological activity. Evaluation of the growth factor

activity normally employs in vitro culture techniques because of their simplicity and convenience, compared with in vivo animal experiments. However, any of in vitro non-degradation systems is not applicable to evaluate the biological activity of the growth factor released, since our release system involves in vivo degradation of hydrogel matrices for the growth factor release. Thus, to assess the activity retention of growth factor, we should pursue the biological events induced by gelatin hydrogels incorporating the growth factor after implantation to animals. It was demonstrated that bFGF incorporation into acidic gelatin hydrogels enhanced angiogenetic (ref. 18,19) and osteogenetic effects (ref. 20), in marked contrast to free bFGF.

As a representative example, results of bone mineral density (BMD) measurement are summarized in Table 1 for the skull defect of rabbits 2, 4, and 6 weeks after TGF-β1 treatment in different dosage forms. Clearly, TGF-β1-incorporating gelatin hydrogels significantly enhanced the BMD of skull defect after 6 weeks of implantation. On the other hand, free TGF-β1 did not exhibit any significant bone regeneration, although BMD tended to be somewhat higher than that of PBS-treated, control rabbits. The BMD at the defect of rabbits treated with bFGF-free, empty gelatin hydrogels was similar to that of control rabbits, indicating that implantation of hydrogels in the defect did not impair bone regeneration at the site. A similar trend was observed for bone formation after 4 weeks of implantation, but the efficacy of the bFGF-incorporating hydrogel in enhancing bone regeneration was not as clear as that of hydrogel implanted for 6 weeks. No significant difference in BMD was observed between these experimental groups after 2 weeks of treatment.

TABLE 1. Bone mineral density (BMD) at the skull defect of rabbits 2, 4, and 6 weeks after treatment with TGF-β1-incorporating gelatin hydrogels.

Treatment with	BMD(mg/cm^2)		
	2 weeks	4 weeks	6 weeks
TGF-β1-incorporating gelatin hydrogel (0.1 μg TGF-β1/rabbit)	58.8± 6.8	81.4±12.7	85.3± 8.5*
TGF-β1 solution (0.1 μg TGF-β1/rabbit)	57.3±10.7	62.6± 6.2	64.4± 4.4
Empty gelatin hydrogel	58.4± 4.2	65.3± 8.1	63.6± 1.9
PBS(-)	57.6± 3.9	57.6± 5.6	56.9± 1.1

* $p<0.05$, significant against rabbit group receiving TGF-β1 solution injection

When implanted into a skull defect, the TGF-β1-incorporating acidic gelatin hydrogel accelerated bone regeneration at the skull defect and closed the defect after 6 weeks of implantation (Fig. 4). On the contrary, insignificant bone regeneration but remarkable ingrowth of soft connective tissues was noticed at the bone defect when rabbits were treated with a TGF-β1-free gelatin hydrogel and free TGF-β1 or left without treatment. The TGF-β1-free gelatin hydrogel neither induced bone formation nor interfered with bone regeneration at the skull defect.

The number of osteoblasts residing near the bone edge of skull defect increased up to 2 weeks and then decreased for both non-treated rabbit groups and those treated with TGF-β1-free, empty gelatin hydrogels. Free TGF-β1 treatment tended to prolong the retention period of cells by a few weeks. On the contrary, rabbit groups treated with TGF-β1-incorporating gelatin hydrogels exhibited a significant increase in the cell number. The high level of cell number was retained for a long time period compared to those with free TGF-β1. It seems that TGF-β1 was released from the gelatin hydrogel retaining the biologically active state and activated osteoblasts to induce bone regeneration at the skull defect. Such a noticeable influence on the osteoblast number was also observed for bone regeneration at the rabbit skull defect by bFGF-incorporating gelatin hydrogels (ref. 20).

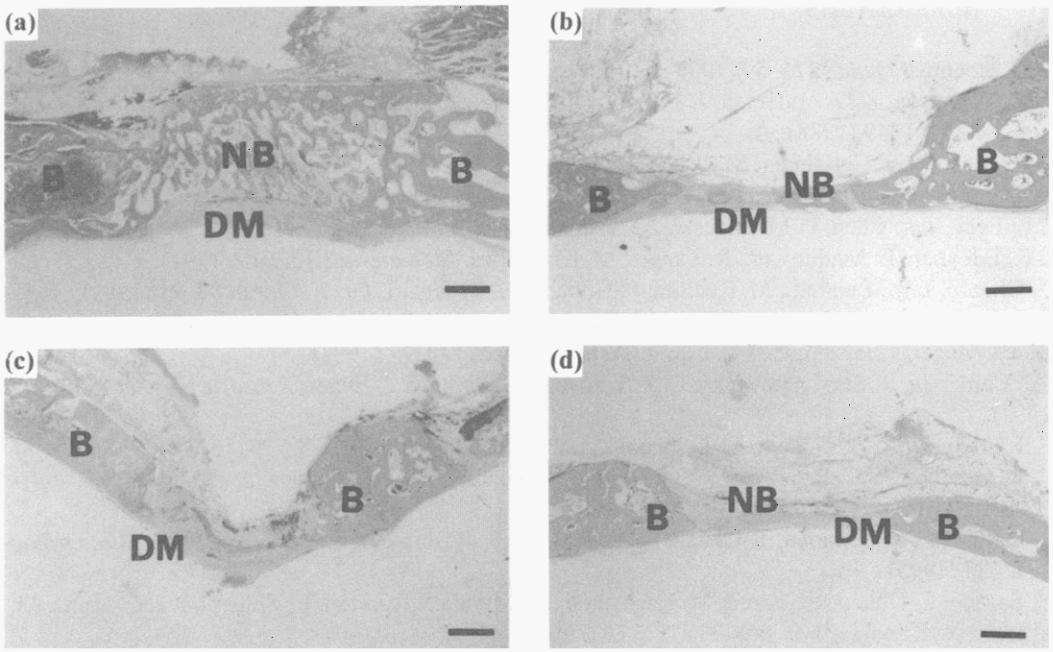

Fig. 4. Histological cross-sections around the skull defect of rabbits 6 weeks after treatment with a TGF-β1(0.1 μg)-incorporating gelatin hydrogel with a water content of 95 wt% (a), 0.1 μg of free TGF-β1 (b), an empty gelatin hydrogel with a water content of 95 wt% (c), and PBS (d): B; bone, DM; dura mater, C; connective tissue, and NB; new bone. (HE staining, x 40) Bar corresponds to 1mm length.

CONCLUSION

The requirement for sustained release of proteins is increasingly becoming important in concert with their production on an industrial scale. However, little has been reported on the technology which facilitates the sustained release of proteins under maintenance of the biological activity. To this end, learning the intact storage manner of bioactive substances in vivo will offer a promising hint for the release design of bioactive proteins. Our technology to release growth factors is based on the polyion complexation, which is commonly observed in the body between growth factors and the extracellular matrix. The interaction of growth factor with biological macromolecules existing in the extracellular matrix enables the growth factor to regulate its biological functions (ref. 27). The gelatin hydrogels which can form polyion complex with growth factors succeeded in facilitating bioloigically active growth factors to release for a desired period of time. In this case, the growth factor release may be mainly governed by the in vivo degradation of matrix hydrogels. In other words, the release period can be regulated by changing the rate of hydrogel degradation which is controllable by the hydrogel water content. Although these conclusions have been deduced from our experimental data on the gelatin hydrogels incorporating bFGF or TGF-β1, this release technology seems to be applicable for any charged biomacromolecules including other proteins and oligo- or polynucleotides. It is theoretically possible for gelatin to form polyion complexes with any type of charged macromolecules although the interaction strength depends on the type of proteins used.

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