

## Development of a New Method to Induce Angiogenesis at Subcutaneous Site of Streptozotocin-Induced Diabetic Rats for Islet Transplantation

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The subcutaneous space is a potential site for clinical islet transplantation. Even though there are several advantages, poor blood supply at this site mainly causes failure of islet survival. In this study, angiogenesis was induced in advance at the diabetic rats subcutis for islet transplantation by implanting a polyethylene terephthalate (PET) mesh bag containing gelatin microspheres incorporating basic fibroblast growth factor (bFGF) (MS/bFGF) and a collagen sponge. The bFGF was incorporated into gelatin microspheres for controlled release of bFGF. As controls, a PET mesh bag with or without either collagen sponges or MS/bFGF was implanted at the subcutaneous site of diabetic rats. Macroscopic and microscopic examinations revealed the formation of capillary network in and around the PET mesh bag containing MS/bFGF and collagen sponges 7 days after implantation when compare with other control groups. When tissue hemoglobin level was also measured, a significantly high level of hemoglobin amount was observed compared with that of control groups. When allogeneic islets mixed with 5% agarose were transplanted into the prevascularized rat subcutis, normoglycemia was maintained for more than 40 days, while other control groups were ineffective. This study demonstrated that combination of gelatin microspheres incorporating bFGF and collagen sponges enabled the mesh to induce neovascularization even at the subcutaneous site of streptozotocin-induced diabetic rats, resulting in improved function of islet transplantation.

Key words: Angiogenesis; Subcutaneous site; Basic fibroblast growth factor (bFGF); Collagen sponge

### INTRODUCTION

Transplantation of isolated islets is an attractive alternative for the treatment of type I diabetes mellitus. Since the first successful isolation of pancreatic islets in 1967 by Lacy et al. (8), many recipient sites have been proposed for islet transplantation. In rodents, good metabolic function and excellent vascularization of islets have been demonstrated in two sites: the liver via portal vein infusion, and in the subcapsular renal space. Islets have also been shown to survive in tissues as diverse as the testis, brain, thymus, submandibular gland, spleen, subcutaneous site, and peritoneal cavity (1). Among these, the subcutaneous site has many advantages for human clinical islet transplantation. Transplantation procedure and retrieval of the graft are easier at this site. However, poor blood supply is the major obstacle for use of this site for cell transplantation. So islet transplan-

tation experiments have commonly been performed in other sites. Angiogenesis could be successfully induced at the subcutaneous site by implanting various artificial biomaterials and growth factor (3–5,9).

In our previous report, we attempted and succeeded in enhancement of angiogenesis at the peritoneal cavity of normal and diabetic rats by implanting a polyethylene terephthalate (PET) mesh bag alone (3,4). These results demonstrated that angiogenesis formation in the PET mesh bag was effective in improving the survival and function of transplanted islets. Few reports have been made on the induction of angiogenesis at the subcutaneous site (7,9). The objective of this study was to induce angiogenesis at the subcutaneous site of diabetic rats for bioartificial pancreas transplantation. We used a combination of gelatin microspheres incorporating basic fibroblast growth factor (bFGF) (MS/bFGF) and a collagen sponge for induction of angiogenesis. The gelatin micro-

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spheres, incorporating bFGF, were found to induce angiogenesis at the subcutaneous tissue in marked contrast to bFGF in the solution form (10). Following subcutaneous implantation of PET mesh bags containing MS/bFGF and a collagen sponge into streptozotocin-induced diabetic rats, angiogenesis in and around the bags was assessed in terms of histological examination and tissue hemoglobin quantitation. We also transplanted allogeneic islets into the prevascularized subcutaneous site to evaluate their function for bioartificial pancreas.

## MATERIALS AND METHODS

### *Materials for Angiogenesis Stimulation at Subcutaneous Site*

PET mesh (Teijinn Co., Japan), which is an artificially made mesh, has been commonly used for preparation of various cardiovascular devices. We used the PET mesh bag ( $2 \times 1.5$  cm), bFGF that was incorporated with gelatin microspheres like a slow drug delivery system (MS/bFGF), and a collagen sponge for scaffolding of cell infiltration. The ability of MS/bFGF and collagen sponge on angiogenesis induction has been already reported. Human recombinant bFGF with the isoelectric point (pI) of 9.6 was supplied from Kaken Pharmaceutical Co. (Tokyo, Japan). Gelatin used in this study was isolated by an alkaline process from bovine bone and had a pI of 4.9 and molecular mass of 99,000 (Nitta Gelatin Co., Osaka, Japan) (10). Twenty microliters containing 50  $\mu$ g bFGF was dropped onto 2 mg freeze-dried gelatin hydrogels for 30 min at room temperature to allow bFGF to sorb into the gelatin hydrogels. Using these three materials, angiogenesis was induced at the subcutaneous site. The order of implantation of the bag was: group I: only PET mesh bag; group II: PET mesh bag containing collagen sponge; group III: PET mesh bag containing MS/bFGF; group IV (experimental group): a collagen sponge was implanted into the PET mesh bag and then the MS/bFGF was injected into the collagen sponge. The four types of angiogenesis-inducing bags were implanted at the subcutaneous site of streptozotocin-induced diabetic rats. After 7 and 14 days, the bags were removed and we investigated the state of angiogenesis in and around the bag by light microscopic examination and histological methods using H&E stain. The prevascularization-inducing effect of the four types of PET mesh bag was assessed 7 days after implantation by determining the amount of tissue hemoglobin level, as a marker of prevascularization (6,10). The extracted hemoglobin was measured using a hemoglobin assay kit (Wako Pure Chemicals Co., Kyoto, Japan).

### *Diabetic Animals*

Diabetes was induced in male Lewis rats weighing 280–320 g by a single IV injection of streptozotocin at

a dose of 55 mg/kg of body weight. Diabetic rats were defined as those with two consecutive nonfasting blood glucose levels of more than 400 mg/dl and fasting blood glucose levels of more than 200 mg/dl after the injection of streptozotocin.

### *Islet Isolation and Transplantation*

Pancreatic islets were obtained from 8–10-week-old male Spargue-Dawley rats by the collagenase (Sigma, type IX) digestion method. Islets were purified by dextran density gradient separation. The islets were cultured overnight in RPMI-1640 medium supplement with 10% fetal bovine serum (FBS) and antibiotics at 37°C, in an atmosphere of 5% CO<sub>2</sub> and 95% air before transplantation. In the preliminary study, transplantation was performed in group III ( $n = 3$ ) and group IV ( $n = 1$ ) 2 weeks after prevascularization. Approximately 2500–2700 isolated islets were mixed with 5% agarose to prepare an immunoisolation membrane and they were transplanted into the angiogenesis bags. After transplantation, fasting blood samples were taken from the lateral tail veins at regular intervals. The body weight changes in both experimental and control group rats were also measured after transplantation.

## RESULTS

### *Light Microscopic Result*

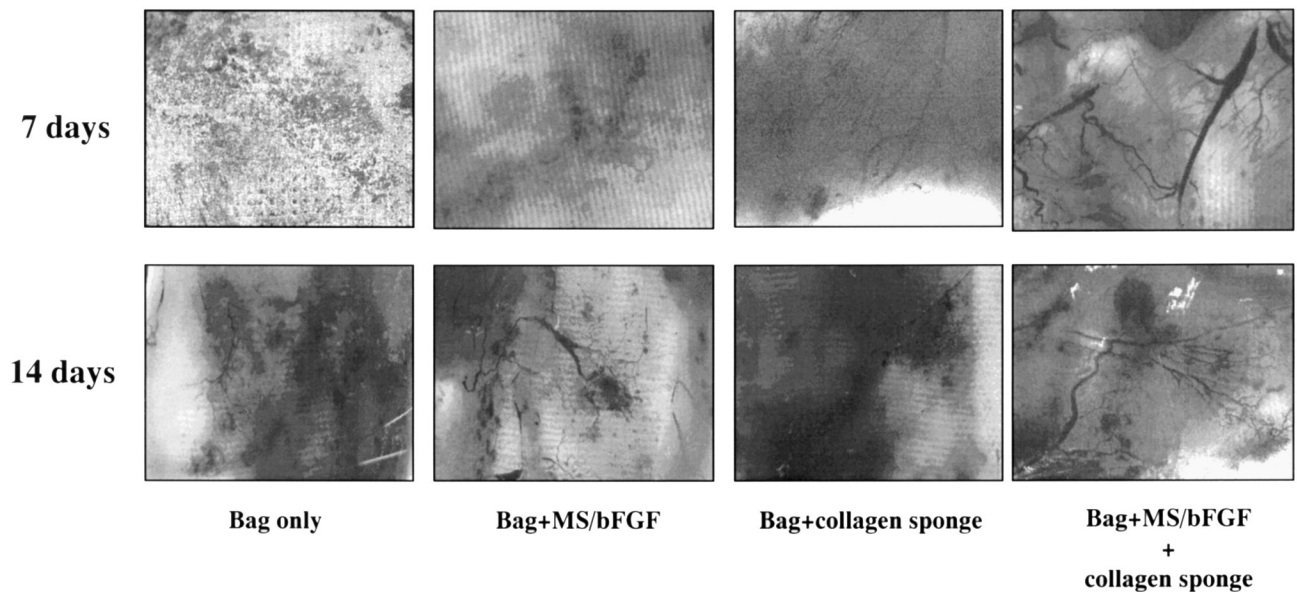
The angiogenesis bags were implanted under the subcutaneous site of streptozotocin-induced diabetic rats, and their effect on neovascularization was evaluated 7 and 14 days after implantation. Only in the PET mesh bag containing collagen sponge (group II) and the PET mesh bag containing both MS/bFGF and collagen sponge (group IV, experimental group) did vascular growth appear around the angiogenesis bag. The PET mesh bag alone and the PET mesh bag containing MS/bFGF were not very effective in inducing neovascularization (Fig. 1).

### *Histological Study*

Histological examination demonstrated that vascularization was remarkable around the bag as well as inside the angiogenesis bag 7 days after implantation in group IV, in contrast to the other three groups. After 14 days of implantation, there were numerous capillaries outside and inside the bags in group IV. Only thin vascular growth was developed around and inside the bags in the other groups (Fig. 2).

### *Tissue Hemoglobin*

The amount of tissue hemoglobin, which is an indirect measure of neovascularization, was estimated in all groups. In all the groups, the hemoglobin levels were increased 7 days after implantation. The hemoglobin

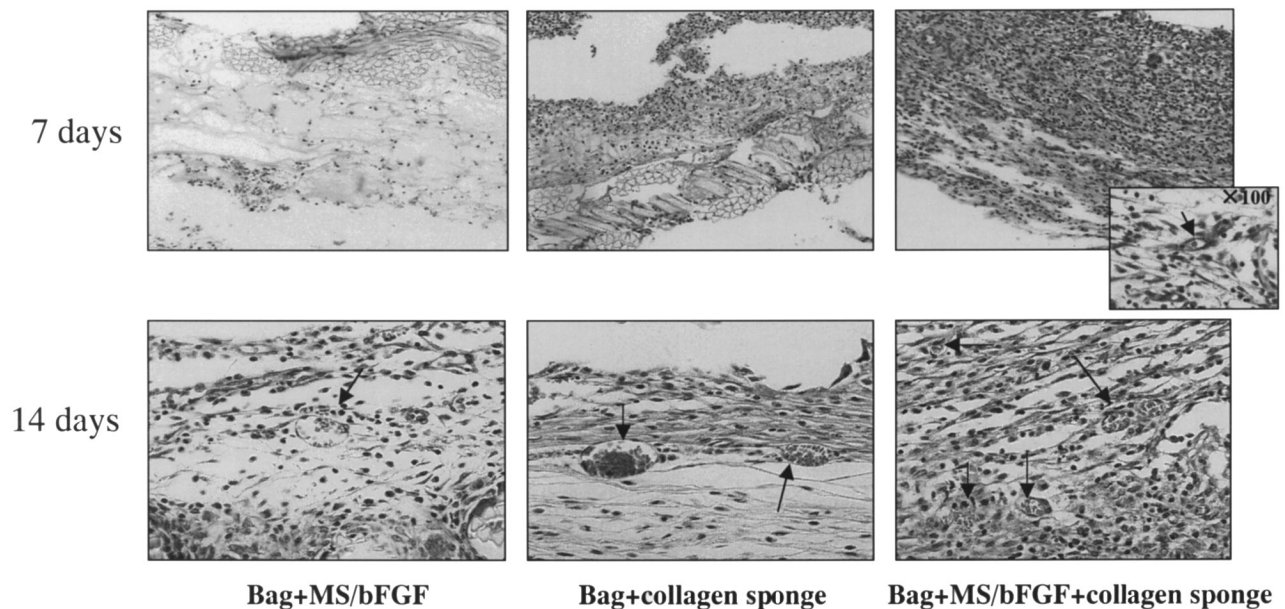


**Figure 1.** Prevascularization around PET mesh bag 7 and 14 days after implantation. The pictures were taken under light microscope (original magnification 10 $\times$ ). MS/bFGF: gelatin microspheres incorporating bFGF.

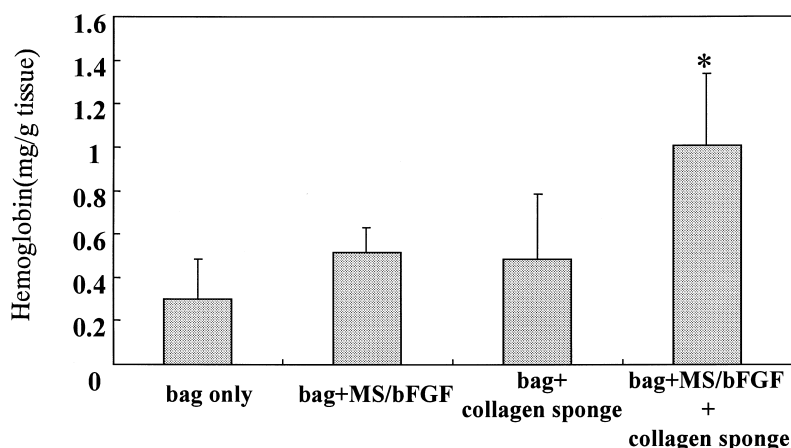
level in group I was  $0.3 \pm 0.2$ , in group II  $0.5 \pm 0.1$ , in group III  $0.47 \pm 0.35$ , and in group IV  $1 \pm 0.37$  mg/g tissue weight. The highest amount of hemoglobin was quantified in group IV when compared with the other groups (Fig. 3).

#### Transplantation Study

In group IV, after allotransplantation of islets, the recipient rats achieved normoglycemia within 2–3 days and maintained it for 40 days. In group III, all three rats failed to maintain normoglycemia for longer than 7



**Figure 2.** Histological examination of PET mesh bag 7 and 14 days after implantation. The arrow indicates the newly formed capillary (original magnification 50 $\times$ ). MS/bFGF: gelatin microspheres incorporating bFGF.



**Figure 3.** These bar diagrams show the level of tissue hemoglobin concentration 7 days after implantation of PET mesh bags. \* $p < 0.05$  compared to bag-only group. MS/bFGF: gelatin microspheres incorporating bFGF.

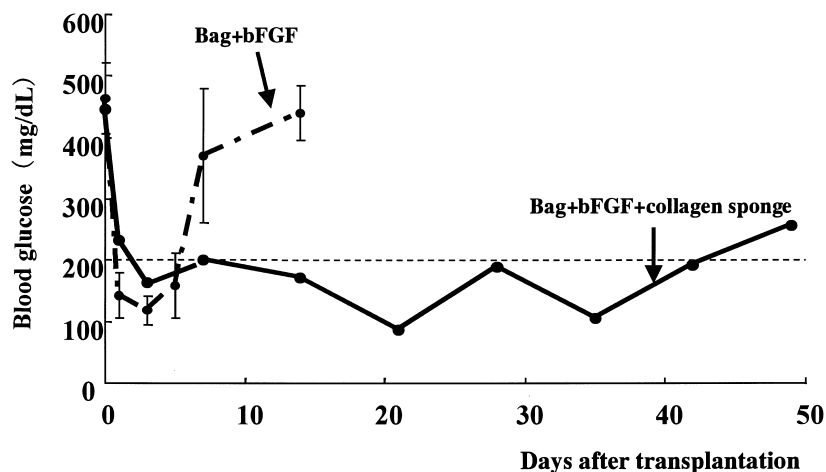
days. The body weight of group IV rats was increased after allotransplantation of islets (Fig. 4).

### DISCUSSION

Various transplantation sites have been proposed in several animal models. Some reports have shown that less prevascularized transplantation sites, especially the subcutaneous site, cause poor engraftment and survival of islets when transplanted directly or in the form of bioartificial pancreas. In our laboratory, we have succeeded in the induction of angiogenesis using a PET mesh bag at the peritoneal cavity site of normal and streptozotocin diabetic rats (4). It was observed that after inducing angiogenesis, the transplanted islet survival

and function were improved at the peritoneal cavity. Various biomaterials and growth factors have been commonly used for induction of angiogenesis (10). In this study, we attempted to induce angiogenesis at the subcutaneous space in order to improve the graft survival after bioartificial pancreas transplantation. We used three kinds of materials to induce neovascularization: PET mesh, MS/bFGF, and collagen sponge.

PET is used clinically in cardiovascular devices (2,11). In our previous study we used the PET mesh bag for induction of angiogenesis and prolongation of the iso- and allografts of islet transplantation was observed (3,4). In this study, we first used the PET mesh alone, which was like the previous peritoneal cavity implanta-



**Figure 4.** Fasting blood glucose levels of diabetic rats that received islet allotransplantation. (---) Group III: PET mesh containing bFGF. (—) Group IV: PET mesh containing collagen sponge and bFGF.

tion study. In group I we induced angiogenesis under the subcutaneous space; only a few blood vessels were formed around the bag 7 and 14 days after implantation. This indicates that induction of angiogenesis at the subcutaneous site is different from the peritoneal cavity. When we used bFGF with drug delivery system (MS/bFGF) alone or a collagen sponge for scaffolding of cell infiltration, they failed to make a network of angiogenesis at the subcutaneous site in diabetic rats. It demonstrated that inducing a network of capillaries is more difficult at the subcutaneous site than it is at the peritoneal cavity in the diabetic animal model. In this study, only in the experimental group (group IV) with the PET mesh bag containing both MS/bFGF and a collagen sponge was vascular growth formed around the angiogenesis bag (light microscope); capillary bed was also observed inside the bag (histological study). The amount of tissue hemoglobin also demonstrated that group IV had a significantly high level of hemoglobin when compared with the other groups ( $1 \pm 0.37$  mg/g tissue weight) 7 days after induction of prevascularization. Our preliminary allograft transplantation study has also demonstrated that, using our new angiogenesis method, we could prolong the graft survival and function after transplantation at the subcutaneous site of diabetic rats.

In conclusion, we found that a PET mesh with basic fibroblast growth factor (MS/bFGF) drug delivery system and a collagen sponge-like scaffolding of cell infiltration could make a network of angiogenesis at the subcutaneous site in diabetic rats. It also demonstrated that this method of angiogenesis induction prolong the graft survival and function of a bioartificial pancreas after transplantation. This study suggests that prevascularized subcutaneous site will be a new site not only for transplantation of bioartificial pancreas but also for other tissue transplantation in the future.

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