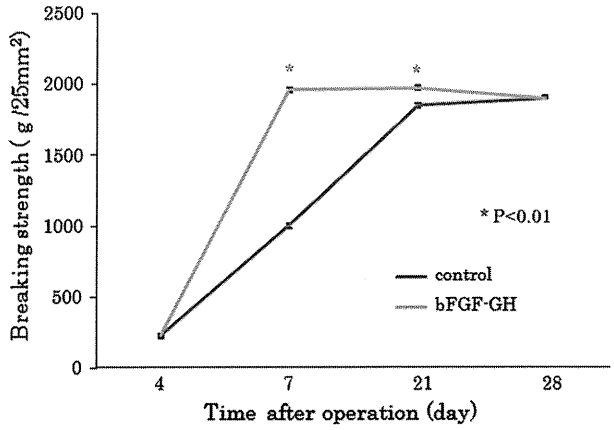

FIG. 3. Time course of breaking strength for the bFGF-GH group and GH-alone group from d 4 to 28.

a similar trend, peaking on d 21. After d 21, the number of positive cells in the bFGF-GH group declined rapidly, reaching a significantly lower level than that in the GH-alone ($P < 0.01$). On the other hand, the TUNEL index in the bFGF-GH group increased rapidly from d 7. and thereafter remained higher than that in the GH-alone group throughout the healing process (Fig. 5) ($P < 0.05$).

Microvessel Density (MVD)

The MVD in the bFGF-GH group increased rapidly from d 7 and reached peak level on d 28. Significant differences between the groups were detected on days 7 ($P < 0.001$), 21 ($P < 0.001$), and 28 ($P < 0.01$) (Fig. 6).

Animal Safety

A systematic acute toxicity assessment during this study confirmed the absence of mortality, treatment-related complications, and local irritation at the site of injection. Furthermore, no evidence of drug-related changes was observed in laboratory investigations, organ weights, or autopsy findings.

However, diffuse fibrosis associated with destruction of acinar cells was found in the remnant pancreas in both groups on d 28.

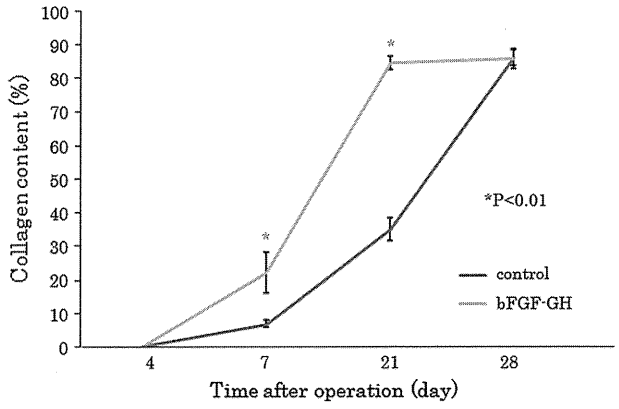

FIG. 4. Time course of collagen content for the bFGF-GH group and GH-alone group from d 4 to 28.

DISCUSSION

Pancreatic fistula (PF) is the most important cause of morbidity after PD [1, 2]. In spite of this, investigators have yet to develop anastomotic techniques capable of reducing the rate of PF to zero. Conjecturing that PF could be eliminated by accelerated healing of PJ, our group proposed an experimental animal study of PJ using a novel system for the controlled-released delivery of bFGF-GH. In a previous study, we demonstrated that bFGF-GH had accelerated the anastomotic healing of PJ by postoperative d 7, in comparison with the healing observed in controls [15].

An important requirement for this study was to select the optimal condition for applying bFGF-GH to the anastomotic site. To accomplish this, we began by establishing 10 μg as the optimal dose of bFGF for delivery within the GH vehicle. The breaking strength in our study plateaued at the 10 μg dose, and treatments at doses of 10 and 100 μg induced more abundant granulation tissue than treatment at a 1 μg dose. Next, we evaluated the shape of the GH and the method of administering the bFGF-GH to the anastomotic site. Ultimately, we decided to use a microsphere rather than a sheet or disc, and designed a method of subserosal injection of bFGF-GH into the anastomotic intestine [15]. We suspected that a sheet or disc would prevent a close connection between the intestine and remnant pancreas, which in turn could lead to anastomotic leakage. We also found, in a preliminary experiment, that a sheet or disc containing bFGF was readily soluble in pure pancreatic juice (data not shown). Our method of subserosal injection of bFGF-GH into the anastomotic intestine has already been confirmed to be effective, in a preliminary study with a rat model [15].

Our sequential analysis of healing revealed that bFGF-GH treatment could bring about a rapid completion of anastomotic healing within 3 wk. We also found, however, that significant differences between the groups in histologic appearance were absent by d 28. And according to microscopic findings by HE and EVG staining, the border zone in the bFGF-GH group on d 7 resembled that in the GH-alone group on d 21. These findings suggested that the bFGF-GH treatment might have promoted a rapid induction and shortening of the proliferating phase, rather than a marked increase in the quantity of granulation tissue. We thus needed to

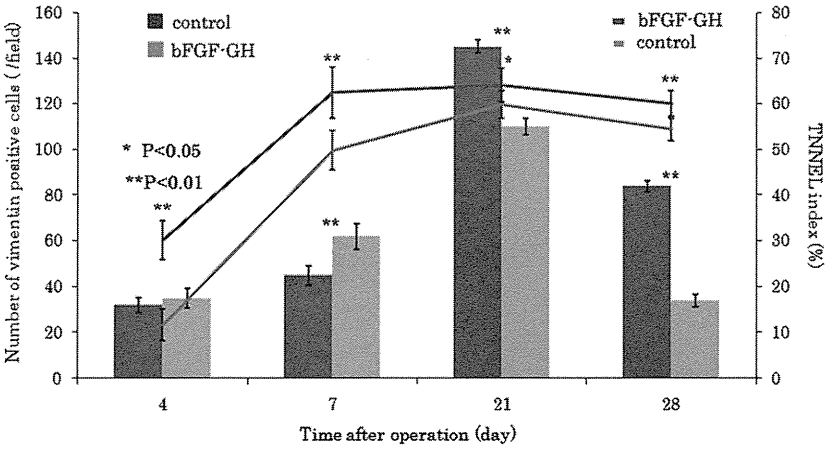

FIG. 5. Time course of the expression level of vimentin-positive cells and TUNEL index in the bFGF-GH group and GH-alone group from d 4 to 28.

conduct further experiments to ascertain whether bFGF-GH administration could improve the quality of the healing as well.

Our examination of the healing demonstrated that bFGF-GH treatment might have helped to improve the quality of granulation tissue formation. First, the bFGF injection promoted the apoptosis of fibroblasts in granulation tissue, ultimately resulting in an abolishment of fibroblasts altogether during the remodeling of the tissue. These findings were supported by a higher TUNEL index and a rapid decline in the number of vimentin-positive cells from d 21 in the bFGF-GH group. Brown *et al.* [18] proposed that treatment with PDGF and IGF-2 accelerates apoptosis and shortens the inflammatory phase in db/db mice. Earlier studies have also reported that elevated levels of TGF-β1 and bFGF may induce fibroblast apoptosis in bFGF-treated wounds [19]. Second, bFGF-GH treatment may suppress excess collagen deposition within granulation tissue in the remodeling period. In our study, semiquantitative evaluations of collagen content and breaking strength showed no significant differences between the groups on d 28. This confirms that the collagen deposition in granulation tissue ultimately takes the same form, with no appreciable differences, and that bFGF-GH treatment induces no adverse effects in the remodeling phase. Akasaka *et al.* [10] found that bFGF administered to an incisional wound may lead to granulation tissue formation and promote a scarless repair process. Third, bFGF-GH is thought to play an important role in healing by promoting angiogenesis in the granulation tissue. In our animals, the MVD in the bFGF-GH group was significantly higher from d 7 onwards. Our results are consistent with previous reports [20, 21], in which bFGF incorporated into gelatin sheets promoted neovascularization compared with controls. Angiogenesis is an absolute requirement for successful healing of anastomosis, regardless of the type of anastomosis [22]. In fact, Garcia-Olmo *et al.* [23] found that TNP-470, an inhibitor of angiogenesis, interfered with the healing of anastomoses and decreased anastomotic resistance in a rat colon model.

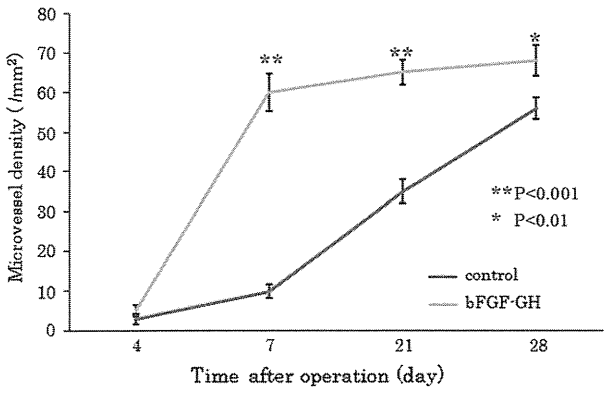

FIG. 6. Time course of the microvessel density (MVD) in the bFGF-GH group and GH-alone group from d 4 to 28.

The absence of treatment-related complications in our study suggests that this novel method is relatively safe. We note, however, that diffuse atrophy and fibrosis of pancreatic parenchyma were detected in the bFGF-GH group on d 28. The bFGF treatment was not likely to have played a significant role in the formation of diffuse fibrosis of the remnant pancreas, given that a similar appearance was observed in the GH-alone group on d 28. We speculate that the fibrotic change may have been attributable to obstructive pancreatitis induced by the stent insertion into the pancreatic duct. Although duct-to-intestinal-mucosa anastomosis would have been recommendable for this type of model, the narrow width of the duct (less than 0.5 mm) in beagle would have made it very difficult to

apply. Thus, further studies using other animals will be required to prepare for clinical use.

In conclusion, bFGF-GH administration can promote the rapid completion of PJ anastomosis, and may help improve the quality of the healing in granulation tissue by conferring potent angiogenesis and accelerating apoptosis.

ACKNOWLEDGMENTS

This study was carried out at Nippon Veterinary and Animal Science University. The authors thank Ms. Otsubo (technician) and Mr. Arai (technician) at Nippon Medical School, and Shidow Torisu, D.VM., Ph.D., Makoto Washizu, D.VM., Ph.D., and the veterinary students at Nippon Veterinary and Animal Science University.

The recombinant bFGF was provided by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan), and the J-VAC drainage system was supplied by Johnson and Johnson Co. (Tokyo, Japan). The GIA device was supplied by Covidien Co. (Tokyo, Japan).

REFERENCES

1. Fuks D, Piessen G, Huet E, et al. Life-threatening postoperative pancreatic fistula (grade C) after pancreaticoduodenectomy: Incidence, prognosis, and risk factors. Am J Surg 2008;197:702.
2. Veillette G, Dominguez I, Ferrone C, et al. Implications and management of pancreatic fistulas following pancreaticoduodenectomy: The Massachusetts General Hospital experience. Arch Surg 2008;143:476.
3. Chen HW, Lai EC, Su SY, et al. Modified technique of pancreaticojejunal anastomosis with invagination following pancreaticoduodenectomy: A cohort study. World J Surg 2008;32:2695.
4. Satoi S, Toyokawa H, Yanagimoto H, et al. A new guideline to reduce postoperative morbidity after pancreaticoduodenectomy. Pancreas 2008;37:128.
5. Nugent MA, Iozzo RV. Fibroblast growth factor-2. Int J Biochem Cell Biol 2000;32:115.
6. Steenfos HH. Growth factors and wound healing. Scand J Plast Reconstr Surg Hand Surg 1994;28:95.
7. McGee GS, Davidson JM, Buckley A, et al. Recombinant basic fibroblast growth factor accelerates wound healing. J Surg Res 1988;45:145.
8. Ono I. The effects of basic fibroblast growth factor (bFGF) on the breaking strength of acute incisional wounds. J Dermatol Sci 2002;29:104.
9. Ono I, Akasaka Y, Kikuchi R, et al. Basic fibroblast growth factor reduces scar formation in acute incisional wounds. Wound Repair Regen 2007;15:617.
10. Akasaka Y, Ono I, Yamashita T, et al. Basic fibroblast growth factor promotes apoptosis and suppresses granulation tissue formation in acute incisional wounds. J Pathol 2004; 203:710.
11. Tabata Y. Regenerative inductive therapy based on DDS technology of protein and gene. J Drug Target 2006;14:483.
12. Tabata Y. Significance of release technology in tissue engineering. Drug Discov Today 2005;10:1639.
13. Marui A, Tabata Y, Kojima S, et al. A novel approach to therapeutic angiogenesis for patients with critical limb ischemia by sustained release of basic fibroblast growth factor using biodegradable gelatin hydrogel: An initial report of the phase I-IIa study. Circ J 2007;71:1181.
14. Igai H, Yamamoto Y, Chang SS, et al. Tracheal cartilage regeneration by slow release of basic fibroblast growth factor from a gelatin sponge. J Thorac Cardiovasc Surg 2007; 134:170.
15. Aimoto T, Uchida E, Matsushita A, et al. Controlled release of basic fibroblast growth factor promotes healing of the pancreaticojejunal anastomosis: A novel approach toward zero pancreatic fistula. Surgery 2007;142:734.
16. Lu Y, Wu Z, Liu C, et al. Hepatic volumetry with PhotoShop in personal computer. Hepatobiliary Pancreat Dis Int 2004; 3:82.
17. Ogawa Y, Chung YS. Microvessel quantitation in invasive breast cancer by staining for factor VII-related antigen. Br J Cancer 1995;71:1297.
18. Brown DL, Kao WW, Greenhalgh DG. Apoptosis down-regulates inflammation under the advancing epithelial wound edge: Delayed patterns in diabetes and improvement with topical growth factors. Surgery 1997;121:372.
19. Funato N, Moriyama K, Shimokawa H, et al. Basic fibroblast growth factor induces apoptosis in myofibroblastic cells isolated from rat palatal mucosa. Biochem Biophys Res Commun 1997; 240:21.
20. Nakajima H, Sakakibara Y, Tambara K, et al. Therapeutic angiogenesis by the controlled release of basic fibroblast growth factor for ischemic limb and heart injury: Toward safety and minimal invasiveness. J Artif Organs 2004;7:58.
21. Miyoshi M, Kawazoe T, Igawa HH, et al. Effects of bFGF incorporated into a gelatin sheet on wound healing. J Biomater Sci Polym Ed 2005;16:893.
22. Enestvedt CK, Hosack L, Winn SR, et al. VEGF gene therapy augments localized angiogenesis and promotes anastomotic wound healing: A pilot study in a clinically relevant animal model. J Gastrointest Surg 2008;12:1762. discussion 1771.
23. Garcia-Olmo DC, Paya J, Garcia Olmo D. Effects of perioperative treatment with TNP-470 on the resistance of colonic anastomoses in rats. Dig Surg 2000;17:154.