Promotion of fibrovascular tissue ingrowth into porous sponges by basic fibroblast growth factor

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Fibrovascular tissue ingrowth into poly(vinyl alcohol) (PVA) sponges of different pore sizes was investigated by incorporating basic fibroblast growth factor (bFGF) into the sponges. The average pore size of PVA sponges used in this study was 30, 60, 110, 250, 350, and 700 μm and gelatin microspheres were employed as release carrier of bFGF. The sponges were subcutaneously implanted into the back of mice after incorporating free bFGF or gelatin microspheres containing bFGF into the sponges. Fibrovascular tissue infiltrated with time into the sponge pores and the extent of fibrous tissue ingrowth showed a maximum at a pore size around 250 μm 1 and 6 weeks after implantation. Significant promotion of the growth of fibrous tissue by bFGF was observed only at 3 weeks post-implantation ($p < 0.05$). New capillaries were formed in the tissue at any time, as long as bFGF was given to the sponges. Both empty gelatin microspheres and phosphate buffered solution neither promoted tissue ingrowth nor induced capillary formation in the sponges. It was concluded that bFGF was essential to induce the fibrovascular tissue ingrowth into the pores of PVA sponges.

1. Introduction

A number of porous biomaterials have been prepared from poly (L-lactide) (PLLA), L-lactide and glycolide copolymers (PLGA), collagen, and hydroxyapatite to demonstrate the efficacy as scaffolds for cell seeding and tissue regeneration in tissue engineering [1–12]. The scaffolds function as temporary substrates for proliferation and differentiation of cells seeded or infiltrated from the surrounding host tissue, and finally are integrated into or degraded in the regenerated tissue. It has been widely accepted that cell-scaffold interaction is greatly influenced by their porous structure, especially by their pore size. It is known that there is an optimal pore size for cell infiltration and host tissue ingrowth; for instance, 5–15 μm for fibroblasts [13], 20–125 μm for adult mammalian skin tissues [14, 15], 100–350 μm for bone tissues [8, 13], and 40–100 μm for osteoid tissues [13].

However, seeded cells do not always survive for a long time period in a scaffold, because oxygen and nutrients are not sufficiently supplied to transplanted cells due to their poor diffusion through the scaffold. Vascularization in the interior of the scaffold seems to be critical to overcome this problem. For example, when seeded into a porous biodegradable scaffold which has been sufficiently pre-vascularized by implantation at the rat mesentery having abundant blood vessels, hepatocytes survived to a significant extent compared with those in the non-vascularized scaffold [9, 10]. Angiogenic growth factors, including bFGF, platelet-derived growth factor, and vascular endothelial growth factor have been used to induce capillary formation in porous scaffolds [11, 12, 16, 17].

The objective of the present study is to investigate the effect of bFGF addition and pore size of a scaffold on the ingrowth of fibrovascular tissue. As the scaffold, poly (vinyl alcohol) (PVA) sponges with different pore sizes were employed while bFGF was selected to induce the fibrovascularization. Two methods were applied for incorporation of bFGF into the sponge: one is by incorporating bFGF into gelatin microspheres and the other is by direct deposition of bFGF from solution into the PVA sponges. The hydrogel microspheres were prepared from ''acidic'' gelatin with an isoelectric point (IEP) of 5.0, as this polymer is able to form polyion complex with ''basic'' bFGF [18]. A recent study demonstrated that the polyion complex could release bFGF in the biologically-active state as a result of gelatin biodegradation [18] and enhanced the angiogenetic effect, in marked contrast to free bFGF [18–20]. The PVA sponges receiving bFGF-containing gelatin microspheres or free bFGF in solution were subcutaneously implanted in the back of mice and ingrowth of fibrous tissues and formation of new capillaries were histologically compared with those without bFGF.

2. Materials and methods

2.1. Materials

Aqueous solution of human recombinant bFGF with an IEP of 9.6 was supplied by Kaken Pharmaceutical Co.

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Ltd, Tokyo, Japan. A gelatin sample with an IEP of 5.0 (Nitta Gelatin Co., Osaka, Japan) was extracted from bovine bone through an alkaline process. PVA sponge sheets with interconnected pores of various average pore sizes but a similar porosity were kindly supplied by Kanebou Co. Ltd, Osaka, Japan (Table I). These sponges were synthesized by formalization of PVA in the presence of starch, followed by starch extraction. The extent of formalization was approximately 80 mol%. Glutaraldehyde (GA), glycine and other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan and used without further purification.

2.2. Preparation of bFGF-containing gelatin microspheres

Gelatin microspheres were prepared through GA crosslinking of gelatin aqueous solution in w/o dispersion as previously described [20]. Immediately after mixing 25 μl of GA aqueous solution (25 wt %) with 10 ml of 10 wt % gelatin solution preheated at 40 °C, the mixture was added dropwise to 375 ml of olive oil under stirring at 420 rpm and 40 °C. Stirring was continued for 24 h at room temperature to allow chemical crosslinking of gelatin to proceed. After addition of 100 ml of acetone to the mixture, the resulting crosslinked microspheres were collected by centrifugation (4 °C, 3000 rpm, 5 min) and washed 5 times with acetone by centrifugation. The washed microspheres were placed in 100 ml of 100 mM glycine aqueous solution containing Tween 80 (0.1 wt %), followed by agitation at 37 °C for 1 h to block residual aldehyde groups of unreacted GA. Then, the resulting microspheres were washed twice with double-distilled water (DDW) by centrifugation, freeze-dried and sterilized with ethylene oxide gas. The water content of gelatin microspheres was calculated from the microsphere volume before and after swelling in phosphate-buffered saline solution (PBS, pH 7.4) for 24 h at 37 °C. The microsphere used in this study had the water content of 95 vol % [19]. The microsphere diameter was measured for at least 100 microspheres by viewing with a light microscope to calculate their volume. The average diameter of microspheres was 100 μm.

bFGF was impregnated into gelatin microspheres by dropping addition of 10 mg/ml of bFGF solution (10 μl) onto 2 mg of freeze-dried gelatin microspheres. They were left at room temperature for 1 h. Empty gelatin microspheres without bFGF were prepared in the same way as above except using DDW as the solution to add. The solutions (10 μl) were completely sorbed into the microspheres during swelling, because the solution volume was less than that theoretically required for the equilibrated swelling of microspheres.

TABLE I The pore size and porosity of PVA sponges used in this study

Average pore size (μm)	Porosity (%)
30	91
60	88
110	88
250	90
350	91
700	90

2.3. Incorporation of sponges with bFGF

PVA sponge sheets 1 mm thick were punched out to obtain sponge discs of 6 mm in diameter. Two PVA sponge discs were overlapped and peripherally sutured together with a 7–0 polypropylene monofilament under a wet condition to make sponges soft for easy sewing and tight suturing. The two-layered PVA sponges (2 mm in thickness, 6 mm in diameter) were dried and sterilized with ethylene oxide gas.

The gelatin microspheres (2 mg) with or without bFGF were suspended in 0.2 ml of DDW and the microsphere suspension was aseptically added into the central portion of two-layered sponges. During this process, excess DDW was diffused out while the microspheres were trapped in the center of sponges, irrespective of the pore size. In addition, 10 μl of PBS containing 100 μg of bFGF was given to PVA sponges to incorporate free bFGF into the sponges. As a control, 10 μl of PBS alone was added to PVA sponges.

2.4. Assessment of fibrous tissue ingrowth and capillary formation in sponges

Sponges with or without bFGF were subcutaneously implanted into the back of female ddY mice, 6 week-age (Shimizu Laboratory Supply, Kyoto, Japan). Each experimental group was composed of 3 mice and the bFGF dose was fixed to 100 μg/mouse. At 1, 3 and 6 weeks post-implantation, mice were sacrificed and the implanted PVA sponges were removed together with their surrounding fibrovascular tissue. The explanted sponges were fixed with 10% of neutralized formalin solution, embedded in paraffin, and sectioned at the center of PVA sponges or at the site as close as possible to the center (2 μm thickness), followed by staining with hematoxylin and eosin (HE).

Photomicrographs of histological sections were taken at a magnification of 40 to evaluate the ingrowth of fibrous tissue and capillary formation in PVA sponges. Six areas were randomly selected from each of microphotographs. The distance from the sponge edge to the front line of the fibrous tissue ingrown into the sponges was measured and the number of capillaries newly formed in the same area was counted and expressed as the mean ± standard error.

2.5. Statistical analysis

All the data were statistically analyzed by Students' *t*-test. Results were expressed as the mean ± the standard error of the mean. Statistical significance was accepted at $p < 0.05$.

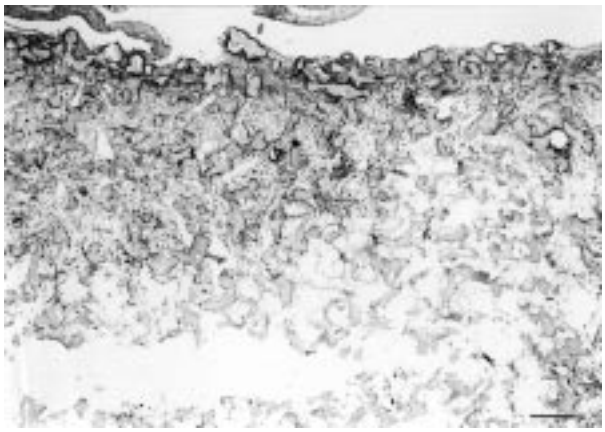
3. Results

3.1. Ingrowth of fibrous tissue into sponges

Fig. 1 shows the typical histological sections of PVA sponges with and without bFGF 3 weeks after implanta-

(a)

(a)



(a)

Figure 1 Histological sections of PVA sponges incorporating bFGF-containing gelatin microspheres (a, b) and PBS (c) 3 weeks after subcutaneous implantation in the back of mice. The pore size of the sponges was 250 (a, c) and 700 μm (b). Arrows indicate newly formed capillaries in PVA sponges.

tion. bFGF was incorporated in gelatin microspheres. Remarkable fibrous tissue ingrowth was observed for the sponge having a pore size of 250 μm, whereas pores were not occupied with the fibrous tissue but with a fibrin-like tissue for the sponge with a large pore size, such as 700 μm.

The time course of fibrous tissue ingrowth is shown in Fig. 2 after subcutaneous implantation of sponges with different pore sizes. It is apparent that tissue ingrowth depended on both the pore size of PVA sponges and their bFGF incorporation. Tissue ingrowth seems to become the highest around the medium pore size (250 μm) at least 1 and 3 weeks after implantation. Except for sponges with pore sizes of 30 and 60 μm, the bFGF entrapped in gelatin microspheres appears to have more markedly promoted the ingrowth of fibrous tissue into sponges than the others at 3 weeks post-implantation, irrespective of the pore size ($p < 0.05$). Empty gelatin microspheres did not contribute to any enhancement of tissue ingrowth. On 6 weeks postoperatively, the pores of PVA sponges were almost completely filled with fibrous tissue, regardless of the presence of bFGF. However, tissue ingrowth virtually did not occur for PVA sponges with the pore sizes of 30 and 60 μm, while less ingrowth was observed for the sponge with the largest pore (700 μm).

3.2. New capillary formation in PVA sponges

Fig. 3 shows the number of newly formed capillaries in the PVA sponges as a function of the distance from their periphery 3 and 6 weeks after subcutaneous implantation in the back of mice. It is apparent that bFGF was effective in promoting neovascularization in the sponges ($p < 0.05$). When bFGF-containing gelatin microspheres had been given to PVA sponges prior to implantation, new capillaries were formed in the sponge more deeply into the interior region than free bFGF. Significantly enhanced capillary formation was noticed for the PVA sponges with pore sizes of 250 and 350 μm than those with pore sizes of 30, 60 and 700 μm. Empty microspheres and PBS did not induce any significant capillary formation in the sponges, irrespective of the pore size. On 6 weeks after implantation, free bFGF promoted capillary formation less than the bFGF-containing gelatin microspheres.

4. Discussion

bFGF was used as an angiogenetic factor in this study, because this growth factor is known to have an inherent ability to let both fibroblasts and capillary endothelial cells proliferate *in vitro*. In addition, bFGF is effective

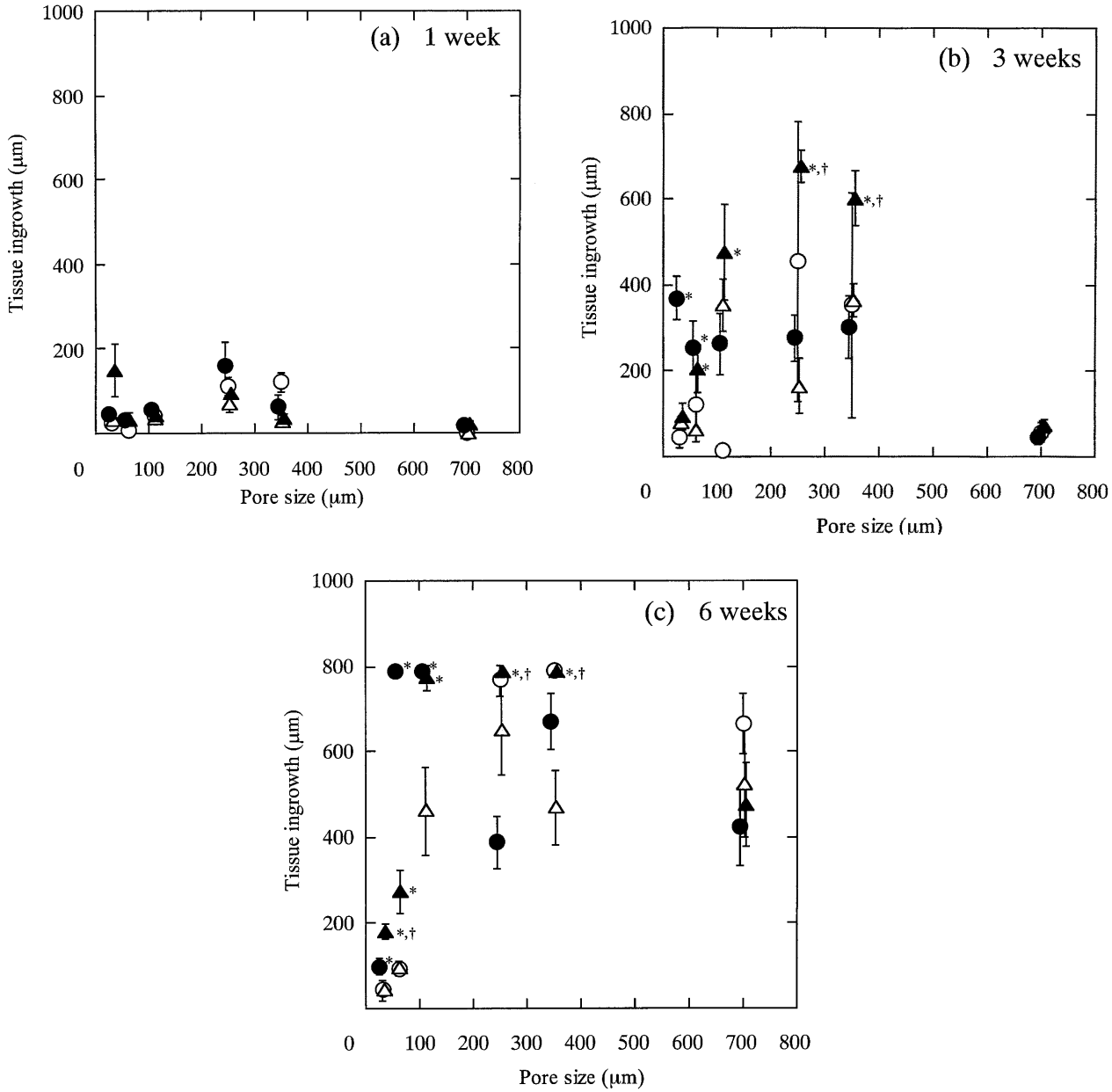


Figure 2 The effect of pore size on the fibrous tissue ingrowth into PVA sponges incorporating PBS (○), free bFGF (●), empty gelatin microspheres (△) and bFGF-containing gelatin microspheres (▲). The sponge sections were viewed 1 (a), 3 (b) and 6 weeks (c) after subcutaneous implantation in the back of mice. The bFGF dose was 100 μg/mouse. The error bars denote the standard error of the mean. *indicates significance of free bFGF or bFGF – containing gelatin microsphere group at $p < 0.05$ against the value of PBS or empty gelatin microsphere group at the corresponding pore size. † indicates significance of bFGF – containing gelatin microsphere group at $p < 0.05$ against the value of free bFGF at the corresponding pore size.

also *in vivo*, as evidenced by enhanced wound healing through vascularization [21, 22]. However, such a high bFGF efficacy is not always achieved when administered directly in the body without any carrier, probably because of its very short half-life in the body. As one trial to effectively induce the biological activity of bFGF, sustained release systems have been created and it was demonstrated that vascularization, granulation and osteogenesis were markedly promoted by sustained release of bFGF, in marked contrast to direct administration of bFGF [18–20, 23]. However, the present study revealed that our release system using gelatin microspheres had a neovascularization effect a little superior to free bFGF in solution. This unexpected result seems to be ascribed to the physical adsorption of bFGF molecules onto the PVA sponge wall.

PVA sponges have an interconnected pore structure with a relatively narrow pore size distribution. As PVA is not biodegradable but bio-inert [24], one can evaluate the fibrovascular tissue ingrowth without taking into consideration the scaffold degradation. A most interesting finding of the present study is that there was an optimum pore size for fibrovascular tissue ingrowth into sponges. Practically no tissue ingrowth was observed in sponges with small pore sizes of 30 and 60 μm even if they were much larger than the fibroblast size. When the sponge size and porosity are fixed, decreased pore size will narrow the pore channels in the sponge. It is possible that the narrow and tortuous pore channels prevent cells from migrating into the interior of sponges. On the other hand, the larger the pore size of sponges, the smaller the surface area for cells to attach to. The surface area inside the sponge is inversely proportional to the pore diameter of sponges with a similar volume and porosity. It is reasonable to assume that there is an optimal surface area required for cell attachment and the subsequent tissue

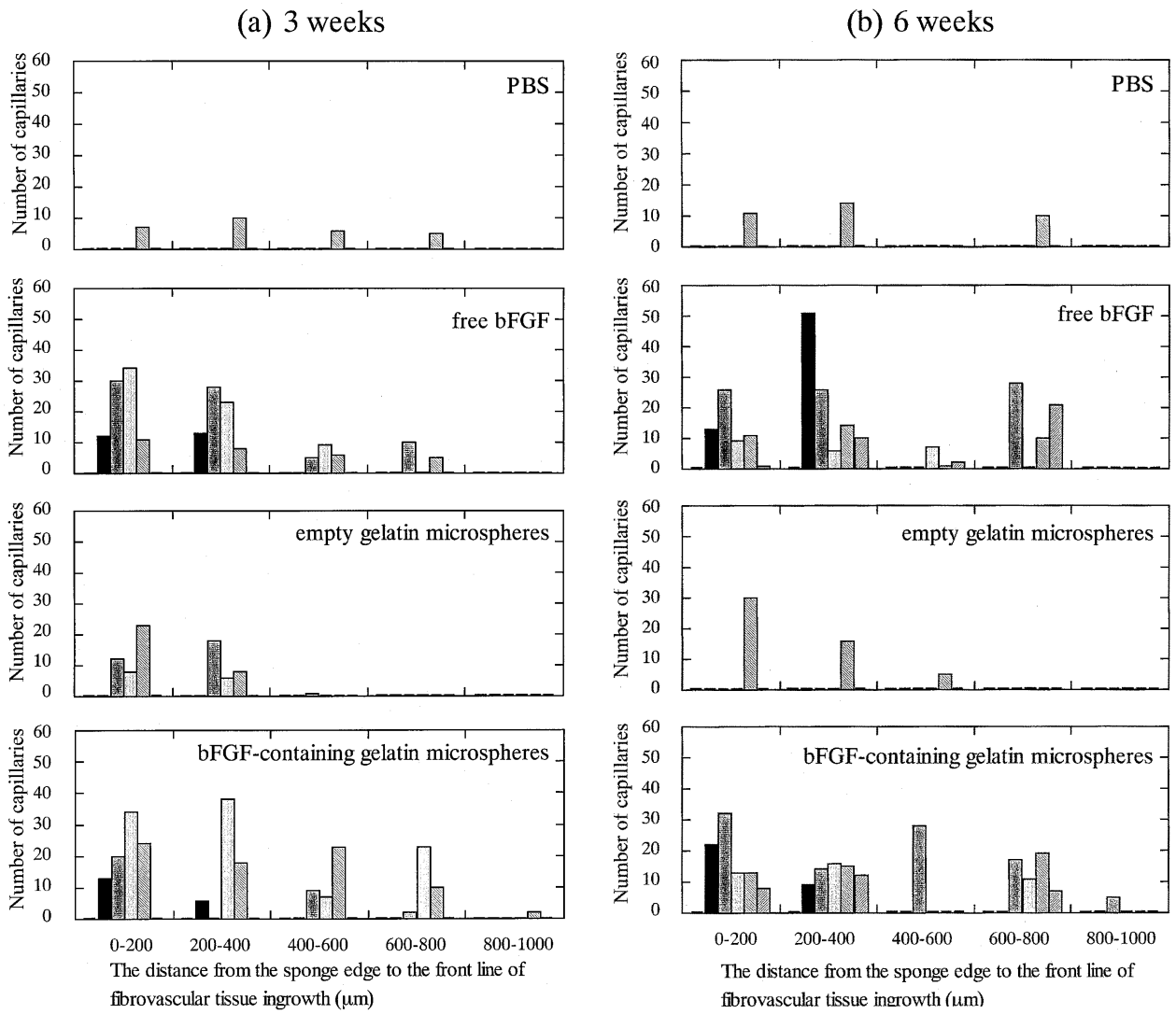


Figure 3 The number of newly formed capillaries as a function of the diameter from the sponge edge to the front line of fibrovascular tissue ingrowth for PVA sponges incorporating PBS, free bFGF, empty gelatin microspheres and bFGF-containing gelatin microspheres 3 (a) and 6 weeks (b) after subcutaneous implantation in the back of mice. The pore size of PVA sponges was 30 (□), 60 (■), 110 (■), 250 (■), 350 (■) and 700 (■) μm.

ingrowth in the sponge. The balance in these two factors, tortuosity of pore channels and surface area of sponges, must be a key factor for tissue ingrowth. As the surface area of sponges decreases with the increasing pore size, the most favorable fibrovascular tissue ingrowth must have occurred in the PVA sponge with the medium pore size ranging from 110 to 350 μm.

The present study has also demonstrated that bFGF incorporation is essential in inducing new formation of capillaries in sponges. The enhanced vascularization by bFGF entrapped in gelatin microspheres is obviously due to sustained release of bFGF [20]. However, the bFGF injected in solution could also promote vascularization, to a little less extent than the bFGF entrapped in microspheres, although free bFGF seems to be rapidly excreted from the injection site. This might be due to protein adsorption to the PVA sponge wall. It is possible that the adsorption has helped the sustained release of bFGF, because of slow desorption of bFGF molecules from the PVA sponge wall. The average number of capillaries in the PVA sponges was larger as the pore size decreased. This suggests that a large space must be required for capillary formation because of canalization, contrary to fibrous tissue ingrowth. This may be also the reason why no vascularization was seen in the PVA sponge with the smallest pore size.

5. Conclusion

Fibrous tissue ingrowth occurred in PVA sponges and the ingrowth rate showed a maximum at pore sizes around 250 μm, probably due to the balance in two factors, the channel size of sponges for cell infiltration and their surface area for cell attachment. Incorporation of bFGF into the sponges promoted the growth of fibrous tissue especially at 3 weeks post-implantation, and more greatly enhanced formation of new capillaries, regardless of the pore size. There was no significant difference in capillary formation between the bFGF entrapped in gelatin microspheres and free bFGF. It was concluded that incorporation of bFGF-containing gelatin microspheres was a potential way to induce fibrovascular tissue ingrowth in the pore of sponges.

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