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The Spine Journal 6 (2006) 397–404

THE SPINE JOURNAL

An in vitro and in vivo analysis of fibrin glue use to control bone morphogenetic protein diffusion and bone morphogenetic protein–stimulated bone growth

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Received 10 December 2004; accepted 14 January 2006

Abstract

BACKGROUND CONTEXT: Recombinant human bone morphogenetic protein-2 (rh-BMP2) has become popular for augmenting spine fusion in the lumbar and cervical spine. Concerns exist, however, over bone morphogenetic protein (BMP)-stimulated soft-tissue swelling and bone growth stimulation in areas where bone is not desired, especially as the material ''leaks'' into such spaces. The most detrimental effects of such leakage might be airway compromise, while heterotopic bone formation into the spinal canal has been reported in animal and human studies. Fibrin glue has been used as a carrier of many osteoinductive materials; however, its efficacy at modulating the clinical effects of BMP are not known. The amorphous nature of fibrin glue makes it a candidate to control diffusion of BMP and possibly limit bone formation by limiting BMP diffusion to areas where such bone is not desired.

PURPOSE: To evaluate the use of fibrin glue to limit BMP diffusion and BMP-stimulated bone growth.

STUDY DESIGN/SETTING: This is an in vitro basic science study and an in vivo prospective randomized animal study.

STUDY SAMPLE: Eighteen Lewis rats.

OUTCOME MEASURES: In vitro study: Enzyme-linked immunosorbent assay measurement of rh-BMP2 concentration in saline. In vivo study: At day 60, rats were evaluated for neurologic deficits before sacrifice. Spines were harvested, and the following studies were performed: 1) manual testing for fusion and bone growth; 2) X-ray evaluation; 3) Micro–computed tomography (micro-CT) scans.

METHODS: In vitro study: Collagen sponges soaked with BMP at two different concentrations were incubated in saline solution with and without encapsulation by fibrin glue. Saline BMP concentrations were measured at consecutive time points. In vivo study: A rat fusion model using rh-BMP2 for fusion has been developed and tested with resultant100% fusion in over 100 rats. Lewis rats were divided into two groups and treated as follows: I: Exposure of L4–L5 transverse processes, decortication, and placement of BMP sponge in the lateral intertransverse space. II: Exposure and decortication as above and placement of fibrin glue before BMP sponge placement.

RESULTS: In vitro study: Peak rh-BMP2 concentrations in saline were 20% and 45% of the maximum possible for fibrin glue encapsulated sponges and controls, respectively, with a more gradual increase to peak concentration in samples encapsulated in fibrin glue. In vivo study: No rats exhibited any neurologic deficits. X-rays revealed at least partial bone formation in all rats. Manual testing of intertransverse fusion spines revealed 100% fusion in rats treated with BMP only, whereas rats treated with fibrin glue before placement of BMP sponges revealed only one possible fusion.

FDA device/drug status: approved but not for this indication (fibrin glue, rh-BMP2).

Funding was provided by the Spine Foundation, Santa Monica, California. No other funds were received from a commercial entity related to this manuscript.

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1529-9430/06/$ – see front matter
doi:10.1016/j.spinee.2005.11.006

Posterior-lateral bone formation was present on X-ray in both groups, and micro-CT imaging revealed bridging bone from transverse processes to the BMP-stimulated bone in the control groups. In spines treated with fibrin glue before rh-BMP2 placement, bone formation could still be seen within the soft tissues; however, bridging bone connecting to the transverse processes was either significantly decreased or not present.

CONCLUSIONS: Fibrin glue can limit rh-BMP2 diffusion. Also, because it limited bone formation at the transverse processes, it can be inferred that fibrin glue can limit bone formation when used to separate areas of desired bone formation from areas where bone formation is not desired.

Keywords: Bone morphogenetic protein; BMP; Rh-BMP2; Fibrin glue; Fusion; Bone; Bone growth; Spine; Rat; Diffusion

Introduction

Recombinant human bone morphogenetic protein-2 (rh-BMP2) is rapidly becoming popular for augmenting spine fusion in the lumbar and cervical spine. Its use has been reported in anterior and posterior lumbar and cervical fusion surgery, as well as transforaminal lumbar interbody fusion and posterior lumbar interbody fusion type procedures. Bone morphogenetic protein (BMP) may, however, stimulate bone growth in areas where bone is not desired, especially as the material ''leaks'' into such spaces. McKay and Poynton have reported such heterotopic bone growth into the spinal canal and neural foramina [1,2]. More recently, a letter of caution by Medtronic-Sofamor Danek implied that rh-BMP2 use may be related to increased soft-tissue swelling in the cervical spine, the most detrimental effect of which would be airway compromise. Though these complications have thus far only been reported with rh-BMP2, they will likely present as use of additional proteins and osteogenic agents increases. While our ability to stimulate bone growth rapidly increases, methods to control such adverse events have not yet been reported or tested.

Fibrin glue has been used as a carrier of many osteoinductive materials including BMP and demineralized bone matrix [3–7] as well as osteogenic cells [8–10]. It has also been used to improve the material handling of bone graft and bone graft substitutes [11–13]. Thus, it appears to have the ability to temporarily contain such materials before implantation, yet release them in vivo over time while itself being completely absorbed. Indeed Hattori postulated that fibrin glue could control the diffusion of BMP [4]. Conversely, fibrin glue should be able to temporarily control the flow of such biologic agents into areas where bone formation may not be desired. This control of bone formation would depend both on the rate of fibrin glue degradation and on the time course of stimulation of osteogenesis. Fibrin glue resorption occurs over the first 7–14 days after implantation [14], whereas rh-BMP2 concentration decreases to less than 50% concentration 2 days after implantation [15,16]. Thus, fibrin glue could potentially protect neural elements from unwanted bone formation during the peak activity of BMP.

Regarding direct osteoinductive properties of fibrin glue, conflicting reports have shown fibrin glue to augment [11,17] and inhibit [18–21] bone healing and bone formation. Though fibrin glue appears to limit BMP diffusion [4], its efficacy at modulating the osteogenic effect of BMP has not been proven.

The purpose of this two-phase study is to evaluate the use of fibrin glue to limit both the diffusion of rh-BMP2 and its in vivo osteogenic effects. The first phase is an in vitro study that measures diffusion of two different concentrations of rh-BMP2 through fibrin glue. The second, a rat study, uses fibrin glue to block bone formation at transverse process fusion sites. The posterolateral rat fusion model was chosen because this is a proven model of transverse process fusion in rats with robust bone formation after simple decortication and application of rh-BMP2. Thus, if fibrin glue can limit bone formation in this model, it is expected to perform similarly in areas where bone formation is less robust.

Materials and methods

The materials used in all aspects of this study were Tisseel fibrin glue (Baxter Healthcare) and commercially available Infuse brand rh-BMP2 (Sofamor Danek) at a concentration of either 0.032 mg/mL or the standard 1.5 mg/mL with the accompanying collagen sponge cut to appropriate size (5×10 mm or 5×5 mm). The concentration approved for human use, 1.5 mg/mL, was used for one segment of the diffusion study. The reduced concentration of BMP was used for rat fusion as it was found to be the minimum concentration required for 100% successful stimulation of rat intertransverse process fusion (unpublished data from previous dose–response studies in our laboratory). At this concentration, transverse process decortication and placement of BMP sponges stimulates fusion 100% of the time without need for local bone or additional treatments.

In vitro study

Fibrin glue was tested for its ability to limit diffusion of BMP. Collagen sponges were soaked with rh-BMP2 in accordance with the package instructions at both 0.032 mg/mL and 1.5 mg/mL concentrations (Infuse,

Medtronic-Sofamor Danek). The 5×5 mm sponges were then submerged in 50 cc of normal saline under the following conditions: 1) Control, BMP sponge placed directly into the saline; 2) Placement of BMP sponge on top of a fibrin glue layer; 3) BMP sponge sealed within a fibrin glue capsule; 4) BMP solution placed directly into the 50 cc of saline without a collagen sponge.

In Group 2, 1 cc of fibrin glue was placed on a glass surface and the BMP-collagen sponge was immediately placed on top. This combination was then placed in the vial to test whether or not equal amounts of BMP would be released as in Group 1; thus, testing if the fibrin glue would bind or absorb the BMP. In Group 3, 1 cc of fibrin glue was sprayed under and over the BMP-collagen sponge in a single application, sealing the sponge as the fibrin glue set. The capsule was then placed in the vial.

The vials were incubated at 37°C and samples of the fluid were taken beginning immediately and at 1, 2, 4, 8, 24, 48, 96, and 192 hours. Fluid samples were analyzed for BMP concentration using enzyme-linked immunosorbent assay (BMP-2 Quantikine enzyme-linked immunosorbent assay kits, R and D Systems, Minneapolis, MN), and compared with the calculated maximum possible BMP concentration if all of the BMP were released by the collagen sponge.

Animal model

Female Lewis rats between 3 and 6 months of age were quarantined and observed for at least 7 days before surgical treatment. Rats were anesthetized with inhaled isofluranebefore shaving and preparation. Surgical procedures were performed as described below, followed by postoperative monitoring. All wounds were closed with 4.0 nylon at both the fascial and skin layers. Immediately postoperatively, rats were given 1 cc of lactated Ringer's solution, 0.2 cc of Baytril antibiotic, and 0.04 mL of Buprenex pain medication. Baytril was also given orally diluted (2 mL per 300-mL bottle) in the drinking water for 1 week. Rats were monitored for 10 weeks with periodic neurologic examinations before sacrifice.

Fusion model

Surgical exposure and decortication at the transverse processes was performed at the L4–L5 spinal level (rats have six lumbar vertebrae). Rats were then treated in one of two methods: A) placement of BMP sponge over the transverse processes and intertransverse space, nine rats; or B) placement of fibrin glue over the transverse processes and intertransverse space before placement of BMP, nine rats (Fig. 1). In Group A the collagen sponge nicely bridged the transverse processes covering them and the intertransverse space completely. In Group B, 0.5 cc of fibrin glue was used for each level; this amount covered the transverse processes and intertransverse space completely in a single layer. Approximately 30 seconds were allowed for the fibrin glue to set before placement of the BMP sponge. Rats were sacrificed at 8 weeks, and spines were harvested for testing and imaging.

Micro–computed tomography (micro-CT) imaging was performed at 1-mm increments and a 0.36-micron in-plane resolution (Scanco Medical AG, Bassersdorf, Switzerland), and images were analyzed for bone formation and connection of bone to the transverse processes using NIH ImageJ 1.34s (National Institutes of Health) software. The maximum area of new bone formation was also measured at each transverse process using the ''Freehand Selection'' tool in ImageJ. Results were tabulated, and the mean, standard deviation, and p value for a two-tailed *t* test were calculated.

Results

In vitro study

The percentage of rh-BMP2 released into the solution as a function of time is shown in Figure 2. The control and

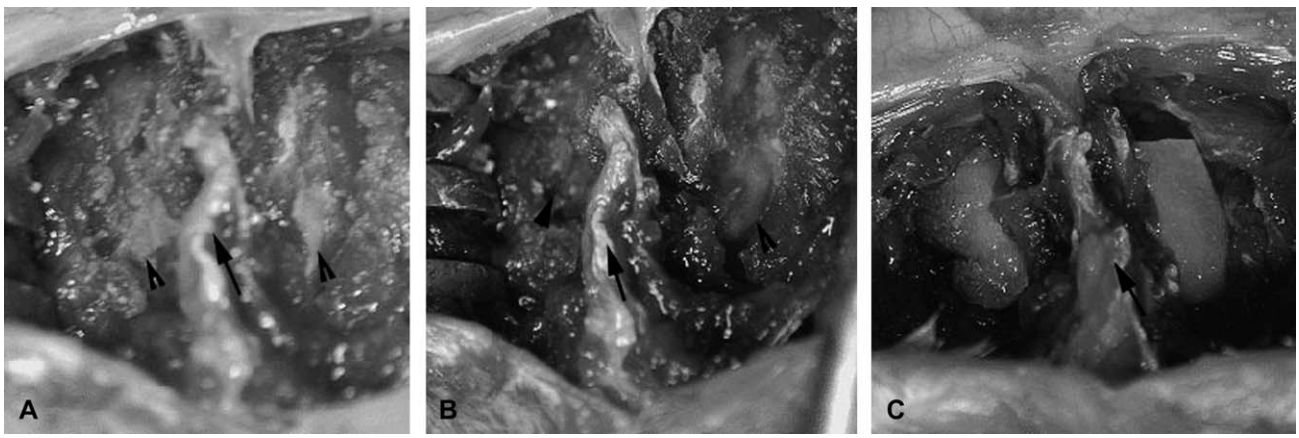

Fig. 1. Rat surgery–fusion. (A) Rat spine exposed showing transverse processes (arrowheads) and spinous process (arrow). (B) Exposed spine with fibrin glue placed over decorticated transverse processes (arrowheads). The recombinant human bone morphogenetic protein-2 sponges were then placed on top of the fibrin glue before closure. (C) Exposed spine with recombinant human bone morphogenetic protein-2 sponges placed at decorticated transverse processes.

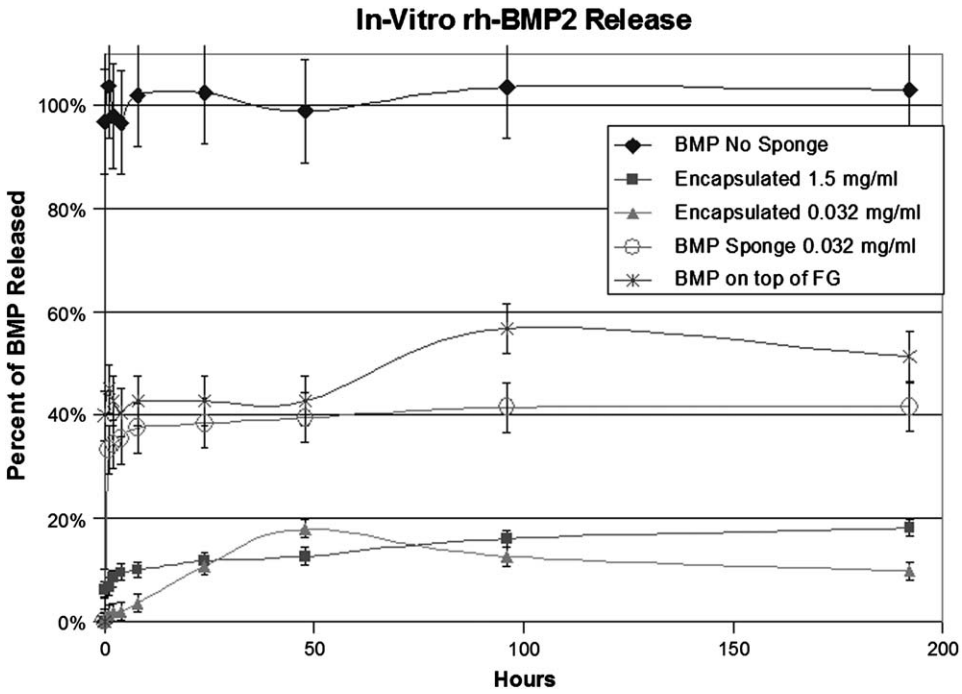

Fig. 2. Diffusion of bone morphogenetic protein (BMP) through fibrin glue. Graphs show averages of control with just bone morphogenetic protein sponge, bone morphogenetic protein sponge on top of a layer of fibrin glue, and bone morphogenetic protein sealed in fibrin glue capsules at 0.032 mg/mL and 1.5 mg/mL concentrations. Note slow increase and significantly lower peak concentration of bone morphogenetic protein in solution when encapsulated with fibrin glue. The top line shows bone morphogenetic protein concentration in solution when the bone morphogenetic protein was simply dripped into the saline solution. Error bars reflect standard error.

fibrin glue with BMP on top curves show a rapid increase to relatively steady high levels of BMP in the solution with nearly 50% release of the BMP into solution. The fibrin glue does not appear to bind the BMP as the curve showing the BMP on top of the fibrin glue has a steady high level similar to the BMP sponge only curve. There was no statistical difference between these two curves at all time points ($p>.10$) except at 100 hours ($p=.043$).

The curves of the BMP sealed in the fibrin glue capsules show slower, steady increases over 4 days and much lower peaks than the control groups, though the 1.5 mg/mL concentration curve increases more rapidly initially relative to the 0.032 mg/mL concentration.

None of the above concentrations were close to the level of BMP in solution when the BMP was placed directly into the saline.

Animal model

No abnormal rat behavior was noted, and no rats appeared to suffer an abnormal postoperative course. None of the rats exhibited any neurological deficits before or after the surgical procedure or at the time of sacrifice.

Fusion model

Manual testing of intertransverse fusion spines with only rh-BMP2 sponges revealed 100% fusion rates, whereas rats treated with fibrin glue before placement of BMP sponges revealed one spine with significantly decreased motion at the treated level. Posterior-lateral bone formation was present on X-ray in both groups, and micro-CT imaging revealed bridging bone from transverse processes to the BMP-stimulated bone in the control groups (Fig. 3). Bridging bone was either significantly decreased or not present in spines treated with fibrin glue before rh-BMP2 placement (Fig. 4). Bone formation could commonly be seen within the soft tissues or at the spinous processes without connection to the transverse processes. Bone area measurements revealed significantly less bone formation when fibrin glue was placed between the transverse processes and the BMP (Table 1).

Discussion

Use of rh-BMP2 in spinal applications has become common in a relatively short time. Although approved by the Food and Drug Administration (FDA) for anterior lumbar interbody use only, BMP use has been reported in posterior lumbar interbody fusion and transforaminal lumbar interbody fusion applications, as well as posterolateral lumbar fusions and cervical fusions. Its safety in such applications has not been thoroughly studied, and many concerns remain. Indeed a letter of caution by Sofamor Danek implied that rh-BMP2 use in the anterior cervical spine can lead to life-threatening airway compromise. Additionally, leakage

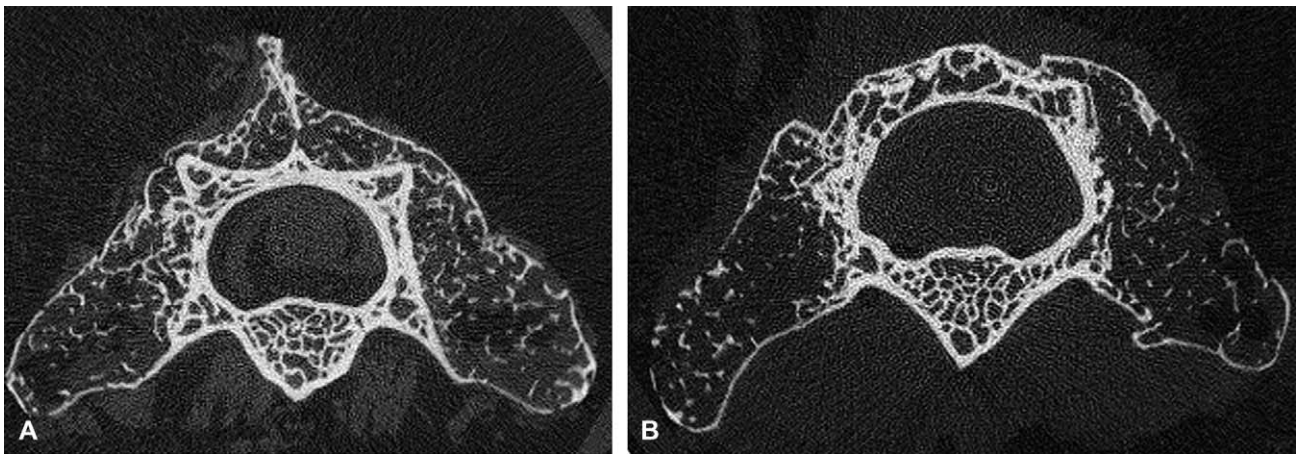

Fig. 3 A and B. Micro–computed tomography images of fusion with bone morphogenetic protein only: Transverse process fusion examples with recombinant human bone morphogenetic protein-2 sponge only. Note exuberant bone formation and connection to transverse processes.

of BMP into the cervical canal could theoretically cause bone formation or ossification of the posterior longitudinal ligament, while in the lumbar canal, leakage from interbody fusions or posterolateral fusions could cause canal and foraminal stenosis. This phenomenon was reported by McKay and Sandhu, in humans, when they aborted a study using cages in a posterior lumbar interbody fusion approach owing to bone formation along the track of cage insertion, including within the spinal canal [2]. Also, in a dog study, Meyer et al. stimulated bone formation in a laminectomy defect by placing rh-BMP2 directly over the dura. Imaging studies showed concavity of the posterior canal in some instances which resolved to the normal convexity by 3 months; however, there was a continued decrease in foraminal size in the rh-BMP2-treated animals that did not resolve over time [22]. Rabbit and mouse models have also shown that BMP-induced calcification of the ligamentum flavum can lead to clinically significant spinal stenosis and myelopathic changes [23–25]. This raises concerns that BMP leakage, especially in the cervical canal, may increase ossification of the posterior longitudinal ligament. Thus, BMP leakage has the potential to cause significant adverse events, including spinal foraminal or canal compromise and significant soft-tissue swelling.

Fibrin glue has two components, fibrinogen and thrombin; when mixed, the thrombin activates the fibrinogen to form a fibrin clot which is very similar to natural blood clot formation. Though introduced in 1909 as a hemostasis

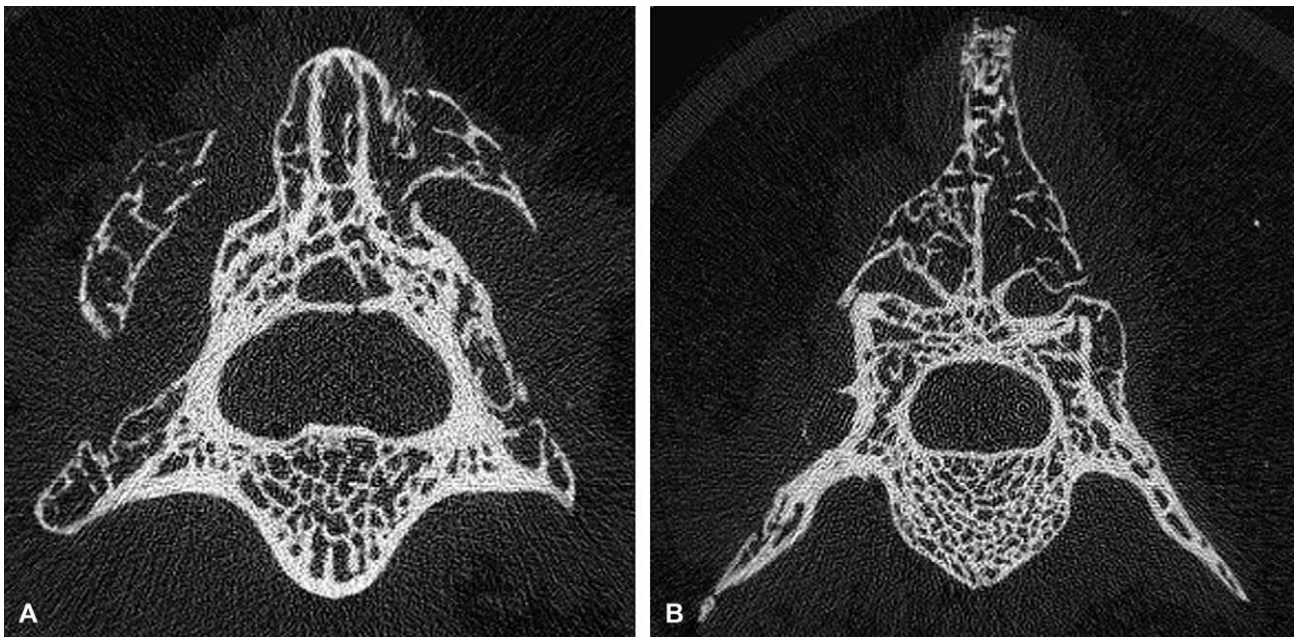

Fig. 4 A and B. Micro–computed tomography images of fusion with fibrin glue before bone morphogenetic protein: Transverse process fusion examples with fibrin glue placed before recombinant human bone morphogenetic protein-2 sponge. Note little to no bone formation at transverse processes yet bone formation at spinous processes and in the soft tissues.

Table 1

	Mean bone area (sq mm)	Standard deviation
Fibrin glue Before BMP	1.72	0.86
BMP only	4.90	1.45
pValue	0.000221957	

rhBMP-2=recombinant human bone morphogenetic protein.
Standard deviation and p value for two tailed *t* test are listed.

agent, it has a long history of use in surgical applications involving bone healing. The modern version of fibrin glue was developed in Vienna in 1972. Arbes and Abiraman have reported that fibrin glue may stimulate bone formation in some human and rat studies [11,26], though other studies refute that it has a positive effect and it may even have a mild inhibitory effect depending on how it is used [18–21,27,28]. At the same time, it did not appear to have a negative effect on bone healing or bone formation in other studies [27,29]. Also, when used with bone and bone substitutes, fibrin glue improves the material consistency for improved handling without any detrimental effects on bone healing [6,13,25]. It has even been used in kyphoplasty type applications with new filler materials [12]. In the end it seems uncertain whether it stimulates, inhibits, or does not affect bone formation directly.

Newer studies have successfully utilized fibrin glue as a carrier for osteogenic cells [9,30], and even BMP [3,31,32]. In Nakamura's study, fibrin glue, BMP, and autograft bone were implanted at pars defects in rats, resulting in exuberant bone formation [5]. Hattori, in a mouse study, postulated that fibrin glue may be useful in controlling the diffusion and delivery of BMP when it controlled the area of osteochondrogenesis [4]. Thus, there is evidence that fibrin glue can control the diffusion of BMP without significantly altering its effectiveness.

In the in vitro aspect of this study, BMP was gradually released from the fibrin glue capsule. Not only was the rise in BMP levels slower, but the peak release of BMP was also significantly lower. Thus fibrin glue slows the diffusion of BMP from the collagen sponge without completely sealing it. There also appears to be a dose–response relationship, as the 1.5 mg/mL curve shows more rapid release before reaching the steady-state levels. This is certainly important as the soft-tissue inflammatory response may be related to BMP concentration.

The fibrin glue does not appear to bind rh-BMP2 as the curves of the control samples (rh-BMP2 sponge only) and the samples with the BMP sponge placed on top of a fibrin glue layer are very similar. This is significant, as a sealant used to protect the neural elements should also not act as a BMP sink.

The real clinical relevance of these in vitro data is worth consideration. It is known that the current FDA-approved concentration of 1.5 mg/mL failed to produce significant amounts of bone in rhesus monkeys [33]. This may have been because the collagen sponges were squeezed by the soft tissues, causing release of the BMP; however, it also implies that the current concentration approved for humans is on the lower end of the effective dose spectrum. Thus, reducing this concentration to under 50% of the original would likely block bone formation in areas where the fibrin glue is placed. The in vivo data support this as the fibrin glue placed between the transverse processes and the BMP did significantly limit bone formation.

Interestingly, in the nonencapsulated samples, the collagen sponge released nearly half of the rh-BMP2 almost immediately. This implies that this BMP was not bound, but simply held in the sponge's absorbed fluid, allowing nearly immediate diffusion into solution. This validates concerns of fluid, and thus BMP, leakage from the sponge into the spinal canal, foramina, and soft tissues. Indeed, in a clinical study, when the fluid that dripped from collagen sponges used for anterior cervical fusions was tested, significant amounts of BMP were found [34]. Thus, one consideration for future research should be the selection of a more ideal carrier for BMP delivery.

The animal model of this study shows significant blockage of bone formation at the rat transverse processes while bone formation in the soft tissues was present in reduced quantities. This shows the ability of fibrin glue to block the connection between the decorticated bony surfaces and the rh-BMP2 sponges. Whether this was from blocking the BMP from reaching the osteogenic cells, or from blocking the osteogenic cells from reaching the BMP is uncertain. Regardless, the connection was significantly disrupted by the fibrin glue. These results are different from previous studies in that fibrin glue has previously been used in conjunction with BMP to *stimulate* bone formation. Hattori, for example, mixed the BMP with the fibrin glue as a means of holding the BMP in place. He found that bone formation was only within the area of the BMP–fibrin glue mixture. Thus, in that combination, he believed the fibrin glue acted as a scaffold for osteogenic cells as well as blocking the diffusion of BMP [4]. Because, in our study, the fibrin glue separated the BMP from the osteogenic cells of the transverse processes, the same diffusion-limiting capacity appears to have blocked the connection, and thus bone formation was decreased.

This reduction in bone formation should caution the user of fibrin glue to keep it separated from decorticated bone surfaces where bone formation actually is desired. An interesting adjunct to this study would also be to place the fibrin glue over the BMP sponge in an attempt to contain the BMP to the vicinity of the bleeding bony surfaces. This may also limit the infiltration of fibroblasts in the initial stages of bone stimulation; thus yielding a greater fusion mass. Unfortunately, this was not studied but is the focus of further research.

This study does have several limitations. The rat model is certainly not the same as a human model, and bone formation may not be the same in humans as in rats. Also, the

BMP concentration used in the animal model was the minimum required for 100% fusion; the commercially available Infuse product rh-BMP2 concentration is 1.5 mg/mL. This higher concentration may more readily diffuse through the fibrin glue and still be in high enough concentrations to stimulate bone formation. Higher concentrations in rat models could certainly be used, but the answers will not really be elucidated until clinical human studies are carried out.

Conclusions

Fibrin glue does not appear to bind BMP when placed adjacent to rh-BMP2-soaked collagen sponges; however, it does limit, without completely stopping, the diffusion of BMP. Fibrin glue inhibited bone formation in the area of robust bone formation; thus, the inhibition of ectopic bone formation can be inferred when fibrin glue is used to separate the areas of desired bone formation from areas where bone formation is not desired.

Acknowledgments

The authors would like to thank Baxter Healthcare for providing the fibrin glue and Medtronic-Sofamor Danek for providing the rh-BMP2.

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