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Slow release of bone morphogenetic protein 2 from a gelatin sponge to promote regeneration of tracheal cartilage in a canine model

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Objectives: We investigated whether bone morphogenetic protein 2, released slowly from a gelatin sponge, could induce cartilage regeneration in a canine model of tracheomalacia and evaluated the long-term results.

Methods: A 1 × 5-cm gap was made in the anterior cervical trachea by removing 5-cm long strips of 10 sequential cartilagines. In the control group (n = 5), the gaps were left untreated. In the gelatin sponge group (n = 5), a gelatin sponge soaked in a buffer solution was implanted in each defect. In the bone morphogenetic protein group (n = 5), a gelatin sponge soaked in a buffer solution containing 12 μg bone morphogenetic protein 2 was implanted in each defect.

Results: Tracheomalacia was observed in the control and gelatin sponge groups but not in the bone morphogenetic protein group. No regenerated cartilage was detected in the control or gelatin sponge groups, even 6 months after surgery. In contrast, regenerated cartilage, which had developed from the host perichondrium, was observed around the stumps of the resected cartilagines in the bone morphogenetic protein group. This regenerated cartilage maintained the integrity of the internal lumen for longer than 6 months. A compressive fracture test revealed that the tracheal cartilage in the bone morphogenetic protein group was significantly more stable than that in the gelatin sponge and control groups (P = .0015 and P = .0001, respectively).

Conclusions: In this canine model of tracheomalacia, cartilage regeneration was induced around the stumps of tracheal cartilagines by bone morphogenetic protein 2 released slowly from a gelatin sponge. This regenerated cartilage was not reabsorbed for longer than 6 months and was strong enough to maintain the integrity of the internal lumen of the trachea.

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Despite the fact that tracheomalacia is not rare, there is as yet no generally accepted treatment for this condition.[1] Although we have developed a method for tracheal transplantation,[2-5] it is not suitable for treating tracheomalacia because too long a segment of trachea is usually affected by the disease. For some years we have been involved in the development of artificial organs, in which regeneration of the host tissue is induced by means of bioabsorbable materials, and have succeeded in regenerating sections of the canine esophagus.[6-8] We are now focusing on inducing the regeneration of host tracheal tissue to form an artificial trachea. The normal trachea is composed of a mucosal layer, a submucosal layer, tracheal glands, a smooth muscle layer, and the tracheal cartilagines, with the most important structures being the mucosa, which inhibits the invasion of exogenous organisms, and the cartilage, which maintains the integrity of the tracheal lumen. The first step in our attempt to produce an artificial trachea was to try to induce the regeneration of tracheal cartilage with bone morphogenetic protein 2 (BMP-2), released slowly from a gelatin sponge. BMP-2 has been identified as a cartilage- and bone-inducing molecule and has been shown to enhance chondrogenesis and osteogenesis in various animal models. It can now be cloned and synthesized by recombinant techniques. Our initial studies were based on the hypothesis that BMP-2 would induce the regeneration of cartilage and bone in an experimentally produced gap in the tracheal cartilage, with the expected outcome that our method would be useful in the treatment of tracheomalacia. In a previous article,[9] we reported the short-term results of implanting a gelatin sponge containing a BMP-2 solution into a tracheal cartilage gap, which demonstrated that such an implant could induce cartilage regeneration. In this article, we report the long-term results of the same study, covering an observation period of longer than 6 months.

Materials and Methods

This experiment was carried out in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996 (http://nap.edu/catalog/5140.html).

Preparation of BMP-2–Containing Gelatin Sponge

The manufacture of the BMP-2–containing gelatin sponge was described in our previous article.[9] Briefly, an aqueous solution of gelatin containing glutaraldehyde was cast in a mold and stored at 4°C for 12 hours. The material was then immersed in glycine solution at 37°C to block any residual glutaraldehyde, washed with distilled water, and finally freeze-dried and sterilized. The resulting gelatin sponge was trimmed into pieces measuring approximately 1.0×5.0 cm, and each was soaked in a solution containing 12 μg BMP-2. This solution was prepared by dissolving BMP-2 in

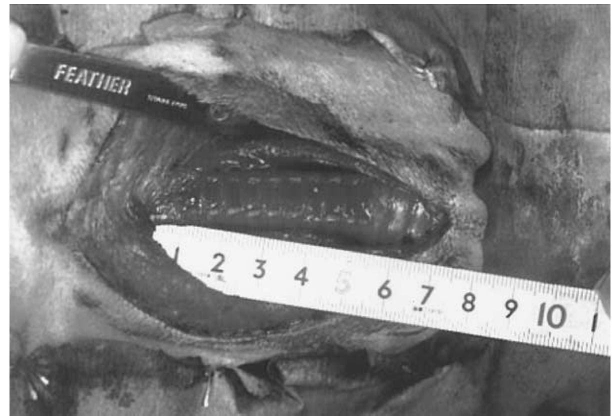

Figure 1. Surgical procedure for control group: 1×5-cm gap in tracheal cartilage was created by removing 5-cm long strips of 10 sequential cartilage rings, taking care not to damage mucosa.

a buffer (5-mmol/L glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% polysorbate 80) at a concentration of 1 μg/1 μL, then adding distilled water to a final volume of 5 mL.

Previous studies have shown that a BMP-2 dose of 10 μg is enough to induce ectopic bone formation in mice.[10] In our laboratory, BMP-2 solution is routinely divided into microtubes containing a 12-μg dose and stored in a refrigerator. We therefore selected this dose partly for convenience and partly to represent a low initial dose.

Surgical Procedures

Fifteen adult mongrel dogs weighting 10 to 14 kg were used in this study. A 1×5-cm gap in the anterior tracheal cartilage was created by removing 5-cm long strips of 10 sequential cartilagines from the middle of the cervical region. The tracheal mucosa was carefully preserved throughout the procedure (Figure 1). In the 5 dogs acting as a control group, the gap was left unfilled. A gelatin sponge soaked in the buffer solution alone was implanted to replace the resected portion of the tracheal cartilage in 5 further dogs (gelatin sponge group). A gelatin sponge soaked in solution containing 12 μg BMP-2 was implanted to replace the resected portion of the tracheal cartilage in the 5 remaining dogs (BMP group; Figure 2).

Follow-up Studies

Routine bronchoscopic examinations were done at 1, 3, 6, 12, and 15 months after the operation. The animals were killed at 1, 3, or 6 months after surgery. The trachea was immediately removed and fixed in 10% formalin, and the cervical trachea was examined histopathologically. In addition, the anterior cartilaginous portion of each trachea was mounted on a Creepmeter (RE 330055; Yamaden Co Ltd, Tokyo, Japan), and a compressive fracture test was performed. The stress required to produce breaks in the cartilage was measured and used as an estimate of the stability of the tracheal cartilage.[11,12] Six preparations were made from 1 animal in each group at 3 months after the operation.

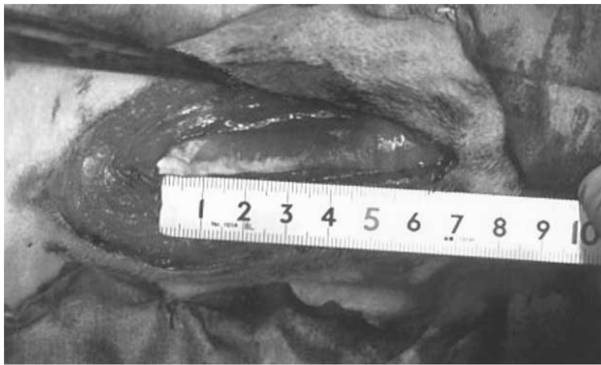

Figure 2. Surgical procedures for gelatin sponge and BMP groups: gap in tracheal cartilage was created as shown in Figure 1. Gelatin sponge soaked in buffer solution alone or solution containing 12 μg BMP-2 was then implanted into gap.

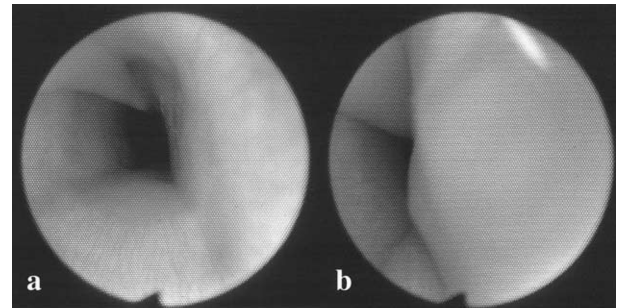

Figure 3. Bronchoscopic findings in control group 6 months after surgery. Tracheal lumen collapsed during coughing. a, At rest; b, during coughing.

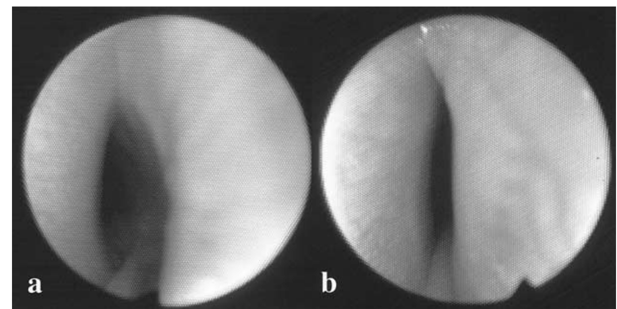

Figure 4. Bronchoscopic findings in gelatin sponge group 6 months after surgery. Tracheal lumen collapsed during coughing. a, At rest; b, during coughing.

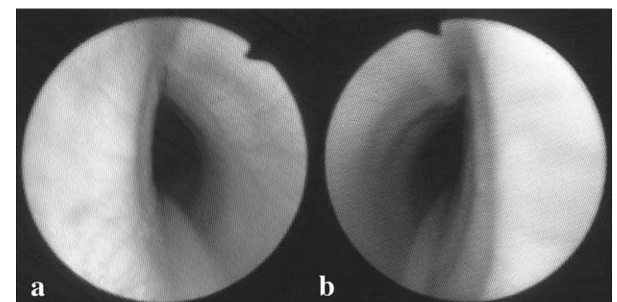

Figure 5. Bronchoscopic findings in BMP group 6 months after surgery. Slight tracheal stenosis was observed, but there was no collapse, even during coughing. a, At rest; b, during coughing.

TABLE 1. Clinical findings

Dog No.	Survival (mo.)	Status	Malacia
Control group			
1	1	Planned death	+
2	3	Planned death	+
3	6	Planned death	+
4	14	Planned death	+
5	6	Alive	+
Gelatin sponge group			
1	1	Planned death	+
2	3	Planned death	+
3	6	Planned death	+
4	6	Alive	+
5	6	Alive	+
BMP group			
1	1	Planned death	−
2	3	Planned death	−
3	6	Planned death	−
4	12	Planned death	−
5	15	Planned death	−

Results

Clinical Findings

All the dogs survived without any major health problems until they were killed as planned (Table 1). However, the dogs in the control and gelatin sponge groups demonstrated stridor during barking or coughing. None of the dogs in the BMP group had stridor.

Bronchoscopic Findings

Throughout the observation period, routine bronchoscopic examinations revealed collapse of the tracheal lumen during coughing in both the control group (Figure 3) and the gelatin sponge group (Figure 4). Although slight stenosis of the tracheal lumen was observed in the BMP group at rest, the tracheal wall was firmly fixed in this group and did not collapse during coughing (Figure 5). The findings for all the groups remained essentially unchanged throughout the observation period, which lasted longer than 6 months.

Microscopic Findings

Control group. Microscopic observations revealed no cartilage regeneration, even at 6 months after the operation, although the ends of the stumps of the cartilagines were covered with perichondrium. The submucosal layer over the cartilage gap was thicker than normal (Figure 6).

Gelatin sponge group. The findings were similar to those in the control group. The gelatin sponge had disap-

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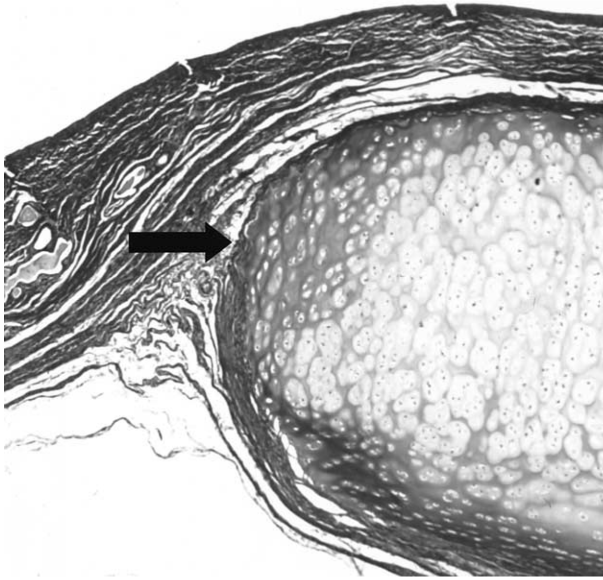

Figure 6. Microscopic findings in control group 6 months after surgery. No cartilage regeneration is detectable around cartilageal stump *(arrow).* (Original magnification ×150).

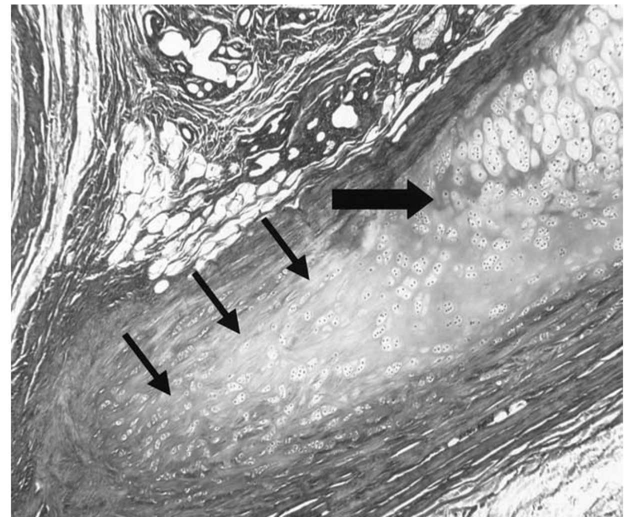

Figure 8. Microscopic finding for BMP group 6 months after implantation. Regenerated cartilage *(thin arrow)* arising from perichondrium can be seen around stump *(thick arrow)* of resected cartilagines. (Original magnification ×150).

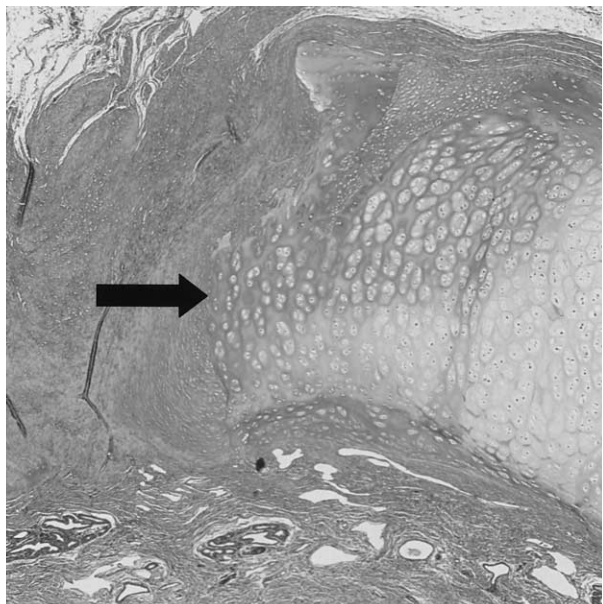

Figure 7. Microscopic finding in gelatin sponge group 6 months after implantation. No cartilage regeneration is detectable around cartilageal stump *(arrow).* (Original magnification ×150).

peared 1 month after the operation; thus the gelatin sponge itself did not induce regeneration of cartilage (Figure 7).

BMP group. Newly formed cartilage arising from perichondrium surrounding the stumps of the cartilagines was observed 1 month after implantation of the sponge. The regenerated cartilage was directed mainly toward the luminal or contraluminal side, rather than toward the center of the gap. The distance between the cartilage stumps was shortened. The gap was filled with amorphous connective tissue that invaded the cartilage gap. The regenerated cartilage was similar to fibrous cartilage but differed from the hyaline cartilage found in the host trachea. It consisted of immature chondrocytes with a poorly developed, nonhomogeneous extracellular matrix. At 6 months after implantation, regenerated cartilage was detected arising from the perichondrium on both sides of the gap, and the gap was filled with fibrous tissue. Again, the direction of regeneration was mainly toward the contraluminal side of the trachea, rather than toward the center of the gap (Figure 8). The regenerated cartilage was covered with perichondrium.

Compressive Fracture Test of Tracheal Cartilage

The stress levels required to produce breaks in the anterior tracheal cartilage (Table 2) were $3.52 \pm 1.03 \times 10^6$ N/m^2 for the normal trachea (n = 6 specimens), $4.92 \pm 1.32 \times 10^6$ N/m^2 for the BMP group (n = 6 specimens), $1.68 \pm 1.26 \times 10^6$ N/m^2 for the gelatin sponge group (n = 6 specimens), and $0.68 \pm 0.43 \times 10^6$ N/m^2 for the control group (n = 6 specimens). The tracheal cartilage was significantly more stable in the BMP group than in the gelatin sponge group (P = .0015) and the control group (P = .0001). In addition, it was significantly more stable than normal trachea in the BMP group (P = .013), and it was significantly more fragile than normal trachea in the control group ($P < .001$) and gelatin sponge group (P = .002).

Discussion

Persistent coughing and stridor during barking were observed in the control and gelatin sponge groups. Bronchoscopy revealed that these dogs had tracheomalacia in the area around the partial resection of the tracheal cartilage. These findings remained essentially unchanged throughout an observation period longer than 6 months. One of the potential causes of tracheomalacia, especially saber sheath–type tracheomalacia, is laceration and weakness of the tracheal cartilage.[13,14] Our experimental procedures were therefore appropriate for constructing a model of this type of tracheomalacia, as typified by the control group. Follow-up microscopic observations in this group revealed no cartilage or bone regeneration, and the gap did not shrink. Similarly, no regeneration of cartilage was observed in the gelatin sponge group, indicating that the gelatin sponge itself does not promote regeneration.

In the BMP group, regenerated cartilage was observed at the ends of the stumps of the host cartilage, with the direction of regeneration toward the luminal or contraluminal side rather than toward the center of the cartilage gap. However, the regeneration process seemed to stop 3 months after implantation, and regeneration was limited to the cartilage stumps even at 6 months. Nevertheless, even this small amount of regenerated cartilage was able to maintain the integrity of the internal lumen of the trachea. The remaining gap was filled not with cartilage, but with amorphous connective tissue that may have represented a postinflammatory reaction.[10] Because our microscopic observations revealed no such tissue in the gelatin sponge and control groups, this phenomenon may have resulted from the wound healing process or a foreign-body reaction caused by the gelatin[10,15] or BMP-2, which may have enhanced any postinflammatory reaction. Gelatin is a biodegradable material that induces fibroblast migration during its absorption.[6-8,16] These migrated fibroblasts become myofibroblasts, which are involved in wound contraction during healing.[6-8] Contrary to our expectations, BMP-2 did not induce the differentiation of such fibroblasts into osteocytes or chondrocytes. Previous studies have shown that a BMP-2 dose of 10 μg or less is enough to induce ectopic bone formation in mice,[10] but not in rats[17] or dogs.[18] In this study, however, cartilage regeneration from the perichondrium was observed around the cartilage stumps in the BMP group but not in the control or gelatin sponge groups, despite the low BMP-2 dose used. These results indicate that even a low dose of BMP-2 can promote cartilage regeneration from perichondrium in dogs, and that slow release of BMP-2 from a gelatin sponge can successfully induce the regeneration of cartilage in a canine tracheomalacia model. Although the regeneration of cartilage was limited, and slight narrowing of the trachea was observed at rest, the internal lumen of the trachea was maintained even during coughing. Furthermore, the regenerated cartilage had not been reabsorbed 6 months after the operation. In addition, a compressive fracture test revealed that the cartilage in the BMP group was significantly more stable than that in the control and gelatin sponge groups, or in normal trachea. Importantly, the implantation procedure is relatively easy to perform and can be carried out in the segmental bronchial area. In the clinical setting of tracheomalacia, the immature cartilage is covered with connective tissue. The direct contact of cartilage with BMP-2 may be essential to form new cartilage.

TABLE 2. Stress for breaks of anterior tracheal cartilage

	Stress (10^6 N/m^2)
Control group	0.68 ± 0.43*†
Gelatin sponge group	1.68 ± 1.26‡§
BMP group	4.92 ± 1.32‖
Normal trachea	3.52 ± 1.03

Data are mean ± SD.
*$P = .0001$ versus gelatin sponge group.
†$P < .001$ versus normal trachea.
‡$P = .0015$ versus BMP group.
§$P = .002$ versus normal trachea.
‖$P = .013$ versus normal trachea.

Conclusion

Long-term observations demonstrated that our control group fits the requirements for a tracheomalacia model. Slow release of BMP-2 from a gelatin sponge induced the regeneration of cartilage in this model. Furthermore, the newly formed cartilage was capable of maintaining the integrity of the internal lumen of the trachea through a long period without being resorbed.

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