

# OP-1 has more effect than mechanical signals in the control of tissue differentiation in healing rat tendons

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Although osteogenic protein 1 (OP-1) is best known for its ability to induce bone formation, it is a differentiation factor with diverse functions in the development of non-bony tissues. It is expressed in developing tendon. We therefore hypothesized that OP-1 might stimulate the differentiation of a tendon callus. Rat achilles tendons were transected and a collagen sponge with or without OP-1 was placed in the defect. OP-1 induced the formation of an ossicle, which reduced tendon strength at 2 weeks postoperatively. Abolition of muscle force by tibial nerve transection or reducing load by forefoot amputation reduced tendon strength by almost half during the same period. Thus, traction forces are potent ten-

don-tissue inducers. OP-1 reduced the strength of denervated tendons even further, but the induced ossicles appeared similar to those in loaded tendons. Thus, both OP-1 and unloading independently reduced tendon strength. In conclusion, the ability of OP-1 to induce bone was greater than the mechanical and environmental signals for a more traction-resistant tissue, indicating that signal proteins may have more direct or stronger effects than mechanical stimuli on tissue differentiation. We also found that a single percutaneous injection of OP-1 reproducibly induced large amounts of bone in this setting, although it is generally believed that BMPs always need to be inserted with a carrier.

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The differentiation of regenerating mesenchymal tissue is governed by local expression of signal proteins (Bostrom et al. 1995) and a direct mechanical input on the differentiating cells (Carter et al. 1996). A cell may start dividing or differentiating either because it has perceived strain directly or because it is exposed to signaling peptides from other cells that are not necessarily secreted as a result of mechanical input. It is not known whether signaling proteins and mechanical stimuli represent two separate control systems or if and how they are integrated with each other. Mechanical signals to regenerating tissue are thought to govern its local differentiation in great detail. The development of a fracture callus can be closely modelled in finite element systems based on local mechanical input (Carter et al. 1988). Many cell types, including osteoblasts, react quickly to mechanical stimuli in vitro by changing their growth and differentiation behavior (Niedlinger-Wilke et al. 1994). Thus, mechanical stimulation could influence cells directly, but it is also possible that they exert their effects indirectly via production of local paracrine or autocrine signaling peptides.

Conversely, differentiation initiated by paracrine factors from the bone morphogenetic protein (BMP)

family may be governed by local mechanical conditions. Although BMPs are mainly known for their ability to induce bone formation, they can also initiate other kinds of differentiation, such as formation of large parts of the urinary system (Vukicevic et al. 1996) and other parts of the body (Reddi 1997). These other effects refer to embryonal development, but can also be reproduced during repair after tissue injury in adults (Vukicevic 1997). In regenerating tissues of the locomotor system, the differentiation induced by BMPs can be modified by local conditions. Implants containing BMP-2 at a damaged joint surface produce bone formation in the deeper layers, but cartilage induction towards the joint cavity (Sellers et al. 1997). Thus, if a BMP is introduced into a local environment which is strongly directed at producing tendon tissue, it could be hypothesized that a BMP would induce a tendon-like tissue. In fact, BMP 7 (OP-1) is strongly expressed in the developing fetal tendon (Macias et al. 1997).

We tested the hypothesis that a mechanical signal which is known to stimulate the differentiation and organization of healing tendon also may direct BMP-induced differentiation towards formation of tendon tissue.

## Animals and methods

### Experimental procedures

We adopted the rat achilles tendon model described by Murrell et al. (1994), with some modifications. Instead of immobilizing the ankle by external fixation, which we found traumatic and difficult, we performed denervation of calf muscles by tibial nerve transection. This yields loss of muscle traction on the achilles tendon, with retention of passive motion. For comparison, we also reduced this muscle force by forefoot amputation in a smaller group of rats.

We used 92 female Sprague Dawley rats (Møllegaard, Copenhagen; Table 1). They were anesthetized with chloral hydrate intraperitoneally (4 mg/kg bodyweight). All rats had an achilles tendon transection, 41 of these were also denervated and 9 had a forefoot amputation. A collagen sponge with or without OP-1 was placed in the tendon defect before the skin was closed. Instead of using a carrier such as collagen, 16 rats were given a local injection with or without OP-1 at the place of the achilles transection. 2 weeks after the operation, the rats were killed with an overdose of pentobarbital.

For tendon transection, a 3 mm transverse skin incision was made beside the right achilles tendon. The surrounding fascia was split longitudinally and the achilles tendon complex was dissected free. Since the plantaris tendon is well developed in rats, it was removed to prevent it from acting as an internal splint. The achilles tendon was cut about 5 mm proximal to the calcaneal insertion and the collagen sponge was inserted before the skin was sutured.

For denervation, a 1.5 cm long posterolateral incision was made just above the knee. The tibial nerve was uncovered and cut off 1–2 mm proximal to the branches to the calf muscle. Thus, the peroneal nerve was left in function.

For amputation, a transverse dorsal incision was made over the tarsometatarsal joints, which were disarticulated. A plantar flap was prepared and sutured to the dorsal skin, so that the foot pad was not damaged under the remaining tarsus.

1 × 2.5 × 2.5 mm pieces of collagen (Helistat, Colla-tec, inc.) were prepared aseptically from larger

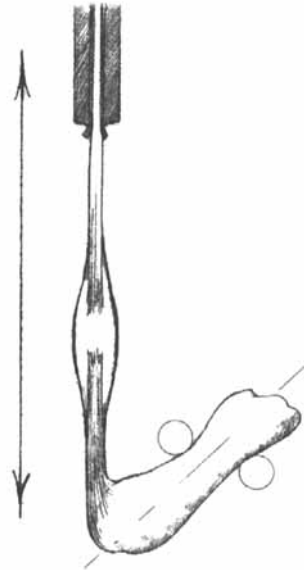


Figure 1. Set-up for tensile strength measurement.

pieces. 10 µg of OP-1 dissolved in 6 µL 5 M acetate buffer or buffer alone was soaked on the collagen, which was then lyophilized before implantation.

6 h after tendon transection, 50 µL OP-1 or buffer was injected subcutaneously into the palpable tendon defects without collagen implants. The dose of OP-1 was 100 µg. During the injection, the rats were lightly anesthetized with ether.

After the rats were killed, the lower limbs were frozen in liquid nitrogen. They were then wrapped in gauze soaked with NaCl and stored in a –20 °C freezer. After about 1 week of storage, the specimens were thawed in NaCl at room temperature. During all handling, the specimens were blinded as to treatment. The calcaneus and achilles tendon were dissected. The complex was fixed between two metal clamps. The angle between the calcaneus and achilles tendon during testing corresponded to 30° dorsiflexion of the foot (Figure 1). The clamps were fixed in a materials testing machine built for this study. A transducer was built from a brass ring and strain gauges in a 4-bridge, and connected to a computer to measure the force applied momentarily. The system was calibrated with

Table 1. Number of animals allocated to different treatment groups. Histology in parentheses. All groups had achilles transection

	Contr. collagen	OP-1 collagen	Contr. inj.	OP-1 inj	Total
Achilles transection only	21 (5)	21 (5)	0	0	42
Denervation	12 (4)	13 (5)	8	8	41
Forefoot amputation	0	0	9	0	9

**Table 2.** Failure load (N) of the rat achilles tendon 2 weeks after transection

Group	n	Mean	SD
Control	13	39	15
Amputation	9	22	7
OP-1	9	24	9
Denervated	8	19	6
Denervated OP-1	8	10	3
Denervated contr. inj.	5	23	7
Denervated OP-1 inj.	7	14	3

weights. The tendon was pulled with a speed of 1.0 mm/s, without preconditioning. Altogether 59 tensile tests were performed.

After fixation, the dimensions of the tendon callus were measured with vernier calipers (maximum frontal and sagittal diameters).

Histological sections were made of 19 specimens in order to measure the size of the bone callus in the healing tendons. The specimens were fixed in buffered formalin, decalcified in Parengy's solution, embedded in paraffin before sectioning (5 µm) and HE staining. Sections were taken every 200 µm through the specimen. In each section, tendon diameter, bone callus diameter and bone callus length were measured using the Videoplan™ system (Kontron Bildanalyse, Esching, Germany). From these serial surface measurements, the volume of the bone callus was calculated.

### Statistics

The effects of denervation and OP-1 were analyzed by 2-way Anova. Other comparisons were tested with the Student's t-test.

## Results

### OP-1 or control on collagen carrier (Table 2)

12 tendons were excluded from the study because they fragmented during freezing and 2 rats died of anesthesia. Amputation reduced the healing tendon strength by 42 % compared to innervated controls ( $p = 0.04$ ). Independent of OP-1 treatment (injections not included), denervation reduced the strength by 61% ( $p = 0.0001$ ). Independently of denervation (injections not included), OP-1 treatment reduced the strength by 44% ( $p = 0.003$ ).

On the histological examinations, bone was never seen without OP-1 treatment, whereas all OP-1 specimens contained a large ossicle (Figure 2). The central part of the ossicle contained bone marrow and fat cells. In the unloaded tendons, there were also large amounts of collagen remnants in the middle of the os-

sicle. This was not found in the loaded tendons. Surrounding the marrow cavity there was a thin bone shell consisting mostly of woven bone. Peripheral to the bone, it was surrounded by obvious cartilage (Figure 3). The cartilage layer was thicker at the proximal and distal ends of the ossicle. There was an abrupt transition from cartilage to the surrounding fibrous tendon callus.

There was no difference in bone callus size between loaded and unloaded OP-1-treated tendons, whereas the product of the two caliper diameters was 24% larger for loaded OP-1-treated tendons compared to unloaded OP-1-treated tendons ( $p = 0.02$ ).

### OP-1 or control injection

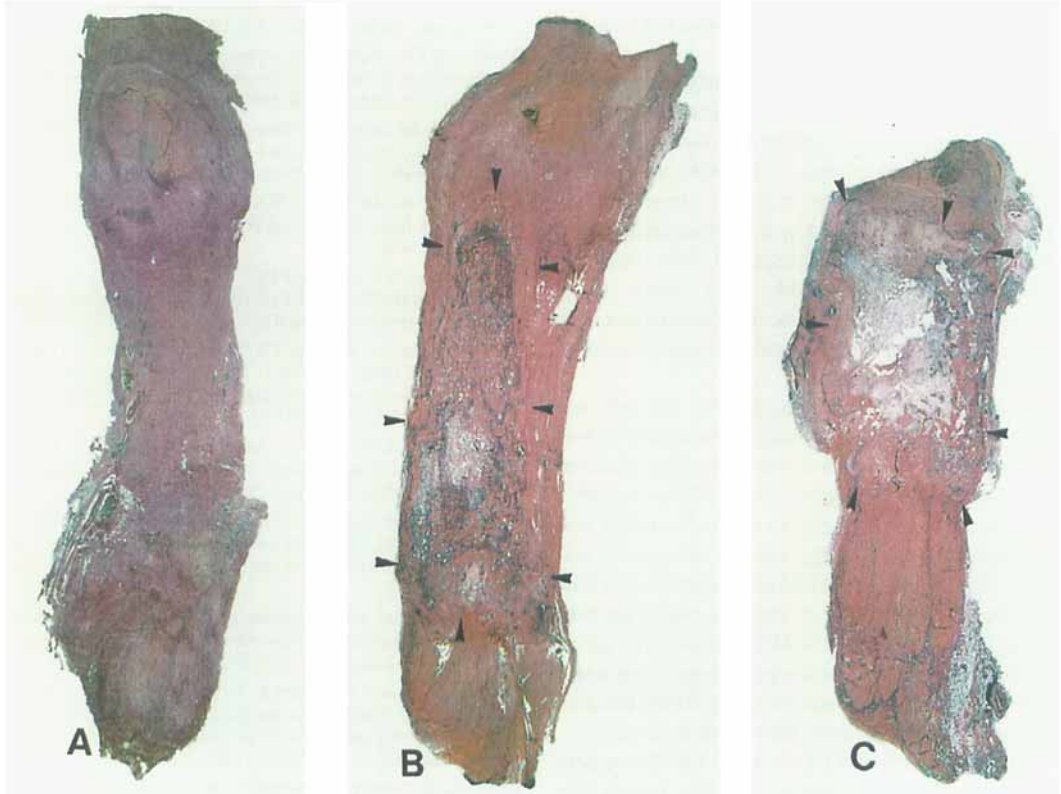
Injection of OP-1 reduced the strength by 39% ( $p = 0.01$ ). Grossly, a clearly larger ossicle had developed, which in most cases had fused with the calcaneus. This fusion was never seen in the other groups.

## Discussion

Our findings support the view that OP-1 in a mesenchymal environment is usually a bone inducer. This property took precedence over the mechanical demand for a more traction-resistant tissue. It appears that the chemical signal had a greater effect than the mechanical signal. It is not known if the mechanical signal affects tissue organization only by direct effects on cells, or if autocrine or paracrine secondary signals are involved. The observation that OP-1 can surpass otherwise powerful mechanical signals might indicate that mechanical signals control differentiation via local para- or autocrine signals likely to involve morphogens of the BMP family. In that case, the secondary signals are unlikely to be mediated by OP-1 in the case of tendon healing. However, a related protein, GDF-5, is thought to be a tendon or ligament inducer (Wolfman et al. 1997). It seems possible that mechanical signals govern the choice of differentiation pathway by regulating the balance between different morphogens like OP-1 and GDF-5. Mice deficient of the GDF-5 or BMP-5 genes show only moderate aberrations in skeletal gross morphology, indicating that these factors can work as fine tuners of skeletal modeling (King et al. 1996).

Our study confirms previous reports that mechanical unloading of the achilles tendon in rats reduces tendon healing, leading to a weaker tendon callus at 2 weeks (Murrell et al. 1994). This has previously been shown using external fixation of the foot versus the lower leg. We found this technically difficult and instead used forefoot amputation to shorten the lever

Figure 2. Achilles tendons 14 days after transection. Calcaneal insertion above and muscle junction below. HE. Total length about 15 mm.



Undenerivated control shows a fibrous regenerate.

Undenerivated OP-1 specimen shows an ossicle (arrow heads) containing a marrow cavity.

Denervated OP-1 specimen is similar to B, but parts of the collagen carrier remain in the marrow cavity.

arm from the loaded part of the planta to the ankle joint. Rats have a foot pad corresponding to the level of amputation. As the rats sustained this amputation with minimum swelling and no other complications, and appeared to bear weight on the foot, we believe that the diminished tendon strength is a result of unloading rather than a nonspecific posttraumatic effect. When instead the calf muscle was denervated, the reduction in strength was similar. We cannot rule out the possibility that the absence of innervation of the tendon callus reduced its healing capacity, but believe it to be more important that the absence of muscle contraction force reduced the tendon healing rate similarly to the unloading by forefoot amputation. We used denervation because it was technically easier. Regardless of whether unloading or absence of callus innervation was responsible, a less optimized healing situation was obtained in our model which would increase the chances of finding a stimulatory effect of OP-1 on tendon callus strength. Even though OP-1 induced bone both in unloaded and loaded tendons, it did not entirely control tissue development. After OP-1 treat-

ment, the loaded tendons were stronger than the unloaded ones although both contained apparently similar ossicles. This is probably explained by the formation of a tendon-like tissue surrounding the ossicle. Using histology, we had the visual impression that this layer was thicker and better organized in the load-

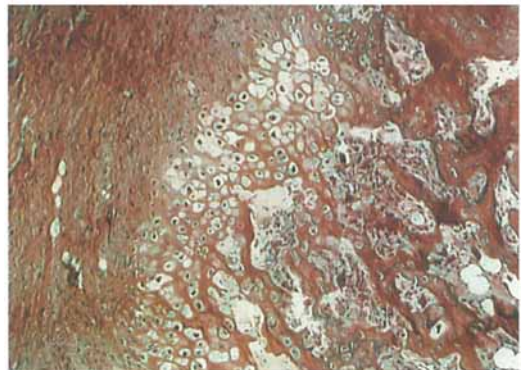


Figure 3. OP-1 injected specimen. The transition zone between bone and tendon regenerate shows cartilage indicating that the ossicle is still growing by endochondral ossification.

ed specimens, but it could not be demonstrated quantitatively in the relatively few histological specimens. However, the loaded tendons were found to be thicker with caliper measurements.

The induced ossicles were definitely larger in the achilles tendon region than in 10 identical intramuscular implants in the abdominal wall (data not shown). This could be due to the more traumatized situation in the achilles region, where a large proliferative response was induced by the tendon transection. Conceivably, more undifferentiated cells were recruited and could be affected by OP-1. This is also in line with previous studies indicating that trauma is an important co-factor for BMP-induced bone formation (Bostrom et al. 1998).

It has generally been thought that recombinant BMPs like OP-1 need a carrier to induce bone formation. In the achilles tendon region, this was not necessary. There could be many reasons. First, the OP-1 dose was large, but not excessively so. Secondly, one could speculate that a large amount of exposed collagen in the tendon transection site could retain BMPs better than the well-perfused muscles, which are usually used for extraskkeletal BMP implantations or injections. Last but not least, the local trauma may again be important to increase sensitivity to BMP stimulus, perhaps by recruiting more undifferentiated cells.

Since other members of the BMP family, namely GDF-5, 6 and 7, are reported to induce tendon-like tissue, even in an unloaded setting (intramuscular implants) (Wolfman et al. 1997), it would, of course, be most interesting to study their effects in a tendon healing model.

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