Collagen Production and Maturation at the Experimental Ligament Defect Stimulated by Pulsing Electromagnetic Fields in Rabbits

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(Received 17 November 1992/Accepted 6 January 1993)

ABSTRACT. Eighty rabbits receiving the square resection (4×4 mm) of both patellar ligaments in full thickness at their center were divided into 4 groups, and each group (20 rabbits) were electromagnetically stimulated with different magnetic intensities, 0 (control group), 2, 10, or 50 gauss (G), for 6 hr daily. Pulse frequency and pulse width were 10 Hz and 25 μ sec, respectively. Five animals of each group were sacrificed weekly from 1st to 4th week after operation and the defect tissue was collected for biochemical and ultrastructural evaluations. Before sacrificing, the blood flow of the central portion of patellar defects were measured. Significant increases in blood flow at the defect were observed in 50 G group compared to those of other groups from 2 to 4 weeks after operation. The collagen content in PEMFs groups showed a significant increase compared to that of control group. Furthermore, those increases were higher according to the increase in magnetic intensity. Electronmicroscopically, massive developmental rough endoplasmic reticulum was seen in the fibroblasts at 2 weeks after operation in 50 G group compared to other groups, which suggests the more active collagen production in this group. These results suggest that PEMFs enhanced the blood flow and increased the fibroblasts at the defect. At the same time, PEMFs directly stimulated the collagen production from the fibroblasts, thus accelerate the healing process of the ligament.—KEY WORDS: blood flow, collagen, electron microscopy, ligament healing, PEMF.

— J. Vet. Med. Sci. 55(4): 527–531, 1993

Using electrical stimulation to promote healing in injuries to the musculoskeletal system is not a new concept. A number of studies on the effect of the electrical stimulation on the bone healing have been reported [4, 6], however, there have been a few reports on its effects on the ligament and tendon healing [1, 8]. From our previous research [14], stimulation by pulsing electromagnetic fields (PEMFs) promotes the early stage of ligament healing in rabbits. Histologically, earlier angiogenesis and more matured prominent longitudinal orientation of the collagen fiber were evident at the defect of the ligament tissue under daily stimulation by PEMFs. Biomechanical investigation also revealed a significant and earlier increase in tensile strength of the regenerated ligament tissue stimulated by PEMFs. These results may suggest that PEMFs enhances neovascularization and maturation of the collagen fibers at an earlier stage of the ligament repairing process. However, precise etiology of the effect of PEMFs on ligament healing is still unclear. This study was conducted to investigate the local blood flow and the maturation of the collagen fibers in the repairing process of the ligament in rabbits.

MATERIALS AND METHODS

Experimental animals: Experimental animals, surgical procedures and methods of electromagnetic stimulation were the same as those of our previous report [14]. Briefly, 80 male Japanese White rabbits received a resection of 4×4 mm square at the center of the patellar ligament in full thickness. They were divided into 4 groups

(20 rabbits each) according to the electromagnetic intensity, 0 (control), 2, 10, or 50 G used for stimulation. Electromagnetic stimulation was performed for 6 hr daily.

Five animals of each group receiving daily stimulation of PEMFs were used weekly from 1st to 4th week after operation for measurement of local blood flow at the defect. After the measurement they were sacrificed and the defective tissue was collected for the analyses of collagen content and its types and ultrastructural observations.

Blood flow measurement: The blood flow at the central part of the defective portion of the patellar ligament was measured by laser Doppler flowmetry (model ALF 21, Advance Co., Ltd., Tokyo). Under the intramuscular injection of xylazine (5 mg/kg) and ketamine hydrochloride (20 mg/kg), the patellar ligament of both sides were exposed through longitudinal parapatellar incision. The probe of laser Doppler flowmetry was fixed to the central part of the defective portion with minimum pressure applied. After the values were stabilized (several min were generally required), blood flow of the regenerated ligament was recorded [9].

Measurement of collagen content: Newly formed tissue at the defect of the patellar ligament was minced into small pieces, crushed in liquid nitrogen and suspended in cold 4.5 M NaCl 0.05 M Tris-buffer (pH 7.5). The sample was then homogenized and centrifuged at 10,000 rpm for 15 min to remove non-collagen ingredients. The residues were extracted using 1.0 M NaCl 0.05 M Tris-buffer (pH 7.5), and divided into neutral soluble, acid soluble and non-soluble fractions [5, 10]. These fractions were then freeze-dried (VD-80, Taitec CO., Tokyo) and preserved.

Table 1. Preparation of SDS-polyacrylamide gel

Solution		Lower gel	Upper gel
Acrylamide solution; ^{a)}	(ml)	4.5	0.81
Water	(ml)	7.5	2.8
10% SDS	(ml)	0.2	0.06
1.5 M Tris-buffer (pH 8.8)	(ml)	5.0	—
0.5 M Tris-buffer (pH 6.8)	(ml)	—	1.5
Ammonium persulfate; ^{b)}	(ml)	0.2	0.06
TEMED	(ml)	0.02	0.006
Urea	(g)	3.6	1.1

a) Acrylamide solution: 22.2 g of acrylamide, 0.6 g of N,N'-methylenebisacrylamide, water to 100 ml.

b) Ammonium persulfate: 100 mg ammonium persulfate per 1 ml water.

Just before the determination of hydroxyproline content, they were hydrolyzed with 6N hydrochloric acid at 130°C for 3 hr. The acid hydrolysate was treated according to the procedure of Woessner's method [20], and the amount of hydroxyproline was read at 557 m μ in a double beam spectrophotometer (Model 124, Hitachi, Tokyo).

Analysis of the type of collagen: Analysis of the type of collagen was carried out using SDS-polyacrylamide gel electrophoresis [18]. The preparation of SDS-polyacrylamide gels was shown in Table 1. Preparation of samples were the same as stated previously for the measurement of collagen. After the removal of non-collagen ingredients, 0.08 ml of sample was added to 0.5 M Tris-buffer solution, 1% SDS, 3M urea, 2% 2-mercaptoethanol and bromphenol blue (BPB), and then incubated at 100°C for 5 min before application to the gel.

The current was kept at 20 mA in SDS-Trisglycine buffer until BPB arrived to the end position of lower gel. The gels were immersed in a staining solution composed of 0.1% of Coomassie Brilliant Blue, 50% methanol and 10% acetic acid for 30 min. The staining solution was decanted out, and the gel was removed and immersed in destaining solution containing 5% methanol, 7.5% acetic acid and water. The existence of each type of collagen band was confirmed by gross appearance.

Ultrastructural observations: Tissue samples were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in epon resin. Thin sections were cut, stained with uranyl acetate and lead citrate, and observed with a JEM-1200EX (JEOL, Tokyo) transmission electron microscope at 80 Kv.

Cross section area of collagen fibril was measured from thin sections of transversely sectioned fibrils. The term fibril here is used to define the collagen structures visible in the electron microscopy. The sections were viewed and photographed using JEM-1200EX electron microscope. The photographs of collagen fibrils were printed on printing paper at $\times 60,000$. The area of horizontal section of collagen fibrils were analyzed using an image analysis system (PIAS LA-500, PIAS Ltd., Osaka) and a computer program (NEC PC-9801VX and Math Coprocessor,

NEC, Tokyo).

Statistical analysis: The results of blood flow, collagen content and cross section area of collagen fibril were statistically analyzed by Student's *t*-test, and a P value less than 0.05 was considered to be significant.

RESULTS

Blood flow: Changes in blood flow at the defective portion of the patellar ligament in each group was shown in Fig. 1. The blood flow reached peak levels at 2 weeks after operation in any of the experimental groups, then decreased thereafter. Among the groups, those in 50 G group always showed significantly higher values than those in any other groups throughout the experimental period. The blood flow in 10 G group at 2 weeks after operation was also significantly higher than that in control group, however, there were no significant differences in blood flow between the rabbits of control and 2 G groups.

Collagen content: Changes in hydroxyproline levels of regenerated tissues at the defect of the patellar ligament were shown in Fig. 2. At any weeks after operation, hydroxyproline contents were significantly higher according to the levels of electromagnetic intensities used, and those of 50 G group were the highest among the groups at any weeks.

Type of collagen: The typical electrophoretic pattern of the regenerated tissues was shown in Fig. 3. The bands of type I and type III collagen were observed in any samples measured in all the groups. Furthermore, there were no

Fig. 1. The changes in blood flow of the regenerated ligament during the experimental period. Vertical bars represent S.D. *: Significantly different from control ($p < 0.05$).

Fig. 2. Time course of collagen content of regenerated ligament calculated by Woessner's method. *: Significantly different from control ($p < 0.05$).

Fig. 3. SDS-polyacrylamide gel electrophoresis of collagen in the regenerated and normal ligament. Lane 1 (control group), Lane 2 (2 G group), Lane 3 (10 G group) and Lane 4 (50 G group) at 4 weeks after operation: components of type I and type III collagen. Lane 5 (normal ligament): components of type I collagen. Type III collagen is not detectable.

Fig. 4. Electron micrographs showing fibroblasts at 2 weeks after operation. Fibroblasts of 50 G group reveal well developed rough endoplasmic reticula among the groups.

Fig. 5. Time course of cross section area of collagen fibrils from 2 to 4 weeks after operation. *: Significantly different from control ($p < 0.05$).

differences in ratio between type I and type III collagen bands by gross appearance at any weeks in all the groups.

Ultrastructural observations: At 1 week after operation, erythrocytes and many fibroblasts could be observed in the regenerated tissues of all the groups. The fibroblasts contained round nuclei, a few rough endoplasmic reticulum and mitochondria, glycogen granules, and fatty granules. However, collagen fibrils were not observed in any tissues of all the groups. At 2 weeks after operation (Fig. 4 a-d), fibroblasts of 50 G group revealed well developed rough endoplasmic reticulum, whereas the development of rough endoplasmic reticulum was moderate in 2 G and 10 G groups, and poor in control groups. In addition, the location of rough endoplasmic reticulum in control group was still in the middle part of cytoplasm, however, those of 2 G and 10 G groups nearly expanded to cell membrane. The position of rough endoplasmic reticulum in 50 G group was close to cell membrane. At this stage, collagen fibrils appeared in all the regenerated tissues.

The reduction of rough endoplasmic reticulum and mitochondria of fibroblasts were observed at 3 weeks after operation in all the groups. Furthermore, the shape of rough endoplasmic reticulum was different among the groups. In control and 2 G groups, the tubular rough endoplasmic reticula could be observed, while in 10 G and 50 G groups, that was cystic in shape. At 4 weeks after operation, the number of rough endoplasmic reticulum and mitochondria decreased progressively in all the groups, and there were no different appearances among the groups. Nucleus membrane was loose in control and 2 G groups, while those of 10 G and 50 G groups showed sharp and jagged.

Cross section area of collagen fibril: The thickness of

collagen fibrils was different among the groups at 2 to 4 weeks after operation. Thus, the cross section area of collagen fibrils was measured, and the results are shown in Fig. 5. The cross section area of collagen fibrils of 50 G group was significantly higher than that of the control group at 2, 3, and 4 weeks after operation. The value of 10 G group at 4 weeks after operation was also significantly higher than that of control group. However, there were no significant differences between 2 G group and control group at any weeks.

DISCUSSION

Electromagnetic stimulation has been reported to enhance the vascularization at the fracture site [7, 15] and the tendon injury [19]. The blood flow was also found to rise under the effect of electrostimulation due to revascularization of the tissue in the fracture region [13]. Our previous study demonstrated the increased vascularity at the defect of the ligament in the rabbits receiving PEMFs stimulation, among which rabbits of 50 G group revealed the earlier and more angiogenesis [14]. In this study, the blood flow at the defect of 50 G group showed the highest increase from 1 to 2 weeks after operation among the groups. This increase coincides with previous histologic results [14], therefore the increase in blood flow was thought to be strongly related to the earlier and more angiogenesis at the defect.

The collagen content of the regenerated tissue stimulated by PEMFs showed a dose-dependent increase in this study. This increase may be the result from the increased vascularity and the blood flow at the defect, however the degree of their increase was not necessarily in accordance with the increase in collagen content. In many different *in vitro* models, various electrical fields have been shown to increase collagen synthesis and/or content [16, 17]. Furthermore, Bassett reported in his *in vitro* study [3] that the effects of electromagnetic stimulation on DNA and collagen synthesis in cultured cells were dependent on its strength. Therefore, the collagen synthesis from fibroblasts at the defect might also be enhanced directly by PEMFs according to its electromagnetic strength.

Electron microscopic findings of fibroblasts revealed massive, well developed rough endoplasmic reticula in 50 G group, whereas in 2 G and 10 G groups, the number of rough endoplasmic reticulum was less than that of 50 G group. In control group, the development of rough endoplasmic reticulum was least among the groups. An extensive development of rough endoplasmic reticulum morphologically is thought to correlate to active synthesis of collagen. Therefore, the stronger magnetic intensity had more effects on synthesis of collagen *in vivo*.

In the meanwhile, tensile strength of the regenerated ligament under PEMFs stimulation in our previous study [14] was significantly higher at 1 and 2 weeks after operation than that of control. Furthermore, tensile strength of the tissue in 50 G group was the highest among the other PEMFs groups. These results may indicate that

increased collagen content in the regenerated ligament receiving PEMFs therapy, accelerates the return of tensile strength.

Thickness of collagen fibrils is also one of the factors relating to mechanical properties of regenerated ligament. In this study, the cross section area of collagen fibrils in 50 G group was significantly higher than that of control group at 2, 3 and 4 weeks after operation. However, these in PEMFs groups did not show a significant dose-dependent increase. The mechanism of an increase in the cross section area of collagen fibrils is still unknown *in vivo*. It was believed that thin collagen fibril reconstituted first, then secondarily the reconstituted collagen fibrils attached to the side of original fibril to increase the diameter of the collagen fibril [11, 12]. In our study, the PEMFs group had higher value of collagen content, which could induce a secondary increase in the diameter of collagen fibrils.

Another major contributing factor of the tensile strength of the regenerated ligament is maturation of collagen. Changes in the types of collagen in the healing ligament with or without PEMFs therapy were evaluated in this study. In the ligament healing process, type I collagen is considered to maintain strength in ligamentous structure, whereas type III contributes to ligamentous elasticity [1]. In this study, differences and ratio changes between type I and type III collagen bands at gross appearance in all the experimental groups were not seen, which may indicate PEMFs therapy does not effect on maturation of collagen. Recently, the cross-links are considered to be the most important factor for mechanical stability of the ligament [2]. In addition, the methods of electrophoresis used in this study did not offer the quantification of collagen types I and III. Further studies will be necessary to clarify the relationships between the tensile strength of the ligament and its collagen types.

In conclusion, the electromagnetic stimulation to the injured ligament increases blood flow at the regenerated ligament. Sufficient blood supply leads to proliferation of fibroblasts and secondarily to production of collagen, in which higher magnetic intensity directly induces the larger collagen synthesis by activating fibroblasts synthetic ability at an earlier stage. These results suggest that stimulation under the proper magnetic intensity (50 G) provides marked stability and earlier healing of the traumatic injuries of the ligament and tendon.

ACKNOWLEDGEMENTS. This investigation was supported in part for Scientific Research No. 02556040 from Ministry of Education, Science and Culture of Japan and Japan Racing Association (JRA).

REFERENCES

1. Akai, M., Oda, H., Shirasaki, Y., and Tateishi, T. 1988. Electrical stimulation of ligament healing. *Clin. Orthop.* 235: 296-301.
2. Akeson, W. H., Amiel, D., Mechanic, G. L., Woo, S. L-Y., Harwood, F. L., and Harmer, M. L. 1977. Collagen cross-linking alterations in joint contractures: changes in the reducible cross-links in connective tissue collagen after 9 weeks of immobilization. *Connect. Tissue Res.* 5: 15-19.
3. Bassett, C. A. L. and Herrmann, I. 1968. The effect of electrostatic fields on macromolecular synthesis by fibroblasts *in vitro*. *J. Cell Biol.* 39: 9a.
4. Bassett, C. A. L., Mitchell, S. M., and Gaston, S. R. 1981. Treatment of un-united tibial diaphyseal fractures with pulsing electromagnetic fields. *J. Bone Jt. Surg., Am.* 63: 511-523.
5. Bornstein, P. and Piez, K. A. 1964. Biochemical study of human skin collagen and the relationship between intra- and intermolecular crosslinking. *J. Clin. Invest.* 43: 1813-1823.
6. Brighton, C. T. and Pollack, S. R. 1985. Treatment of recalcitrant non-union with a capacitively coupled electrical field. *J. Bone Jt. Surg., Am.* 67: 577-585.
7. De Haas, W. G., Watson, J., and Morrison, D. M. 1980. Non-invasive treatment of united fractures of the tibia using electrical stimulation. *J. Bone Jt. Surg., Br.* 62: 465-470.
8. Frank, C., Amiel, D., Schachar, N., Dittich, D., DeHass, D., and Edwards, G. 1981. The electromagnetic stimulation of ligament healing in a rabbit model-collagen analysis by gel electrophoresis. *Orthop. Trans.* 5: 474-475.
9. Frank, J. S., Thomas, A. L., Narc, F. S., and Tony S. K. 1987. The use of laser doppler flowmetry to evaluate anterior cruciate blood flow. *J. Orthop. Res.* 5: 150-153.
10. Igarashi, M. 1983. Collagen analysis. *Orthop. Trauma. Surg.* 26: 1375-1377 (in Japanese).
11. Kajikawa, K. 1966. Fibroblast and fiber formation. *J. Lab. Morphol.* 20: 1-17.
12. Kajikawa, K. and Kakiyama, S. 1974. Odontoblast and collagen formation: An ultrastructural and autoradiography. *J. Electromicrosc.* 23: 9-17.
13. Landa, V. A. and Yanovskaya, E. M. 1980. Changes of blood flow in the regenerating bone tissue in the electro-stimulation of the reparative osteogenesis. *Ortop. Travmatol. Protez.* 8: 8-10.
14. Lin, Y. C., Nishimura, R., Nozaki, K., Sasaki, N., Kadosawa, T., Goto, N., Date, M., and Takeuchi, A. 1992. Effects of pulsing electromagnetic fields on the ligament healing in rabbits. *J. Vet. Med. Sci.* 54: 1017-1022.
15. Nishimura, R., Sato, Y., Lin, E., and Takeuchi, A. 1988. Experimental and clinical studies of PEMFs on the fracture repair in dogs. *J. Jpn. Bio-Electrical Res. Soc.* 1: 53-54 (in Japanese with English summary).
16. Norton, L. 1982. Effects of a pulsed electromagnetic field on a mixed chondroblastic tissue culture. *Clin. Orthop.* 167: 280-290.
17. Norton, L. 1985. Pulsed electromagnetic field effects on chondroblast culture. *Recon. Surg. and Trauma.* 19: 70-86.
18. Sykes, B., Puddle, B., Francis, M., and Smith, R. 1976. The estimation of collagens from human dermis by interrupted gel electrophoresis. *Biochem. Biophys. Res. Comm.* 72: 1472-1480.
19. Watkins, J. P., Auer, A. J., Morgan, S. J., and Gay, S. 1985. Healing of surgically created defects in the equine superficial digital flexor tendon. *Am. J. Vet. Res.* 46: 2097-2103.
20. Woessner, J. F. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys.* 93: 440-447.