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Effects of elastin-derived peptide on Achilles' tendon healing: An experimental study

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Different matrix macromolecules modulate the tendon healing process. Elastin contains sequences which exhibit chemotactic activity both *in vitro* and *in vivo*. We analyzed the effects of synthetic elastin-derived peptide Val-Gly-Val-Ala-Pro-Gly suspended in a gel solution on the healing process of Achilles' tendon in a rat model.

A total tenotomy at the middle 3rd was performed in 32 rats. During the suture repair the gel with (Group A) or without (Group B) the elastin-derived peptide was applied to the tendon stumps. Four animals for each period and group were killed at 10, 30, 60 and 90 days after surgery. The scar tissue was processed for histochemical, immuno-histochemical and morphometric analysis. An improved healing process with increase in cellularity and vascularity, especially at the early stage of the Achilles' tendon healing process was observed in Group A compared to Group B. The fiber alignment was also positively influenced by the factor. Immunolabeling with HAM 56 and lisozyme revealed a stronger reaction for the presence of monocyte/macrophage in Group A *vs* Group B especially in early stages. Chondral metaplasia and endochondral ossification occurred in the healed tissue of both group at 60 and 90 days.

Introduction

Many researchers have attempted to elucidate the tendon healing process in its biological [1–3] and mechanical aspects [4–8]. Tendon repair is influenced by different matrix macromolecules, such as collagens [9], laminin [10] and/or growth factors such as epidermal growth factor [11], fibroblast growth factor [12], platelet derived growth factor [11], insulin-like growth factor-I [13], growth and differentiation factors [14].

The elastic fibers, being of mesodermic origin, are one of the most important components of the extracellular matrix in the musculoskeletal tissues [15]. It is known that elastin contains sequences which exhibit biological activity and are able to modify cellular behavior; elastin-derived peptides (EDPs) were found active as chemo-attractants, particularly for monocytes [16] and able to stimulate fibroblast proliferation and neoangiogenesis both *in vivo* and *in vitro* [17, 18]. Among the different chemotactic EDPs, the hexapeptide Val-Gly-Val-Ala-Pro-Gly (VGVAPG) was firstly isolated [17]. Recently we evaluated the structure-activity relationships of elastolytic fragments of elastin and synthetic derivatives [19, 20].

In a previous study on Achilles' tendon healing in a rat model we observed a high amount of elastic fibers during the early phase of reparative fibrillogenesis [21] and hypothesized a possible contribution to those cell-matrix interactions crucial to the healing process. However, the role of elastin fragment or derived peptide on the tendon healing process is still unknown. The aim of the present study was to determine the effects of elastin-derived hexapeptide VGVAPG on Achilles tendon healing.

Materials and methods

The synthesis of the elastin-derived hexapeptide VGVAPG was performed by a solid-phase automatic peptide synthesiser, model 431A of Applied Biosystem, according to the manufacturer's instructions [19, 20]. Hydroxyethylstarch (HES) (Sigma, Italy) was used as a factor delivery vehicle.

25 μl of water containing 4 μg of the elastin peptide (VGVAPG) was added to 25 mg of HES and the mixture obtained was used for each rat. A mixture of 25 mg of HES and 25 μl of water was used for the control group (see below).

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The study was performed on 32 six-month-old Male Wistar rats, weighing 250–280 g. All animals were housed in standard cages, four animals per cage, and a standard 12 h light and 12 h dark cycle was used. Animals were fed rat chow and water *ad libitum*.

The operative procedure was performed under general anaesthesia achieved by intraperitoneal injection of Diazepam (Valium Roche) (20 mg/100 g) and intramuscular injection of Ketamina (Ketalar, Parche-Davis) 2.5 mg/100 g in sterile conditions. A 2 cm midline incision was made in the skin overlying the Achilles tendon of the right hindlimb. Blunt dissection was used to free the plantaris and Achilles tendon from the surrounding fascia, and a total tenotomy of Achilles' tendon at the middle 3rd was performed. The tendon ends were approximated with suture repair and simultaneously the HES with (Group A) or without (Group B) the peptide was applied among the tendon stumps. The skin was closed in a subcuticular fashion with a 5-0 absorbable Vicryl (polyglactin) suture. Four animals for each period and for each group were killed at 10, 30, 60 and 90 days after injury by intracardiac injection of sodium pentobarbital (Pentothal, Abbott, Italy) and both tendons were removed.

The scar tissue was processed for histochemical, immuno-histochemical and morphometric analysis.

Histochemistry

For light microscopy specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 24 h and decalcified in 4 N formic acid and sodium citrate for 72 h, embedded in paraffin, cut into longitudinal sections (3–5-thick), and stained with haematoxylin-eosin and with Verhoeff's iron haematoxylin.

Immunohistochemistry

The anti-lysozime (Dako, Italy) and anti HAM 56 antibodies (Dako, Italy) for immunohistochemistry were used. Tissues samples were paraffin embedded and cut into consecutive sections (8–10 μm thick). They were dewaxed with xylene and rehydrated with a graded series of ethanol immersions. Intrinsic peroxidase activity was blocked by immersion in distilled water containing 3% hydrogen peroxide for 6 min. The samples were treated with 0.1% trypsin (Dako) for 30 min at 37 °C, and several washes in water and 0.05 M Tris/HCl were performed. Primary antibody for lysozime and HAM 56 were prepared (Dako diluent with background reducing components), at working dilution respectively of 1 : 1000 and 1 : 100, and were incubated on the slides overnight at 4 °C. After three Tris/HCl washes, Dako LSAB 2 kit peroxidase, and DAB chromogen (Sigma, Italy) were used following the manufacturer's recommendations. Stainings were viewed and photographed with a Leica microscope (Leica Cambridge Ltd., England). Control sections were treated with rabbit normal immunoglobulins, instead of specific antibodies.

Histomorphometry

Computerized morphometric analysis was performed using the Leica Q500MC image analysis system (Leica Cambridge Ltd., England) as previously described [22]. In brief, based on a calibration factor determined by a calibration procedure in the set-up menu, the system calculates the area fraction (Aa%) occupied by cells on the whole field, i.e. pixels of cells/256^2 pixels. Nine fields were studied in each specimen. Data are expressed as mean $\pm$ SD of the mean and the statistical evaluation was carried out by a nonpaired, two-tailed t test. Significance was established at $p < 0.05$.

Results

Histochemistry

10 days after surgery the EDP-treated tendons (Group A) showed numerous cells in a disarranged dense matrix; numerous vessels were dispersed in the scar tissue. In control tendons (Group B) the tissue scar assumed the same arrangement as in Group A, but appeared more lax. Moreover a reduced number of cells and vessels were evident.

30 days after surgery the cellular component was still well-represented among the reparative tissue in Group A tendons, while the number of vessels decreased in comparison with early stage of the healing process. The fibers and cells alignment assumed a predominantly longitudinal orientation parallel to the major axis of the tendon (Fig. 1(a)). In Group B the scar tissue appeared disarranged, less oriented and dense (Fig. 1(b)) in comparison with Group A.

After *60 days* it was difficult to detect the lesion in Group A tendons because of the almost normal aspects of the scar. The tendon crimp resulted more evident in comparison with the early stage. In Group B tendon scar the histological, pictures were quite similar to those observed in Group A, however the reduction in cellularity and a still disarranged matrix were detectable.

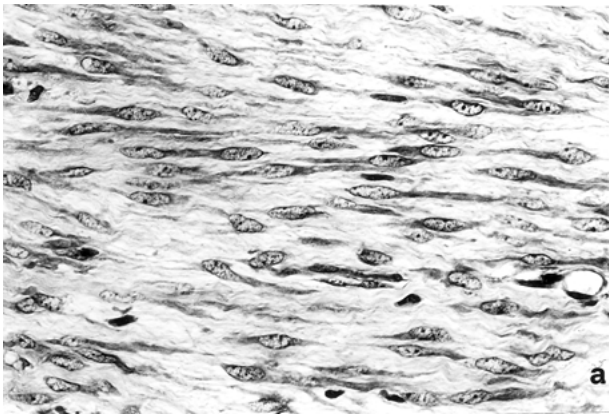
90 days after surgery numerous areas of chondral metaplasia and endochondral ossification inside the tendineous tissue were impressive in both Group A and B (Fig. 2).

Immunohistochemistry

The analysis was performed at 10 and 30 days after surgery.

After *10 days* the EDP-treated tendons showed a stronger immuno-reaction for both lysozime and HAM 56 compared to the sham specimens. Histomorphometry showed a statistically significant difference ($p < 0.001$) in area fraction occupied by positive cells among treated and sham tendons.

30 days after surgery the EDP-treated tendons showed a light immuno-reaction for lysozime and HAM 56 similar to that observed in the sham specimens. No statistically significant differences ($p > 0.001$) were detected at histomorphometric analysis.



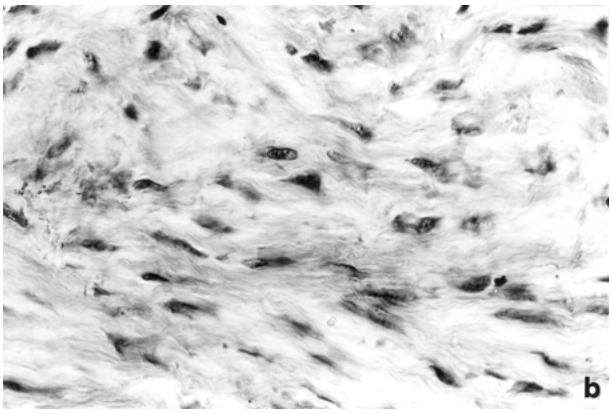


Figure 1 Tendon scar at 30 days after tenotomy (Verhoeff's iron haematoxylin, × 400). (a) The cellular component is well-represented among the reparative tissue in EDP-treated tendons. The fibers and cells alignment assume a predominantly longitudinal orientation parallel to the major axis of the tendon; (b) in controls, the scar tissue still appears disarranged and less dense in comparison with EDP-treated tendons.

Histomorphometry

As shown in Fig. 3, there was an increase in cellularity in all treated tissue samples compared with controls. In particular, there was statistically significant differences $(p < 0.001)$ in area fraction occupied by cells among treated and sham tendons at 10 and 30 days after surgery.

Figure 2 Tendon scar at 90 days after tenotomy (H-E, × 100). Chondral metaplasia (C) and endochondral ossification areas (B) are easily detectable among tendon reparative tissue (*T*).

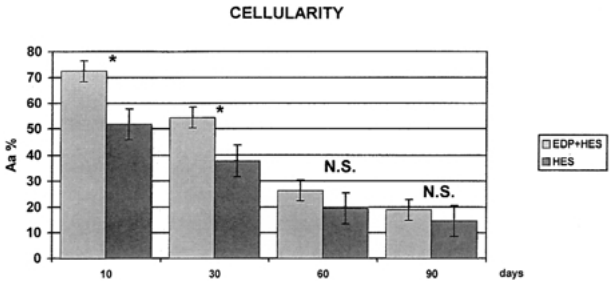


Figure 3 Histomorphometric analysis of area fraction (mean Aa% ± SD) occupied by cells in rat Achilles tendon healing. (Aa%, area fraction; $*p < 0.001$; N.S., not significant; HES, Hydroxyethylstarch; EDP, elastin-derived peptide).

Discussion

A variety of growth factors modulate connective tissue healing [23] and it is generally accepted that monocytes/macrophages are essential to normal wound healing [24]. In sites of tissue injury monocyte/macrophage accumulation follows neutrophil influx and marks the transition between the early and late phases of inflammation. Moreover monocyte/macrophage releases a plethora of biologically active substances, such as vasoactive mediators, chemotactic factors, growth factors, and enzymes [25].

While neutrophils appear early at the site of injury, monocytes appear after a 12–24 h lag and continue to accumulate after neutrophil influx has ceased. This implies some differences in chemotactic signals for these two cell types. Monocyte migration is stimulated by degradation products of the extracellular matrix proteins, such as collagen [26] and elastin [17, 19] whereas neutrophil migration is not [25].

Our previous observations on Achilles' tendon healing in a rat model showed high amounts of elastic fibers during the early phase of reparative fibrillogenesis [21]. The knowledge that elastin contains sequences which exhibit biological property, being active as chemoattractants, particularly for monocytes [16] and able to stimulate fibroblast proliferation and neoangiogenesis both *in vivo* and *in vitro* [18] led us to hypothesize a possible role of synthetic EDPs on Achilles tendon healing.

In this study a single topical application at the time of injury of EDP resulted in an improved healing process, with a better organization of the new fibrous tissue. In particular, histologic evaluation revealed an increase in cellularity and vascularity of the Achilles' tendon in the experimental specimens when compared to the sham specimens. This was most evident at the early stage of the healing process. The fiber alignment was also positively influenced by the factor. Moreover, immunohistochemistry revealed a high amount of positive cells (i.e. monocytes/macrophages) quantitatively evaluated by morphometry.

Because of the positive migration assays of monocytes produced by EDPs [19] it is possible that these cells produce their own mitogenic and/or chemotactic factors, theoretically increasing fibroblast amount and eventually resulting in more collagen production at the injury site [27].

In previous experimental studies on this animal model, even in the more advanced phases of the healing process,

the reparative scar did not show metaplastic tissues [15]. In the present study, the evidence of chondral metaplasia and endochondral ossification in the healed tissue of treated and sham specimens at 90 days after surgery, lead us to conclude that the delivery vehicle for EDP, i.e. hydroxyethylstarch, may be the cause of this metaplasia; thus, in this case, it is inadequate as delivery system.

In conclusion, the elastin-derived hexapeptide VGVAPG seemed to improve tendon healing process. However, some aspects are still obscure. Further studies will have to establish a better delivery vehicle for EDP and evaluate a possible improvement of the biomechanical properties of the repaired tendon.

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