

The promoting effect of pentadecapeptide BPC 157 on tendon healing involves tendon outgrowth, cell survival, and cell migration

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Submitted 16 August 2010; accepted in final form 6 October 2010

Chang CH, Tsai WC, Lin MS, Hsu YH, Pang JH. The promoting effect of pentadecapeptide BPC 157 on tendon healing involves tendon outgrowth, cell survival, and cell migration. *J Appl Physiol* 110: 774–780, 2011. First published October 28, 2010; doi:10.1152/jappphysiol.00945.2010.—Pentadecapeptide BPC 157, composed of 15 amino acids, is a partial sequence of body protection compound (BPC) that is discovered in and isolated from human gastric juice. Experimentally it has been demonstrated to accelerate the healing of many different wounds, including transected rat Achilles tendon. This study was designed to investigate the potential mechanism of BPC 157 to enhance healing of injured tendon. The outgrowth of tendon fibroblasts from tendon explants cultured with or without BPC 157 was examined. Results showed that BPC 157 significantly accelerated the outgrowth of tendon explants. Cell proliferation of cultured tendon fibroblasts derived from rat Achilles tendon was not directly affected by BPC 157 as evaluated by MTT assay. However, the survival of BPC 157-treated cells was significantly increased under the H₂O₂ stress. BPC 157 markedly increased the in vitro migration of tendon fibroblasts in a dose-dependent manner as revealed by transwell filter migration assay. BPC 157 also dose dependently accelerated the spreading of tendon fibroblasts on culture dishes. The F-actin formation as detected by FITC-phalloidin staining was induced in BPC 157-treated fibroblasts. The protein expression and activation of FAK and paxillin were determined by Western blot analysis, and the phosphorylation levels of both FAK and paxillin were dose dependently increased by BPC 157 while the total amounts of protein was unaltered. In conclusion, BPC 157 promotes the ex vivo outgrowth of tendon fibroblasts from tendon explants, cell survival under stress, and the in vitro migration of tendon fibroblasts, which is likely mediated by the activation of the FAK-paxillin pathway.

BPC 157; tendon healing; outgrowth; migration; FAK; paxillin

TENDON INJURY is one of the most common injuries in sport activities. Tendon is composed of cells (tendon fibroblasts) and extracellular matrix, which contains type I collagen mostly, type III collagen, and glycoproteins. Most tendon injuries result from the tear of tendon fibers due to overuse or aging. The injury can happen gradually or suddenly. Healing ability of the ruptured tendon can be poor. Surgical repair of the completely torn tendon is often important. The process of tendon healing is classified into three overlapping stages: inflammation, regeneration, and remodeling. In the regeneration stage, tendon fibroblasts migrate into the injured site, proliferate, and produce different types of collagens and glycoproteins to form the extracellular matrix (3, 17).

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Many growth factors such as epidermal growth factor (EGF), transforming growth factor- β (TGF- β), and bone morphogenetic proteins (BMPs) have been used to improve the healing of torn tendon in the lab (2, 31). However, the short duration of these easily digested growth factors hampers their clinical usage. Gastric pentadecapeptide BPC 157 is a partial sequence of human gastric protein BPC, which has been discovered in and isolated from gastric juice (5). It is highly stable and resistant to hydrolysis or enzyme digestion, even in the gastric juice. Besides, it is easily dissolved in water and needs no carrier for its application. Experimentally it was demonstrated to enhance the healing of different wounds, such as gastric ulcer (19, 32), skin (4, 15), cornea (13), muscle (28), colon-colon anastomosis (22), colocolutaneous fistula (11), and segmental bone defect (21). It was also found to accelerate the healing of transected rat Achilles tendon (12, 29) and medial collateral ligament of knee (8). Currently it is in clinical trial for treating inflammatory bowel disease (25).

The mechanism by which pentadecapeptide BPC 157 accelerates healing is not clearly understood. It has been suggested to include upregulation of growth factors (15), proangiogenic effects (6, 22, 27), and modulation of nitric oxide (NO) synthesis (26). BPC 157 may also control functions of collagen fragments (30). It has been shown to have a connection with bone morphogenetic proteins (15, 21). However, none of these functions have been proved experimentally in tendon healing. The present ex vivo and in vitro study attempted to elucidate the potential mechanism by which BPC 157 works on tendon fibroblasts to improve tendon healing.

MATERIALS AND METHODS

Outgrowth of tendon fibroblasts from tendon explants. This study has been approved by the Institutional Animal Care and Use Committee before the procedures were performed. Achilles tendons were harvested from male Sprague-Dawley rats weighing 200–250 g. Tendons were carefully and equally excised into 20 pieces, divided into two groups, and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco/BRL, Grand Island, NY). Pentadecapeptide BPC 157 (GEPPPGKPADAGLV, mol wt 1,419) was synthesized and purchased from Kelowna International Scientific, Taiwan, Republic of China. BPC 157 at the concentration of 2 μ g/ml was added to the experimental group, and the ex vivo outgrowth of tendon fibroblasts was examined and compared with the control group, to which no BPC 157 was added. The total number of cells migrating out of the tendon explants in the control or BPC 157-treated groups was counted at the 7th day.

Primary culture of rat tendon fibroblasts. Male Sprague-Dawley rats, weighing 200–250 g, were used as the source of tendon fibroblasts in this study. Achilles tendons were harvested from these rats by aseptic procedures first. Each tendon was chopped into pieces at a size

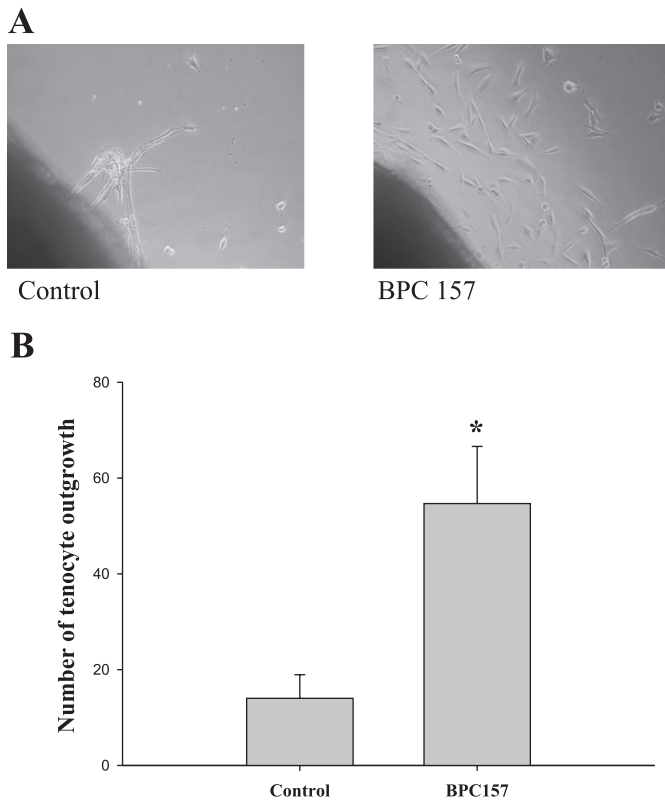


Fig. 1. BPC 157 promoted the cell outgrowth from tendon explants. To investigate the outgrowth of tendon fibroblasts from tendon explants, tendon explants were treated with or without 2 $\mu\text{g/ml}$ BPC 157 for 7 days. Representative pictures were shown that the outgrowth of tendon fibroblasts from tendon explants was significantly increased in BPC 157-treated group compared with control group (A). In B, total numbers of outgrown tendon fibroblasts after 7 days of ex vivo culture from tendon explants were counted. * $P < 0.05$ vs. control.

of about 1.5–2.0 mm³ and separately put into six-well culture plates. Then 3-ml culture medium made of Dulbecco's modified Eagle's medium with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin was added to each well. After migrating out from the explants, tendon fibroblasts started to grow rapidly. After reaching confluency, the cells were subcultured by trypsinization at a 1:3 dilution ratio. Tendon fibroblasts between passages 2 and 4, having proper growth rate and normal fibroblast shape, were used in the following experiments. Each experiment was repeated three times using tendon fibroblasts isolated from different rat.

MTT assay. Tendon fibroblasts (1×10^5) were seeded in each well of 24-well culture plate, which contained culture medium made of 0.5 ml of DMEM, 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin in each well. BPC 157 was added into each well at the concentration of 0 (control group), 0.5, 1, and 2 $\mu\text{g/ml}$. After incubation at 37°C in a humidified atmosphere of 5% CO₂-95% air for 24 h, cells were washed once with 1× PBS, followed by adding 1 ml DMEM containing 0.05 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). After incubation at 37°C for 1 h, the media were removed and formazan crystals in the cells were solubilized in 1 ml DMSO and processed for OD reading at 570 nm using a spectrophotometer. The experiments were performed in triplicate.

Transwell filter migration assay. Tendon fibroblasts (2×10^5) treated with BPC 157 at concentration of 0 (control group), 0.5, 1, and 2 $\mu\text{g/ml}$ for 24 h were seeded on each transwell filter with 8.0- μm pores (Costar, Cambridge, MA). The upper chamber was filled with 250 μl serum-free DMEM and the lower chamber was filled with 600

μl DMEM with 10% FBS. These cells were allowed to migrate at 37°C in an atmosphere of 95% air-5% CO₂ for 3 h. Cells that migrated through the pores were stained with Liu's stain and washed with 1× PBS twice. Then the cells on the lower surface of the filter were counted under four random high-power microscopic fields (HPF; 100×) per filter, and the mean number of cells that migrated through the filter was calculated for each condition. The experiments were performed in triplicate.

Cell spreading experiment. Tendon fibroblasts (1×10^5) were seeded into a 6-cm culture dish and treated with BPC 157 at concentration of 0 (control group), 0.5, 1, and 2 $\mu\text{g/ml}$ for 24 h. Then cells

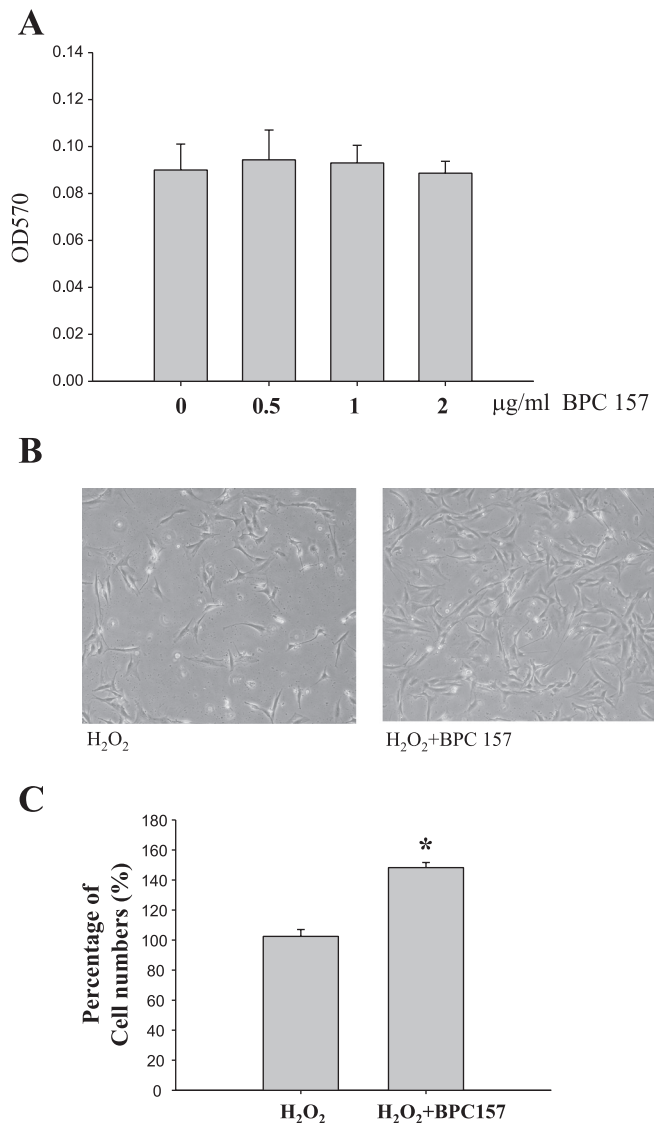


Fig. 2. BPC 157 did not exert direct effect on the proliferation of tendon fibroblasts, but increased cell survival under oxidative stress. In A, the numbers of tendon fibroblasts cultured in media containing BPC 157 at concentrations of 0, 0.5, 1, and 2 $\mu\text{g/ml}$ for 24 h were measured by MTT assay. In B and C, the protective effect of BPC 157 on tendon fibroblasts from oxidative damage was studied by treating tendon fibroblasts with 0.1 mM H₂O₂ for 24 h in the absence or presence of 2 $\mu\text{g/ml}$ BPC 157. Representative photos were shown (B) that the morphological change of tendon fibroblasts due to H₂O₂ treatment was much less obvious in BPC 157-treated group compared with H₂O₂ alone group. In C, total numbers of tendon fibroblasts were counted after H₂O₂ treatment for 24 h. Experiments were done in triplicate. * $P < 0.05$ vs. H₂O₂ alone.

were harvested by trypsinization and reseeded. The adhesion and spreading of cells were observed and counted at 30 min after seeding.

Staining of F-actin with FITC-conjugated phalloidin. Tendon fibroblasts were seeded onto cover slides and treated with 2 $\mu\text{g/ml}$ BPC 157 for 24 h. The cover slides were fixed with 10% formaldehyde for 15 min. Tendon fibroblasts were washed with $1\times$ PBS and fixed in methanol at -20°C for 5 min. After being washed with $1\times$ PBS again and then incubated in 0.1% Triton X-100 in $1\times$ PBS for 1 min, the slides were stained with FITC-conjugated phalloidin (0.5 $\mu\text{g/ml}$ in $1\times$ PBS) for 15 min. Slides were again washed with $1\times$ PBS and mounted. The formation of F-actin stained by FITC-conjugated phalloidin was observed under fluorescent microscope.

Western blot analysis. Cell extracts were prepared in lysis buffer containing Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM PMSF, and 1% Triton X-100 followed by ultrasonication. Protein concentration of the cell extracts was determined by Bradford assay (Bio-Rad Laboratories). Samples with same amount of proteins were then separated by 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was incubated at room temperature in blocking solution (1% bovine serum albumin, 1% goat serum in $1\times$ PBS) for 1 h, followed by incubation in blocking solution containing an appropriate dilution of primary monoclonal antibody (NeoMarks, Fremont, CA) for 2 h. After being washed three times in $1\times$ PBS, the membrane was then incubated in $1\times$ PBS containing goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO) for 1 h. Membranes were washed three times in $1\times$ PBS, and positive signals were developed with enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK).

Statistical analysis. All data were expressed as means \pm SE. Quantification of FAK and paxillin expression was performed by calculating the band density using 1D Digital Analysis Software (Kodak Digital ScienceTM, Eastman Kodak, Rochester, NY). Comparisons between every assay of the BPC 157-treated and control cells

were performed using Kruskal-Wallis test. A Mann-Whitney test was used to identify where the difference occurred. The level of statistical significance was set at a P value of 0.05.

RESULTS

BPC 157 promoted the ex vivo outgrowth of tendon explants. To clarify the potential mechanism of BPC 157 on promoting tendon healing, the initial outgrowth of tendon fibroblasts from tendon explants cultured with or without 2 $\mu\text{g/ml}$ BPC 157 was examined and compared. At the 2nd day after implantation, tendon fibroblasts migrating out from the tendon explant were observed in 5 of 10 tendon explants in the BPC 157 group compared with 2 of 10 tendon explants in the control group. At the 5th day, the outgrowth of tendon fibroblasts was observed in all the tendon explants in two groups. This result indicated that BPC 157 could accelerate cell outgrowth from the tendon explant. In addition, the total number of tendon fibroblasts outgrowth from the explants after 7 days of incubation was significantly increased in the BPC 157 group (Fig. 1B). Representative pictures from the two groups are shown in Fig. 1A.

BPC 157 increased the survival of tendon fibroblasts under oxidative stress. To further investigate the effect of BPC 157 on the proliferation of in vitro tendon fibroblasts culture, we treated the cells with BPC 157 at concentrations of 0, 0.5, 1, and 2 $\mu\text{g/ml}$ for 24 h, and the viability of tendon fibroblasts was determined by MTT assay. The result shown in Fig. 2A indicated that BPC 157 did not exert a direct effect on accelerating the cell proliferation of cultured tendon fibroblasts. Similar results were obtained from cultures of tendon fibroblasts in the absence or presence of BPC 157 treatment for 3

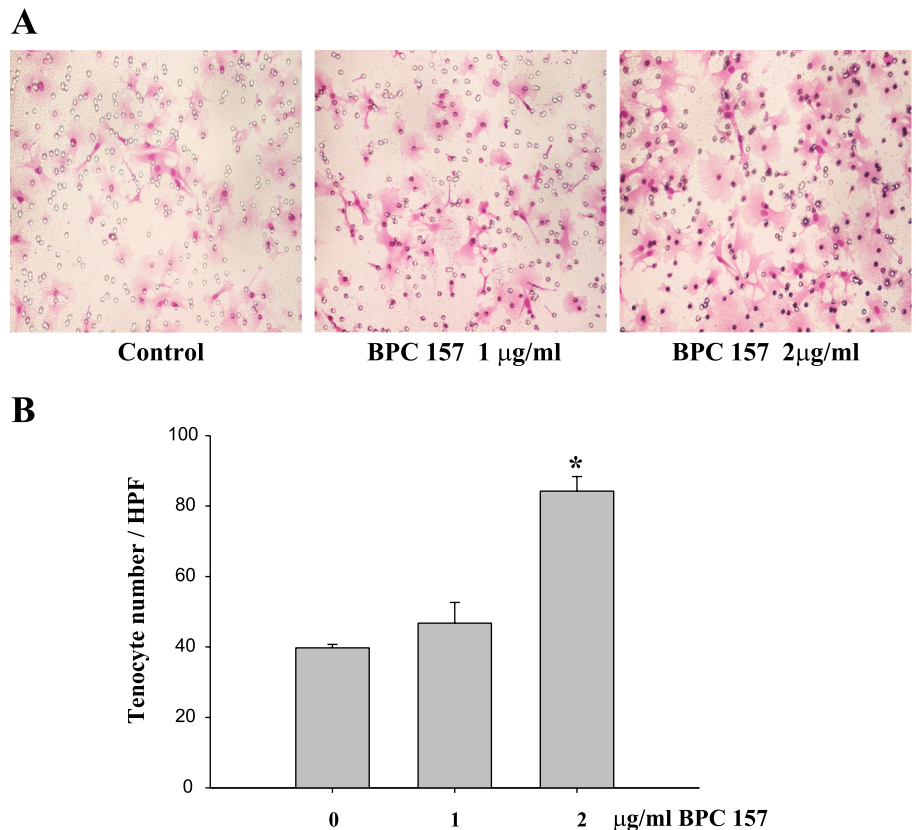


Fig. 3. BPC 157 accelerated the migration of tendon fibroblasts. Tendon fibroblasts were treated with BPC 157 at concentration of 0, 0.5, 1, and 2 $\mu\text{g/ml}$ for 24 h, and the migration of cells was studied by transwell filter migration assay. A: representative pictures of cells migrating through the transwell membranes. B: quantitative data were calculated from 3 independent experiments. * $P < 0.05$ vs. control.

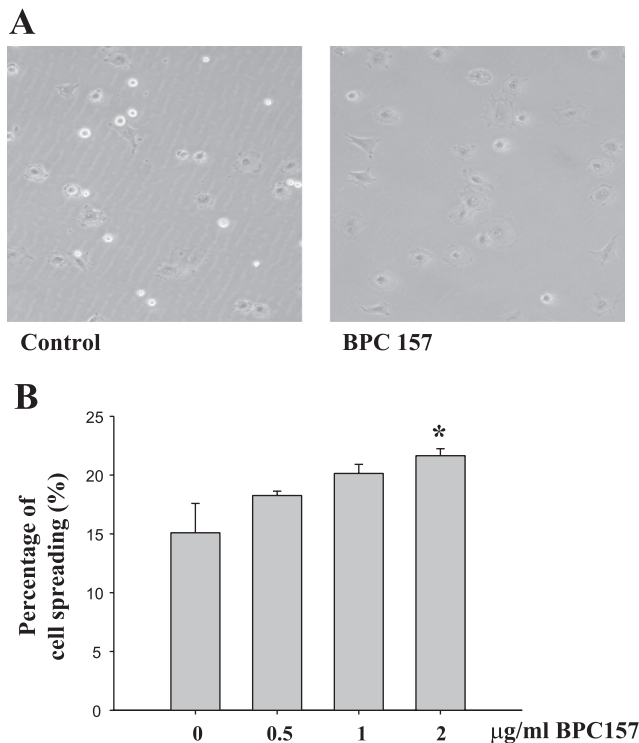


Fig. 4. BPC 157 induced the spreading of tendon fibroblasts. Tendon fibroblasts were treated without or with 1 µg/ml or 2 µg/ml BPC 157 for 24 h, and the spreading of cells was observed at 30 min after reseeding the cells on culture dishes. *A*: representative photos of cells adhered and spread out on the dishes. *B*: number of spread cells was counted and compared between control and BPC 157-treated group. Experiments were done in triplicate. * $P < 0.05$ vs control.

days (data not shown). It is known that oxidative stress may hamper the healing of tendon (14); we then tested the effect of BPC 157 on the damage of tendon fibroblasts caused by H_2O_2 treatment. Dose-dependent cell loss was found in H_2O_2 -treated tendon fibroblasts for 24 h (data not shown) as determined by MTT assay. The coinubation of tendon fibroblasts with 2 µg/ml BPC 157 significantly increased the number of viable cells in tendon fibroblasts treated with 0.1 µM H_2O_2 for 24 h (Fig. 2, *B* and *C*), suggesting a protective role of BPC 157 on the injury of tendon fibroblasts under oxidative stress.

BPC 157 accelerated the cell migration of tendon fibroblasts. Except for cell proliferation, cell migration is another important possibility that can determine the rate of tendon outgrowth. Tendon fibroblasts with or without BPC 157 were cultured for 24 h and then subjected to transwell filter migra-

tion assay. Results demonstrated that BPC 157 could increase the migration speed of tendon fibroblasts in a dose-dependent pattern (Fig. 3*A*). BPC 157 at 2 µg/ml could significantly increase the migration of tendon fibroblasts up to 2.3-fold (Fig. 3*B*).

BPC 157 accelerated induced the cell spreading of tendon fibroblasts. Tendon fibroblasts with or without BPC 157 treatment for 24 h were trypsinized and reseeded onto new culture plate with fresh growth medium. Within 5–10 min, cells started to adhere to the culture plate and the spreading of cells was first observed in BPC 157-treated tendon fibroblasts 15 min later. The number of cells that have spread out at 30 min was markedly increased in BPC 157-treated tendon fibroblasts in a dose-dependent pattern (Fig. 4, *A* and *B*).

BPC 157 induced the F-actin formation in tendon fibroblasts. Cell migration is an actin-dependent process, and many proteins that regulate F-actin formation have now been implicated in controlling migration, particularly the spreading process (16). We then compared the formation of F-actin in tendon fibroblasts with or without BPC 157 treatment for 24 h by staining F-actin with FITC-conjugated phalloidin. The formation of the F-actin was revealed by the green fluorescent polymerized fibers that were weakly detected in control tendon fibroblasts but notably increased in the tendon fibroblasts treated with BPC 157 (Fig. 5).

BPC 157 activated the FAK-paxillin signaling pathway. To further reveal the mechanism of BPC 157 on inducing the migration of tendon fibroblasts, the protein expression and activation of FAK and paxillin were examined by Western blot analysis. Results shown in Fig. 6*A* demonstrated that the protein expression of proliferating cell nuclear antigen (PCNA), a well-known cell proliferation marker, in tendon fibroblasts was not affected by the treatment of BPC 157 for 24 h. The total expression levels of FAK and paxillin was not changed either (Fig. 6*A*). However, the activation of FAK and paxillin as indicated by the levels of phosphorylation was significantly increased by BPC 157 also in a dose-dependent pattern (Fig. 6, *B* and *C*).

DISCUSSION

Body protection compound (BPC, mol wt 40,000) was first discovered and isolated in gastric juice and later a stable 15-amino acid fragment (Gly Glu Pro Pro Gly Lys Pro Ala Asp Asp Ala Gly Leu Val, mol wt 1,419, called BPC 157) with apparently no sequence homology to other known peptides, was found to be essential for BPC's activity (8a). Although the detailed mechanism is poorly understood, BPC 157 appears to

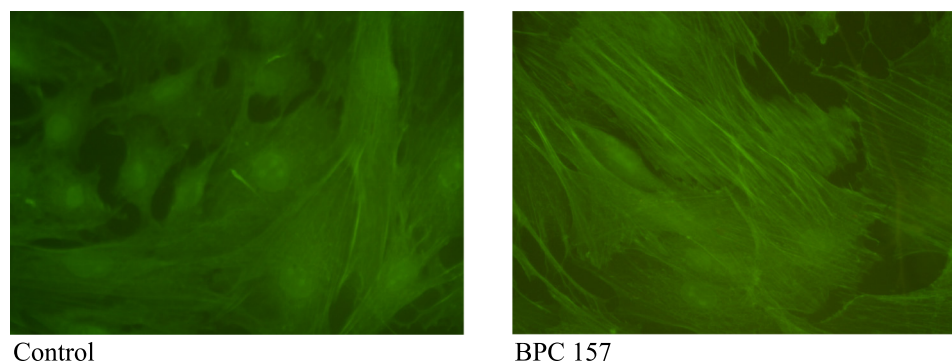


Fig. 5. BPC 157 induced the F-actin formation of tendon fibroblasts. Tendon fibroblasts were treated without or with 1 µg/ml or 2 µg/ml BPC 157 for 24 h, and the F-actin formation was revealed by staining with FITC-conjugated phalloidin and observed under fluorescent microscope. Representative photos of cells without or with BPC 157 treatment were shown.

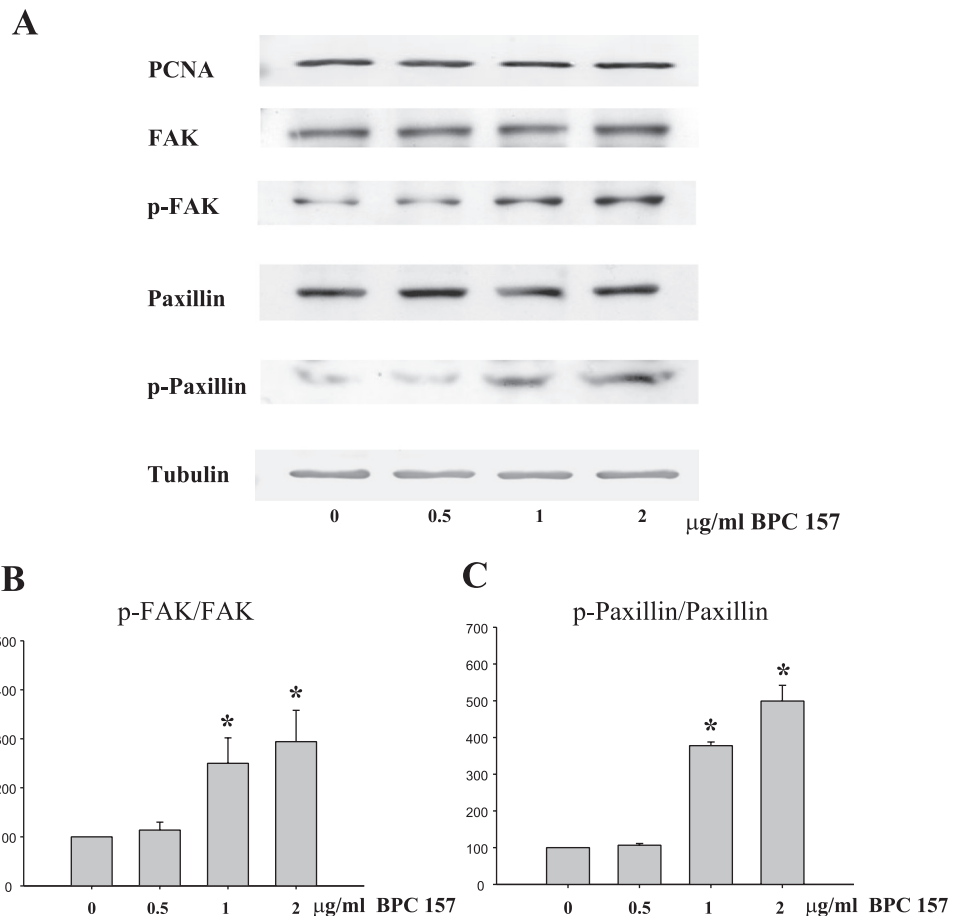


Fig. 6. BPC 157 activated FAK and paxillin in tendon fibroblasts. Tendon fibroblasts were treated without or with 1 $\mu\text{g/ml}$ or 2 $\mu\text{g/ml}$ BPC 157 for 24 h. The protein expressions, including proliferating cell nuclear antigen (PCNA), FAK, phospho (p)-FAK, paxillin, p-paxillin, and tubulin, were analyzed by Western blot analysis and results are shown in A. The ratio of phosphorylated FAK to total amount of FAK (B) and that of phosphorylated paxillin to total paxillin (C) were calculated by direct densitometric analysis of the blot. * $P < 0.05$ vs. control.

be beneficial to almost all organ systems in many species when very low dosages (mostly ng/kg to $\mu\text{g/kg}$ range) after intraperitoneal, intragastric, and intramucosal (local) application are used. The effect is usually apparent already after one application, while long-lasting activity has also been demonstrated. Except the effects on various gastrointestinal lesions, the healing-promoting effects of BPC 157 have also been reported on pancreas, liver injuries, endothelium, heart damage, and pseudoarthrosis (23). The effect of BPC 157 is particularly prominent when applied simultaneously with noxious agents or in already pathological conditions. Previous studies have also proven that there is no side effect or toxicity found in BPC 157 usage; therefore it could be a useful new class of drugs for organ protection. Gastric pentadecapeptide BPC 157 is a very stable water-soluble peptide that is resistant in human gastric acid for at least 24 h. In the literature, it exhibits both antiulcer and anti-inflammatory effects. Like other growth factors (such as TGF- β 1 and EGF), it can improve the healing of injured tendon in animal experiments. It is therefore suitable for local or systemic usage.

Healing of the injured tendon involves a lot of complex pathways. It progresses through overlapping stages of inflammation, regeneration, and remodeling. The whole process is slow, and the strength of the healed tendon is inadequate. It heals by scars, which take at least 1 year for maturation. The scar tissue has reduced mechanical strength and renders the tendon susceptible to reinjury (16). This process of regeneration is believed to occur either extrinsically by infiltration of

external cells or intrinsically by tendon fibroblast proliferation or both. Migration and proliferation of cells seem to be fundamental for tendon healing.

The beneficial effect of BPC 157 was confirmed by the acceleration of initial outgrowth of tendon fibroblasts from tendon explant. This experiment was designed to mimic the very early stage of tendon regeneration, during which period tendon fibroblasts migrate from either epitendon or endotendon (9, 20). This influence was also confirmed by the transwell filter migration experiments. Tendon cells with 24 h treatment with BPC 157 significantly increases their migratory speed up to 2.3-fold at the concentration of 2 $\mu\text{g/ml}$.

In our experiments, BPC 157 had no direct effect on promoting the proliferation of an in vitro culture of tendon fibroblasts, which could also be verified by the unaltered expression of PCNA protein after 24 h of treatment with BPC 157. Since cell proliferation is an important process of tendon healing, the result was unexpected. However, we understood that the experimental condition using in vitro culture of tendon fibroblasts could not mimic the real environment of tendon. During the course of in vivo healing, other cells such as leukocytes and stem cells may also interact with each other and contribute to this complicated process. It is possible that the healing-accelerating effect of BPC 157 may act on other cells and exert an indirect effect on promoting the proliferation of tendon fibroblasts. An additional explanation is that the effect of BPC 157 has been shown to be restricted to diseased conditions, and therefore the proliferation of normal tendon

fibroblasts is not affected. In a study carried out by Staresinic et al. (29), the proliferation-enhanced activity of BPC 157 was only demonstrated in HNE-damaged cells but not in normal cells. We also demonstrated that BPC 157 could increase the cell survival of tendon fibroblasts under the oxidative stress of H₂O₂. However, the underlying protective mechanism of BPC 157 needs to be further investigated.

The interesting finding in the present study was the positive effect of BPC 157 on the migrating and spreading of tendon fibroblasts. The activation of migration and spreading can be strengthened by the observation of increased F-actin formation in tendon fibroblasts after BPC 157 treatment. BPC 157 was further proven in the present study to be capable of activating the phosphorylation levels of FAK and paxillin in tendon fibroblasts. FAK is a non-receptor protein tyrosine kinase (PTK) localized to focal contact structures. FAK functions as part of a cytoskeleton-associated network of intracellular signaling proteins that facilitate the generation of integrin-stimulated signals to downstream targets to play a role in the regulation of cell migration (30). FAK is one of the upstream regulators for paxillin. After phosphorylation of FAK, it can combine with Src and then induce phosphorylation of paxillin (7). Phosphorylation of paxillin can activate the downstream effector molecules such as CRK and CAS, which are important in cell motility (1). Paxillin is believed to have a relation with cell migration. It is an intracellular protein located at the site of focal adhesion. After activation of integrin following engagement with the extracellular matrix, the signals produced will be propagated by focal adhesions to actin cytoskeleton. The activation of FAK and paxillin is correlated well with the increased migration of tendon fibroblasts after 24-h incubation with BPC 157. This implies that BPC 157 may increase the ability of migration of tendon fibroblasts by increasing FAK phosphorylation, which in turn increases the activation of paxillin.

This is the first report to show that the healing effect of BPC 157 could be mediated by activating a cellular FAK-paxillin signal pathway. However, it is still mysterious how exactly BPC 157 generates the integrin-stimulated signals and consequently activates the downstream FAK. Whether BPC 157 can act as a ligand to bind directly to a membrane receptor will be of great interest to study in the future.

In conclusion, BPC 157 accelerates the ex vivo outgrowth of tendon explant in culture and increases the migratory potential of cultured tendon fibroblasts. The underlying mechanism involves the activation of a FAK-paxillin pathway. BPC 157 may play an important role in promoting the tendon healing, and potential clinical usage in the future is expected.

ACKNOWLEDGMENTS

We thank National Science Council of Taiwan and Chang Gung Memorial Hospital for supporting this work.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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