Dose-related cellular effects of platelet-derived growth factor-BB differ in various types of rabbit tendons in vitro

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ABSTRACT – Cellular responsiveness to growth factors that can affect tendon healing may be site-specific. We have compared the dose-response effects of platelet-derived growth factor-BB (PDGF-BB) on proteoglycan, collagen, noncollagen protein and DNA synthesis between intrasynovial intermediate and proximal segments of deep flexor tendons, and extrasynovial peroneal tendons of rabbits during short-term cultures. PDGF-BB stimulated matrix and DNA synthesis of the three types of tendon segments in a dose-dependent manner in the range from 0.1 to 100 ng/mL. PDGF-BB stimulated collagen synthesis and noncollagen protein synthesis (calculated from LogED$_{50}$) in proximal intrasynovial tendon segments more than in extrasynovial peroneal tendon segments, and DNA synthesis less in proximal than in intermediate intrasynovial tendons. However, the estimated maximal stimulations (E$_{max}$) by PDGF-BB were similar in the three types of tendon segments. These findings show that PDGF-BB stimulates DNA and matrix synthesis differently in various types and regions of tendons during short term explant culture and suggests that there may be differences in cellular responsiveness during tendon healing.

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Healing of sutured flexor tendons in the intrasynovial region of the hand may be site- or donor-specific. Inferior biomechanical and clinical results have been reported in Zone II injuries—i.e., in the tendon sheath region of digits (Kleinert et al. 1973, May and Silfverskiold 1993). Survival and cellular responsiveness may also differ between extra- and intrasynovially-derived flexor tendon grafts (Seiler et al. 1993, Ark et al. 1994). These differences may be related to variations in morphology, vascularity, and biochemistry in different types of intra- and extrasynovial tendons or responsiveness to growth factors (Okuda et al. 1987, Abrahamsson et al. 1989, Abrahamsson and Lohmander 1996).

During tissue repair, growth factors induce specific biological responses, including cell proliferation and migration, matrix synthesis, angiogenesis and release of growth factors (Lynch et al. 1989, Pierce et al. 1991). Platelet-derived growth factor (PDGF) is an about 30kDa dimer of two polypeptide chains, A and B, with 3 isoforms, PDGF-AA, -AB and -BB, depending on cell-surface receptor specificity. PDGF is stored in the alpha-granules of platelets and secreted by locally-activated cells, including macrophages, fibroblasts, and endothelial cells, during tissue repair (Bowen-Pope and Ross 1984, Pierce et al. 1991). These isoforms have several similar modes of action, but they may also differ. PDGF-BB has been shown to be the strongest stimulator of cell proliferation in various tissues and cell types in vitro (Lepistö et al. 1992, Wroblewski and Edwall 1992). However, its stimulatory effect on synthesis of matrix components has been considered less marked (Nakashima 1992, Bartold 1993).

Release of insulin-like growth factor-I (IGF-I) during tissue repair may enhance the synthesis of matrix components and the effects of PDGF-BB (Luyten et al. 1988, Abrahamsson 1991). Since IGF-I may be present in tendons and its stimulatory effects on cell proliferation and matrix synthesis differ in various types of tendons, one might speculate that the effects of PDGF-BB may vary in different types of tendons as well (Abrahamsson et al. 1994, Abrahamsson and Lohmander 1996, Murphy and Nixon 1997).

We compared the stimulatory effects of PDGF-BB on cell proliferation and matrix synthesis in different regions and types of rabbit tendons in vitro.

Animals and methods

Tissue preparation

6 mixed gender Swedish Loop rabbits, weighing 2.2–2.8 kg, were used as experimental animals. The rabbits were anesthetized with an intramuscular dose of fentanyl-fluanison (Hypnorm; 0.5 mL/kg of body weight) and killed with an intravenous injection of phenobarbital (8 mL; 60 mg/mL). With the use of operating facilities, the intrasynovial part of the deep flexor tendons (FDP) and the extrasynovial peroneal tendons from both of the hindpaws were removed and rinsed in physiological saline solution.

One segment from the proximal region of the deep flexor tendon at the level of the metatarsophalangeal joint (FDPp) and one segment from the intermediate region at the level of the proximal phalanx (FDPi) were each divided and split into 6 mm-long segments making twice 16 segments of each animal (Abrahamsson et al. 1989). The peroneal tendons (PER) were removed from the paratenon and divided into 6 mm long segments to produce 16 segments in each animal.

After an additional rinse, the segments were placed in MCDB 105 (M 6395, Sigma BioSciences, MO; McKeehan et al. 1978), supplemented with gentamicin (50 μg/mL), ascorbic acid (50 μg/mL) and bovine serum albumin (1 mg/mL) before culture.

Tendon culture and labeling protocols

When synthesis of matrix components was studied, 48 of the tendon segments were divided into 8 groups with one tendon segment/rabbit represented in each group. These segments were placed in 24-multidish plates, 1 segment per well (A/S Nunc, Roskilde, Denmark) and 1 mL of supplemented medium, MCDB 105, was added to each well.

Tendon segments were preincubated for 24 h at 37 °C in a water-saturated atmosphere of 2% CO_2. On the second day, the medium was replaced by fresh medium. Recombinant human platelet-derived growth factor-BB (PDGF-BB; Genzyme, Cambridge, MA) was added (0.1, 0.3, 1.0, 3.0, 10.0, 30.0, 100.0 ng/mL) to 7 of the 8 groups of tendon segments, respectively. On the third day, the procedure was repeated and tendon segments were labeled for 24 h with ^{3}H-proline (10 μCi/mL) and ^{35}S-sulfate (40 μCi/mL; Radiochemical Centre, Amersham, England). On the fourth day, the segments were rinsed once and chase-incubated twice for 30 minutes in supplemented medium and L-proline (50 μg/mL). All tendon segments were then stored at –20 °C, pending analysis. Dry weight was determined after lyophilization.

When cell proliferation was studied, the same protocol was used, but non-radioactive thymidine (1.2 μg/mL) was added to the medium in each well on the second and third days of culture (Puzas and Brand 1986). The tendon segments were labeled with ^{3}H-thymidine (10 μCi/mL; Radiochemical Centre, Amersham, England) and then rinsed in medium with non-radioactive thymidine (50 μg/mL).

Determination of incorporation rates

Dried tendons, labeled with ^{3}H-proline and ^{35}S-sulfate, were hydrolyzed in 6 M hydrochloric acid at 104 °C for 24 h, evaporated and dissolved in 0.1 M hydrochloric acid. The samples were then separated on a column of Aminex A6 (BioRad) eluted with citrate buffer, pH 3.75, at 60 °C. Radioactivity in peaks corresponding to ^{35}S-sulfate, ^{3}H-hydroxyproline and ^{3}H-proline were measured with a radioactivity flow-detector (Radiomatic Instruments and Chemical, Tampa, FL, USA). Results of incorporation were expressed as disintegrations per minute per milligram of dry-weight tendon tissue (dpm/mg dwt). The macromolecular content of ^{35}S-sulfate and ^{3}H-hydroxyproline was used to measure new synthesis of proteoglycan and collagen, respectively. The incorporation of ^{3}H-proline, corrected for the known ratio of proline for hydroxyproline in collagen, represents the de novo synthesis of noncollagen protein (Diegelmann and Peterkofsky 1972). For a full description of protocols, see Abrahamsson and colleagues (1989).

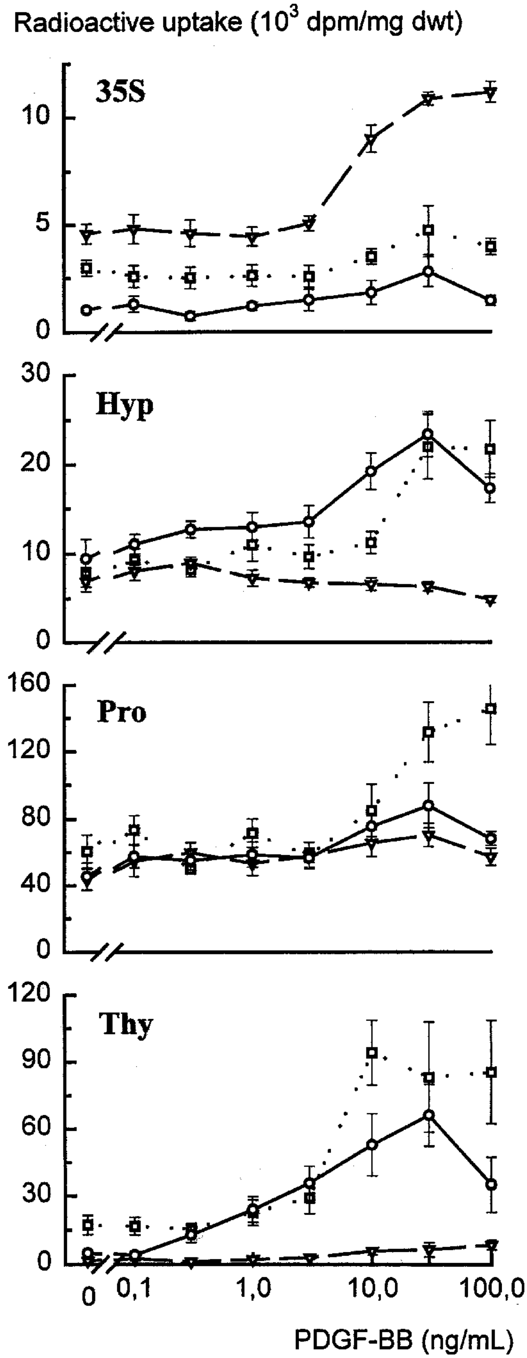
Tendons labeled with ^{3}H-thymidine were dissolved in 1 M potassium hydroxide at 37 °C for 24 hours, neutralized and cooled on ice. Nucleic acids were precipitated by adding trichloroacetic acid (TCA). After centrifugation, the supernatant was discarded and the procedure repeated. The final washed pellet was dissolved in sodium hydroxide, neutralized and mixed with liquid scintillant and counted in a scintillation counter. The content of ^{3}H-thymidine was used to measure the new synthesis of DNA (cell proliferation). The results were expressed as disintegrations per min per mg of dry-weight tendon tissue (dpm/mg dwt).

Statistics

E_{max} (estimated maximum response; %) refers to the maximum response of an added factor (max; dpm/mg dwt) given as a percentage of the response without the factor (con; dpm/mg dwt; E_{max} = max/con × 100). Linear regression was calculated by the method of least squares and in all curves was limited to the pure ascending part. $LogED_{50}$ refers to the logarithm of the estimated dose of an added factor eliciting the half-maximal effect {(max-con)/2+con}. It was calculated from the equation of the regression line. When the maximal response was obtained by the highest dose of PDGF-BB, the dose-response curve lacked endpoint and thus neither a sure E_{max} could be stated nor a sure $LogED_{50}$ could be calculated. Unless stated otherwise, the results are presented as mean ± SD. Neuman-Keul's test (ANOVA) was used when comparing multiple groups and the Student's t-test when comparing two groups with each other. A value of $p < 0.05$ was considered significant.

Results

The rate of proteoglycan synthesis was represented by the mean radioactive uptake of ^{35}S-sulfate. The effects of PDGF-BB on the rate of synthesis of proteoglycan in intermediate and proximal intrasynovial flexor tendon segments were dose-dependent between 0.1–30 and 0.1–100 ng/mL, respectively, and for extrasynovial peroneal tendon segments between 0.1–30 ng/mL (Figure). The level of E_{max}, representing the quantitative response of PDGF-BB on the rate of proteoglycan synthesis,



Log dose effects of PDGF-BB on proteoglycan (35S), collagen (3H-Hyp), noncollagen protein (3H-Pro) and DNA synthesis (3H-Thy) in intermediate (FDPi; ●) and proximal (FDPp; ▼) intrasynovial deep flexor and extrasynovial peroneal (PER;) tendon segments of rabbits. Values are mean radioactive uptake (10^3 dpm/mg dwt) and bars indicate SEM (n 6).

Synthesis stimulated by PDGF-BB. Synthesis of proteoglycan (35S), collagen (Hyp), non-collagen protein (Pro), and DNA (Thy) in intermediate (FDPi) and proximal (FDPp) intrasynovial flexor and extrasynovial peroneal (PER) tendons following short-term culture in medium with PDGF-BB in increasing concentrations

	FDPi	FDPp	PER
E_{max} [a]			
35S	293 (122)	258 (61)	206 (98)
Hyp	250 (50)	168 (64) [c]	337 (124)
Pro	197 (12)	218 (81) [c]	289 (105)
Thy	1504 (611)	828 (402)	874 (246)
$LogED_{50}$ [b]			
35S	1.03 (0.66)	0.91 (0.12)	0.97 (0.20)
Hyp	0.99 (0.43)	-1.68 (1.28) [c d]	1.15 (0.24)
Pro	1.01 (0.32)	0.64 (0.31) [c d]	1.23 (0.10)
Thy	0.61 (0.47)	1.31 (0.45) [d]	0.91 (0.22)

[a] Emax – the maximal response of PDGF-BB given as percentage (%) of the response of control tendons (without PDGF-BB), mean of E_{max} (SD) (n 6).
[b] $LogED_{50}$ – the logarithm of the dose in nanograms per milliliter medium (ng/mL) eliciting the half-maximal response given as mean $LogED_{50}$ (SD) (n 6).
[c] E_{max} obtained by the highest dose of PDGF-BB and corresponding $LogED_{50}$.
[d] indicates significant difference at $p < 0.05$.

and the level of $LogED_{50}$, which is used to calculate the potency of the stimulation by PDGF-BB, were similar in the three types of tendon segments (Table).

The synthesis of collagen in tendon segments was represented by the mean radioactive uptake of ^{3}H-hydroxyproline. The effects of PDGF-BB on the synthesis of collagen in intermediate and proximal intrasynovial flexor tendon segments increased in a dose-dependent manner between 0.1–30 ng/mL and 0.1–0.3 ng/mL, respectively, and in extrasynovial peroneal tendon segments between 0.1–30 ng/mL (Figure). The level of E_{max}, representing the quantitative response of PDGF-BB on the rate of collagen synthesis, did not differ significantly between the three types of tendon segments. But the level of $LogED_{50}$, which is used to calculate the potency of the stimulation by PDGF-BB, was significantly lower in the proximal intrasynovial flexor tendon ($p = 0.041$; Table).

The synthesis of noncollagen protein in tendon segments was represented by the mean radioactive uptake of ^{3}H-proline. The effects of PDGF-BB on synthesis of noncollagen protein in intermediate and proximal intrasynovial flexor tendon segments were dose-dependent between 0.1–30 ng/mL, and in extrasynovial peroneal tendon segments between 0.1–100 ng/mL (Figure). The levels of E_{max}, representing the quantitative response of PDGF-BB on the rate of noncollagen protein synthesis, were similar in the three types of tendon segments (Table). However, the level of $LogED_{50}$, which is used to calculate the potency of the stimulation by PDGF-BB, was significantly lower in the proximal intrasynovial flexor tendon than in the extrasynovial peroneal tendon segments ($p = 0.032$, Table).

The rate of DNA synthesis was expressed as mean radioactive uptake of ^{3}H-thymidine. The effects of PDGF-BB on DNA synthesis in intermediate and proximal intrasynovial flexor tendon segments were dose-dependent between 0.1–30 and 0.1–100 ng/mL, respectively, and extrasynovial peroneal tendon segments between 0.1–10 ng/mL (Figure). The levels of E_{max}, representing the quantitative response of PDGF-BB on the rate of DNA synthesis, were similar in the three types of tendon segments (Table). The level of $LogED_{50}$, which is used to calculate the potency of the stimulation by PDGF-BB, was significantly higher in proximal intrasynovial flexor tendon segments than in intermediate intrasynovial flexor tendon segments ($p = 0.028$; Table).

Discussion

This study shows that PDGF-BB dose-dependently stimulates matrix synthesis and cell proliferation in intermediate and proximal intrasynovial flexor and extrasynovial peroneal tendon segments of rabbits in vitro. We found that the rate of matrix synthesis and cell proliferation, represented by a quantitative response of PDGF-BB (E_{max}), did not differ in the three types of tendon segments. In contrast, the potency of PDGF-BB (calculated from $LogED_{50}$) stimulating synthesis of matrix components and cell proliferation varied in different types of tendon segments. The highest potency of synthesis of collagen and noncollagen protein and lowest potency of cell proliferation were seen in the proximal intrasynovial tendon segments. These results correlate with previous findings that fibrocartilaginous-like regions in proximal intrasynovial flexor tendons and extrasynovial tendons have different morphological and biochemical characteristics and suggest that the sensitivity to PDGF-BB differs in various regions and types of tendon segments (Okuda et al. 1987, Abrahamsson 1991, Abrahamsson and Lohmander 1996).

Comparing maximal stimulatory effects, we found that PDGF-BB stimulated DNA synthesis more than matrix synthesis, the rates of 3H- thymidine uptake ranging from 8- to 15-fold that of controls and the rates of uptake of matrix components ranging from 2- to 3-fold that of controls. Although experimental studies have shown that PDGF can stimulate cell proliferation and matrix synthesis in various connective tissues, others have shown that PDGF may inhibit or be unable to stimulate synthesis of collagen and proteoglycan in vitro (Nakashima 1992, Lepistö et al. 1992). We observed only minor stimulatory effects of PDGF on collagen synthesis in proximal intrasynovial flexor tendon segments, suggesting that PDGF is mainly a potent mitogen, but a modulator of matrix synthesis in different rabbit tendon segments in vitro. The characteristic effects of PDGF-BB on various tissues and sites may depend on a local down- or upregulation at the cellular level, concentration of PDGF-BB or other growth factors, or distribution of PDGF isoform receptors (Rechler and Nissley 1985, Marx et al. 1994).

Other growth factors, including IGF-I, may affect cell proliferation and matrix synthesis in various connective tissues (Lynch et al. 1989, Murphy and Nixon 1997). IGF-I dose-dependently stimulates cell proliferation and matrix synthesis with equal potencies, but with varying maximal responses in different tendon segments of rabbits (Abrahamsson and Lohmander 1996). In this study, PDGF-BB dose-dependently stimulated cell proliferation and matrix synthesis with equal maximal responses, but varying potencies. These differences between stimulatory effects of IGF-I and PDGF-BB may be due to specific potency and responsiveness of each growth factor, and be related to the number, distribution, affinity and expression of each receptor at different sites in tendons (Rechlar and Nissley 1985, Marx et al. 1994).

PDGF can influence the major activities required for a normal tissue repair response. However, endogenous PDGF has not conclusively been shown to play a critical role in tissue repair, nor to act independently of other polypeptide cytokines (Pierce et al. 1991). Other growth factors are known to play important roles individually and have specific synergistic actions with PDGF in promoting or modulating cell proliferation and matrix synthesis during tissue repair of connective tissue (Lynch et al. 1989). On the other hand, mechanical load acted synergistically with dose-dependent effects of PDGF-BB on DNA synthesis of flexor tendon cells in vitro (Banes et al. 1995). Thus, a synergistic or additive mechanism via the biochemical response, including growth factors and mechanical stimulation, may be important in the enhancement of cellular activity during flexor tendon healing.

PDGF has a short-time clearance, $t_{1/2}$ of 4.2 to 12 hours in different tissues and less than 2 minutes in plasma, and acts in the early stage of wound healing (Bowen-Pope and Ross 1984, Sprugel et al. 1987, Lynch et al. 1991). PDGF induces synthesis of other growth factors including IGF-I and regulates the presence of other receptors (Bowen-Pope and Ross 1984, Pierce et al. 1991). These observations support the hypothesis that PDGF may be a key growth factor and stimulant during the early stages of tendon healing, acting with other growth factors. In addition, the findings suggest clinical use of PDGF in enhancing intrinsic tendon metabolism during tendon surgery. In this study, we found that the effects of PDGF-BB on matrix synthesis and cell proliferation varied different types and sites of tendons, suggesting divergent effects when given in vivo. However, since adhesion formation between the tendon and surrounding tissues may also be affected, the role of exogenous PDGF-BB and other growth factors in promoting tendon repair requires further investigation.

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