# GROWTH FACTOR EXPRESSION IN HEALING RABBIT MEDIAL COLLATERAL AND ANTERIOR CRUCIATE LIGAMENTS

Joe Lee, M.D.
Frederick L. Harwood, B.S.
Wayne H. Akeson, M.D.
David Amiel, Ph.D.

## ABSTRACT

**Exogenously administered growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-ß) and basic fibroblast growth factor (bFGF) have been shown to affect connective tissue healing in vivo[6,8,17,21,22,23], but their intrinsic role in the healing response has not been established. In the present study, immunohistochemistry with antibodies directed against these growth factors showed that expression of PDGF, TGF-ß1and bFGF was increased in and around the wound site in the rabbit medial collateral ligament (MCL) seven days following surgical injury. The strong expression of PDGF correlated with the observed increased cellularity consistent with this growth factor's mitogenic and chemotactic properties. Expression of these growth factors was also increased in wounded rabbit anterior cruciate ligaments (ACL) at seven days following surgical injury, but such expression was limited to the edge of the ACL injury site and was of lesser intensity relative to the MCL. This study suggests that PDGF and TGF-ß1, and to a lesser extent bFGF, are actively involved during the early stage of MCL healing, but have a more limited presence in the injured rabbit ACL.**

## INTRODUCTION

Growth factors are proteins that have been noted to enhance wound healing of cartilage, bone, skin and ligaments *in vivo*[1-6], and have been localized in normal as well as in healing tissue[1,8,10]. Platelet-derived growth factors (PDGF-A, PDGF-B), transforming growth factor beta (TGF-ß), and basic fibroblast growth factor (bFGF) have been found in healing tendons[18], skin lesions[1], platelets[2,3], and macrophages[4,5,27].

Previous *in vitro* studies from our laboratories demonstrated that a combination of the growth factors PDGF-BB, TGF-ß1, bFGF, plus bovine insulin increased the outgrowth of cells from tissue explants after three and six days in culture[20]. In the present study, immunohistochemistry was used to investigate whether there was increased expression of the growth factors PDGF, TGF-ß1, and bFGF in injured rabbit knee medial collateral and anterior cruciate ligaments during the first two weeks following ligament injury.

## MATERIALS AND METHODS

### Animal Model

Fifteen adult New Zealand white rabbits were used for this study. Four unoperated animals served as normal controls. Under general anesthesia and sterile conditions, a medial parapatellar skin incision was made on one side of the eleven study rabbits as described in previous studies[25,34]. The medial collateral ligament (MCL) was exposed through a fascial incision. The MCL was first freed from the surrounding tissue and then lifted from the underlying tissue with a pair of forceps. A transverse full-thickness defect was made in the MCL with a number 61 square-edged microsurgical (Beaver 6100) blade with care being taken not to enter the joint or damage the meniscus. A medial parapatellar fascial incision allowed lateral patellar dislocation. The knee joint capsule was incised and opened, providing exposure of the anterior cruciate ligament (ACL). A similar transverse full-thickness defect was created in the midsubstance of the ACL. Approximately 60% of the ligament in the medial to lateral diameter was disrupted and the ligament was disrupted entirely through the anterior to posterior diameter. The wounds were washed with sterile saline and closed.

Department of Orthopaedics
University of California San Diego
La Jolla, CA 92093-0630 USA

Please address correspondence to:
David Amiel, Ph.D.
Professor of Orthopaedics
University of California San Diego
Orthopaedic Connective Tissue Biochemistry
9500 Gilman Dr. Dept. 0630
La Jolla, CA 92093-0630 USA
Tele (619) 534-2676
FAX (619) 534-5304

Postoperatively the animals were allowed unrestricted cage activity in standard metal cages following recovery from anesthesia. Six of the animals were sacrificed at seven days post-injury and five at 14 days post-injury. All of the animals survived the healing interval. The two ligaments were dissected without disrupting the wound site. The ACL and MCL were then placed into chilled phosphate-buffered saline. The ligament insertion sites were removed, and the ligaments were divided longitudinally through the wound site. The ACL and MCL were mounted parallel to each other in OCT Compound (Miles, Inc.; Elkhart, IN) with the wound site reproducibly oriented. The mounted tissue blocks were snap-frozen with liquid nitrogen and stored at -70°C until use.

### Positive Control Tissue

Epidermal and dermal tissues from an immature and a mature rabbit were used as positive control tissues to test the crossreactivity of these antibodies to rabbit tissue, as PDGF, TGF-ß1 and bFGF have been found in skin[1,9,29].

### Immunohistochemistry

The frozen tissue blocks were warmed to -20°C prior to staining as previously described[25]. Eight micron thick cryosections were made, and the sections were mounted on microscope slides and fixed in 4°C acetone for five minutes. Following drying, the sections were hydrated with a solution of TRIS-buffered saline (TBS) + 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO) for ten minutes.

Blocking was accomplished with 10% normal goat serum (Vector Laboratories, Burlingame, CA) in TBS + 0.1% BSA for ten minutes. A second blocking step was performed with 10% rabbit serum in TBS + BSA for 15 additional minutes. Sections were then incubated with appropriate dilutions of the primary antibodies overnight at room temperature in a humidified container. The sections were then washed three times in TBS + BSA, which was used for all subsequent washes. Incubation with biotinylated goat anti-mouse IgG for mouse monoclonals and biotinylated goat anti-chicken for chicken antibodies (Vector Laboratories, Burlingame, CA) was performed for 30 minutes at a concentration of 10 mg/ml.

Sections were again washed three times and then incubated for 30 minutes with a 1:75 dilution of alkaline phosphatase conjugated streptavidin (Vector Laboratories, Burlingame, CA). After washing, Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) was added to the sections and incubated for 25 minutes in the dark. The sections were examined with a Zeiss Sedival inverted-type transmitted-light microscope and photographed with a Canon EOS camera.

### Antibodies

The monoclonal antibodies 1F-133 directed against human PDGF-BB and 3E-205 reactive against human PDGF-AA were obtained from Dr. Glenn F. Pierce, Amgen, Inc., Thousand Oaks, CA. The source of these antibodies was mouse ascites, and these antibodies were capable of cross-reacting with various forms of PDGF at dilutions of up to 1:500. For these studies, dilutions of 1:150 for 1F-133 and 1:160 for 3E-205 were utilized. The anti-human bFGF monoclonal F155C was obtained from Dr. Andrew Baird, Whittier Institute, La Jolla, CA. A concentration of 25 mg/ml was used.

For TGF-ß, the polyclonal chicken anti-human TGF-ß1 neutralizing antibody was purchased from R&D Systems, Minneapolis, MN, and reacts only to the active form of TGF-ß1. A working stain concentration of 15 µg/ml was used.

## RESULTS

### Expression in MCL and ACL

*Normal tissue:* No detectible staining to PDGF or TGF-ß1 antibodies was observed in either normal MCL or normal ACL. However, a slight presence of bFGF was seen in these tissues (Figure 1).

*Seven day post-injury tissue:* On gross examination seven days post-injury, the MCL wound was consistently filled in with translucent granulation tissue, while the ACL defect remained quite visible. This was previously demonstrated by our laboratories[35]. The MCL wound and the surrounding area also appeared to be more vascular than the ACL wound and demonstrated a greater amount of adherent tissue.

Microscopically, increased expression of all growth factors was seen in both MCL and ACL wounds (Figure 2). In the ACL wound, PDGF staining was limited to one edge of the injury site, while for the MCL wound, the staining was noted in the entire area surrounding the wound site. All six animals sacrificed seven days after injury demonstrated increased staining for PDGF in the MCL wound site. Only two of the animals showed increased staining in the ACL wound site. In both tissues, PDGF staining was seen in the pericellular region and in the extracellular matrix. Increased cellularity was observed within the regions where PDGF was present.

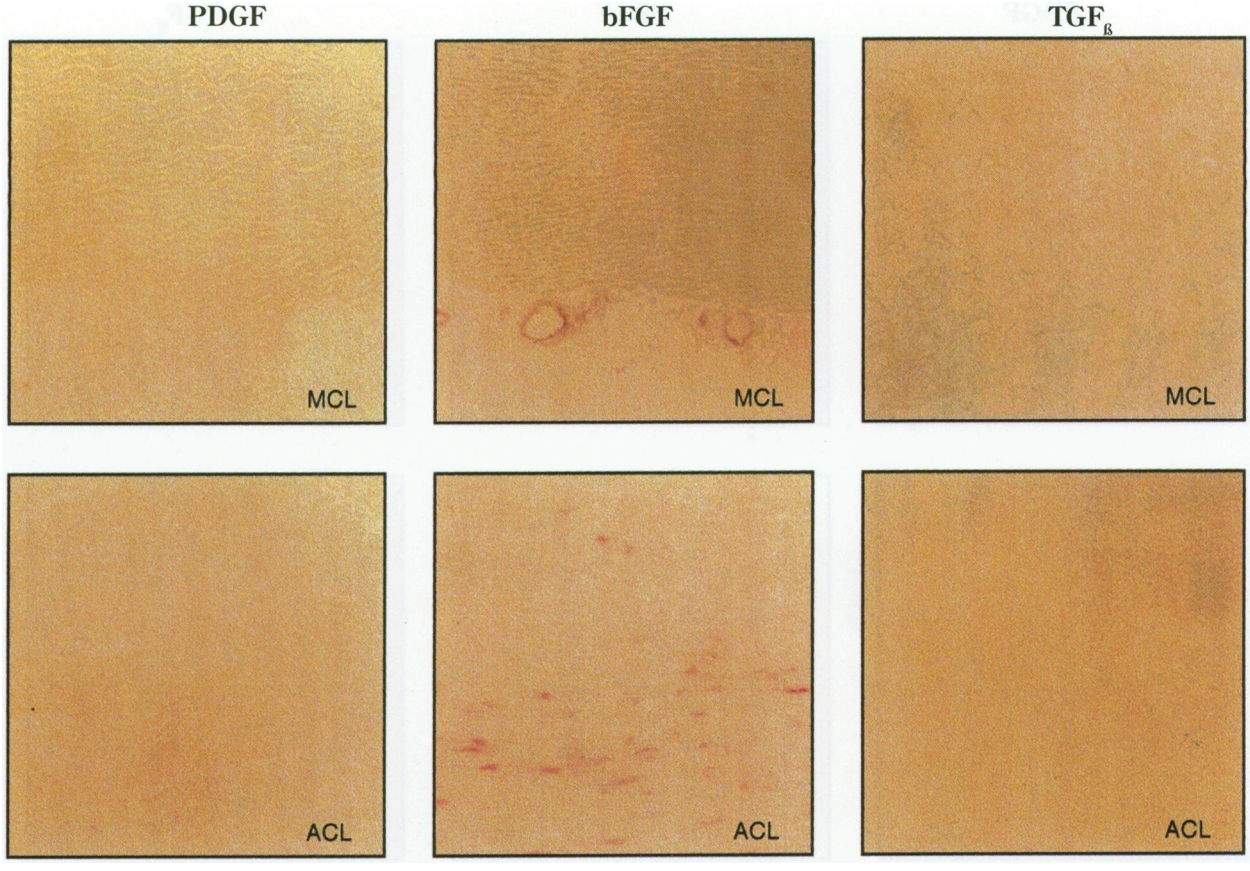


**Figure 1. Immunohistochemical staining of PDGF, TGF-ß1 and bFGF in normal MCL and ACL; original magnification = 20X.**

Expression of TGF-ß1 was observed in and around the MCL wound site and on one edge of the injured ACL. The expression of this growth factor was found in the same region that expressed PDGF.

At seven days, weak expression of bFGF was observed in the ACL, and was limited to vascular structures in the MCL wound. Staining appeared to be associated with vascular structures in both the MCL and ACL and also around cells in the ACL.

*Fourteen day post-injury tissue:* On gross examination at 14 days, the MCL wounds were filled in, while little healing was noted in the ACL wounds. With the exception of bFGF in the ACL, the intensity of staining for all growth factors in both tissues was less than that seen at seven days (Figure 3). PDGF-A antibody staining demonstrated a slight decrease in intensity between seven and 14 days. PDGF-B antibody staining intensity appeared to have an even greater decrease from seven to 14 days. The ACL wound in Figure 3 shows a small region with intense staining of PDGF. Most other regions resembled the Figure 1 ACL stained for PDGF. The oval structure in Figure 3 is a vascular structure, and that may explain why it stained so intensely. PDGF and TGF-ß1 were both localized around vascular structures, but only PDGF could be found in the pericellular regions of the MCL wounds. In the case of the ACL wounds, expression of bFGF was slightly increased at 14 days relative to that observed at seven days, and was found primarily around cells rather than vascular structures.

## DISCUSSION

The results from this study demonstrate that, relative to normal tissues, expression of growth factors PDGF, TGF-ß1, and bFGF was increased in wounds of surgically injured MCL's and ACL's. The growth factors could be found within and around the MCL injury site, but the expression was limited in the injured ACL. The intensity of staining, especially for the growth factors, was generally less in the injured ACL relative to the injured MCL, especially for PDGF and TGF-ß1 at seven days post-injury. The presence of PDGF and TGF-ß1 in the wounded MCL suggests that they may have a role in the early healing response of the rabbit MCL. The

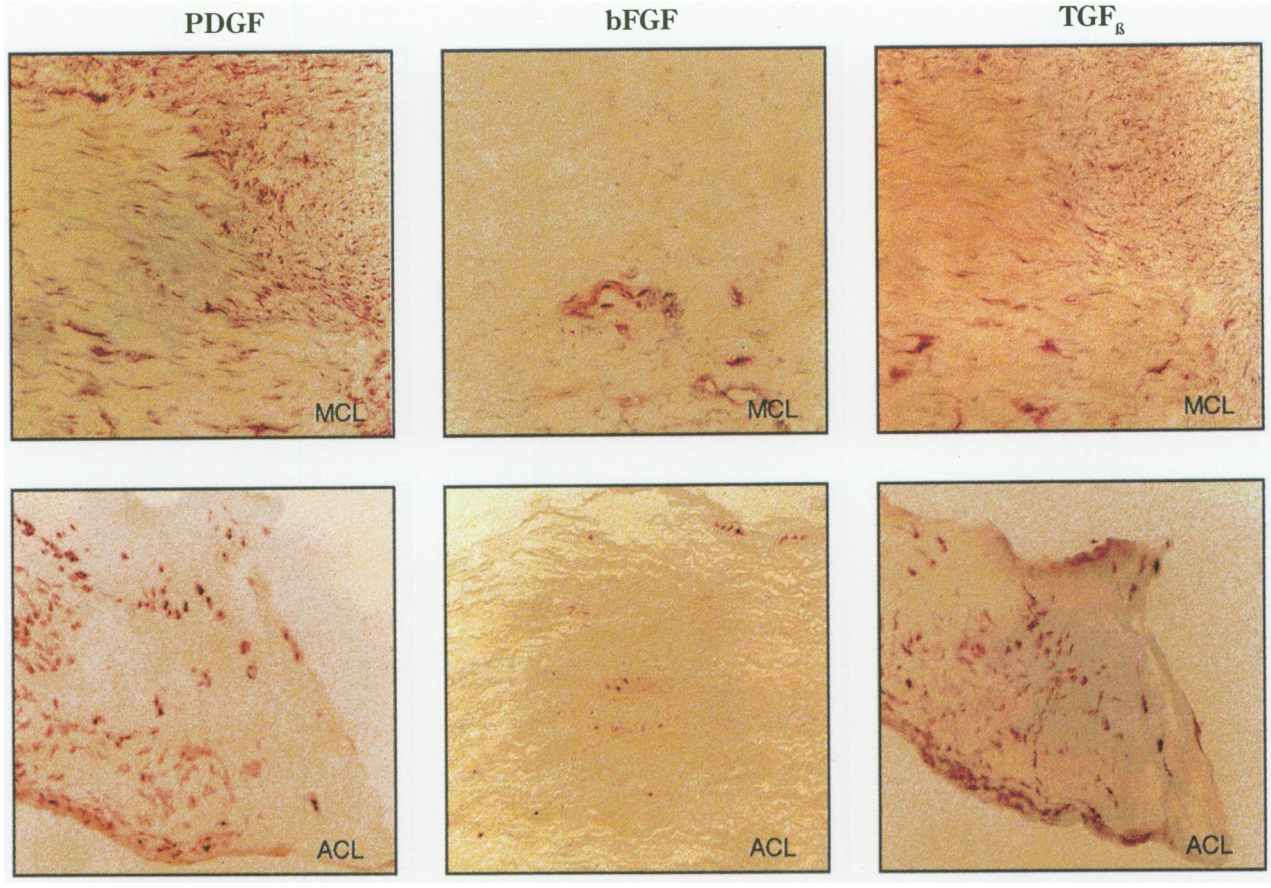


**Figure 2. Immunohistochemical staining of PDGF, TGF-ß1 and bFGF in injured MCL and ACL seven days post-surgery; original magnification = 20X.**

lower expression of these growth factors in the injured ACL relative to the MCL, combined with differences in intrinsic properties between MCL and ACL cells, may contribute to the dissimilar healing responses seen in these tissues. More specifically, the decreased presence of PDGF and TGF-ß1 in the ACL might be due to the reduced vascular supply within the ACL compared to the MCL[32]. The reduced blood supply in the ACL might limit the number of platelets, macrophages and lymphocytes which produce these growth factors[2-4] in the wound site. Decreased staining for growth factors in the ACL wound may also be secondary to bathing of the ACL wound in synovial fluid, inhibiting clot formation and allowing for the elution of growth factors away from the wound site. Therefore, the healing response at the ACL injury site could be diminished from reduced amounts of growth factors for a variety of reasons.

We have performed other studies and have presented results showing that, beginning at five days post-injury, there is an influx of T lymphocytes into the MCL scar and the surrounding ligamentous tissue[19]. This influx occurred prior to the increase in the staining of the growth factors examined in this experiment. The MCL scar tissue did not stain strongly for PDGF or TGF-ß at five days post-injury. In the injured ACL, the influx of T lymphocytes was much less. There was no significant influx of B lymphocytes or macrophages into either wounded ACL or MCL. Double staining showed that PDGF and TGF-ß were found in the region where the T lymphocyte influx had occurred.

The location of these growth factors can be correlated with increased cellularity within and around the MCL wound site, which is consistent with their chemotactic and mitogenic effects. PDGF is known to be chemotactic for fibroblasts, monocytes and granulocytes[26,28]. TGF-ß1 has been shown to be chemotactic for monocytes[31]. These growth factors have also been found to increase the expression of integrins by various cells[7,11,13,15,16]. Certain specific integrins, e.g. the ß1 subunit, are associated with cell motility[7] and others, e.g. the α6 subunit, with vascular structures in injured ligaments[25]. The prior study from our laboratory also found a marked increase in the expression of integrin sub-

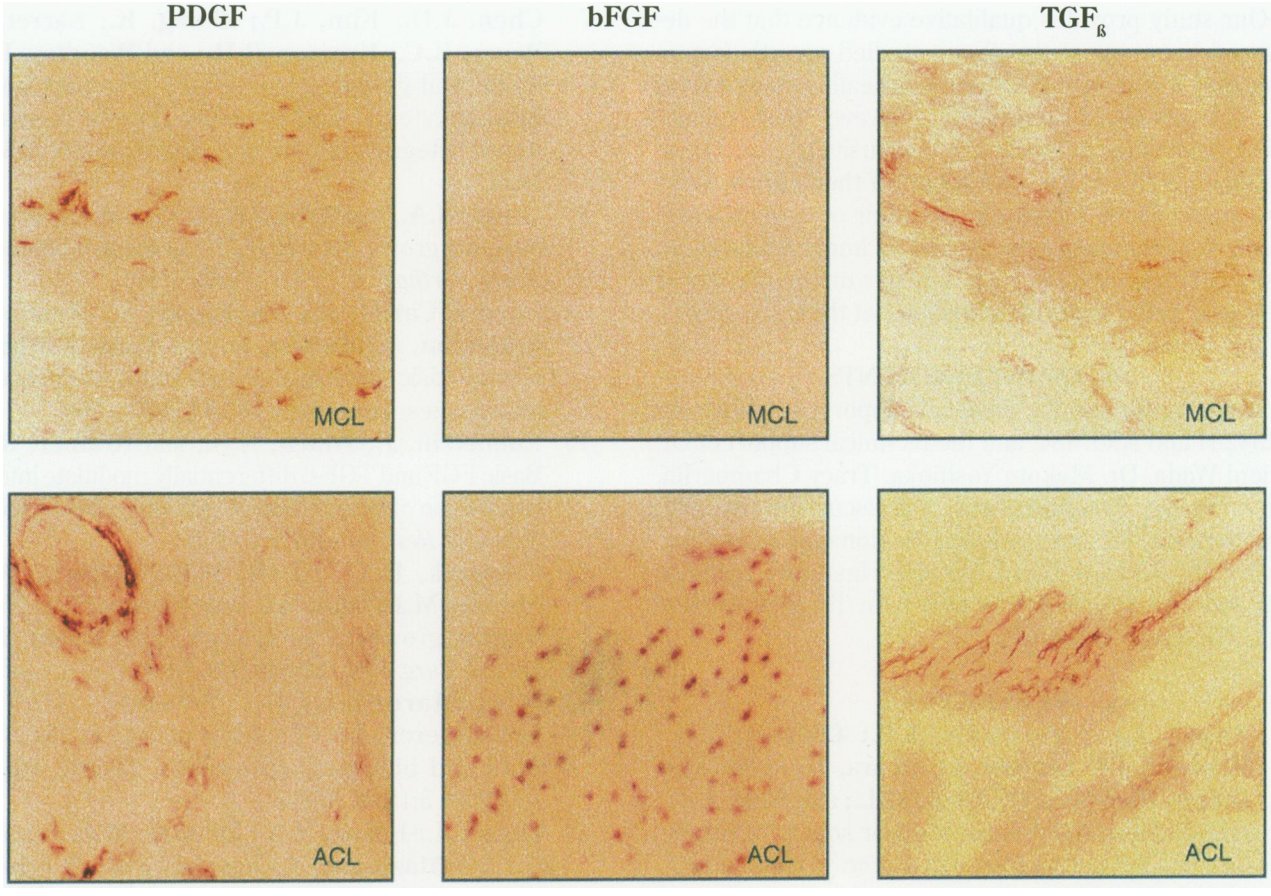


**Figure 3. Immunohistochemical staining of PDGF, TGF-ß1 and bFGF in injured MCL and ACL 14 days post-surgery; original magnification = 20X.**

units in the injured MCL, in particular the ß1 subunit, during the early healing process. There was no similar increase in the expression of these integrins during the same period in the injured ACL. Finally, other known properties of these growth factors include stimulation of neutrophil and monocyte granule release by PDGF, promotion of extracellular matrix formation by TGF-ß1, and stimulation of angiogenesis by bFGF.

Studies similar to ours have been performed on injured tendons. These growth factors have been found in the flexor digitorum profundus tendon within zone II following a surgically created sagittal laceration. PDGF was localized intracellularly in tenocytes and epitenocytes, and maximal staining was seen by post-injury day four. TGF-ß1 was found in the extracellular matrix on post-injury days one and two, but decreased with time in this region. Intracellular staining of TGF-ß1 increased in epitenocytes throughout the tendon and endotenocytes at the site of repair and reached a maximum on post-injury day seven. bFGF antibodies strongly stained the extracellular matrix of the tendon and weakly stained the tenocytes and reached a maximum on post-injury day 28. bFGF was noted to accumulate only in the extracellular matrix surrounding the tenocytes and epitenocytes that were active in the tendon repair process[18].

It has also been shown that reduced growth factors, in particular the PDGF isoforms, are present in some chronic nonhealing dermal wounds[23]. Treatment of patients with chronic pressure ulcers with recombinant human PDGF showed greater healing response with a dose of 100 µg/ml compared to controls[24].

Previous *in vitro* work has shown that individual growth factors alone do not promote cell outgrowth from ligamentous tissue explants. A combination of growth factors, however, seems to promote cell outgrowth from rabbit ligament explants. In fact, the most outgrowth was obtained in a medium containing 10% fetal bovine serum[20]. This serum contains various growth factors, but the major one is considered to be PDGF[2].

Our study provides qualitative evidence that the decreased presence of the above studied growth factors may play a role in the differential healing response of the ACL compared to the MCL. However, more quantitative means may be needed to conclusively prove these findings. In the future, extraction of these growth factors from injured ligaments and their quantification in tissue extracts using enzyme-linked immunoabsorbent assay (ELISA) could provide a better understanding of their role in the differential healing of these ligaments.

## ACKNOWLEDGMENTS

We acknowledge the financial support of NIH grants AR34264 and AR07484, and the technical support of Dr. Yuichi Wada, Dr. Makoto Yoshioka, Tracy Chamberlin, Mike Furniss, Fran Shepherd, and discussions with Dr. Paul Schreck. We acknowledge the donation of antibodies by Dr. Andrew Baird, Whittier Institute, La Jolla, CA, and Drs. Glenn Pierce and John Tarpley, Amgen, Inc., Thousand Oaks, CA.

## REFERENCES

1. **Ansel, J.C.; Tiesman, J.P.; Olerud, J.E.; Krueger, J.G.; Krane, J.F.; Tara, D.C.; Shipley, G.D.; Gilbertson, D.; Usui, M.L.;** and **Hart, C.E.:** Human keratinocytes are a major source of cutaneous platelet-derived growth factor. *J. Clin. Invest.*, 92:671-678, 1993.
2. **Antoniades, H.N.:** Human platelet-derived growth factor (PDGF): Purification of PDGF-I and PDGF-II and separation of their reduced subunits. *Proc. Natl. Acad. Sci.* USA, 78:7314-7317, 1981.
3. **Assoian, R.K.; Komoriya, A.; Meyers, C.A.; Miller, D.M.;** and **Sporn, M.B.:** Transforming growth factor-ß in human platelets: Identification of a major storage site, purification and characterization. *J. Biol. Chem.*, 258:7155-7160, 1983.
4. **Assoian, R.K.; Fleurdelys, B.E.; Stevenson, H.C.; Miller, P.J.; Madtes, D.K.; Raines, E.W.; Ross, R.;** and **Sporn, M.B.:** Expression and secretion of type ß transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci.* USA, 84:6020-6024, 1987.
5. **Baird, A.; Mormede, P.;** and **Bohlen, P.:** Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests identity with macrophage-derived growth factor. *Biochem. Biophys. Res. Comm.*, 126:358-364, 1985.
6. **Batten, M.L.; Hansen, J.C.;** and **Dahners, L.E.:** Influence of dosage and timing of application of platelet-derived growth factor on early healing of the rat medial collateral ligament. *J. Orthop. Res.*, 14(5):736-741, 1996.
7. **Chen, J.D.; Kim, J.P.; Zhang, K.; Sarret, Y.; Wynn, K.C.; Kramer, R.H.;** and **Woodley, D.T.:** Epidermal growth factor (EGF) promotes human keratinocyte locomotion on collagen by increasing the α2 integrin subunit. *Exp. Cell Res.*, 209:216-223, 1993.
8. **Conti, N.A.,** and **Dahners, L.E.:** The effect of exogenous growth factors on the healing of ligaments. *Trans. Orthop. Res. Soc.*, 18:60, 1993.
9. **Cordon-Cardo, C.; Vlodavsky, I.; Haimovitz-Friedman, A.; Hicklin, D.;** and **Fuks, Z.:** Expression of basic fibroblast growth factor in normal human tissues. *Lab. Invest.*, 63:832-840, 1990.
10. **Enenstein, J.; Waleh, N.S.;** and **Kramer, R.H.:** Basic FGF and TGF-ß differentially modulate integrin expression of human microvascular endothelial cells. *Exp. Cell Res.*, 203:499-503, 1992.
11. **Cromack, D.T.; Sporn, M.B.; Roberts, A.B.; Merino, M.J.; Dart, L.L.;** and **Norton, J.A.:** Transforming growth factor ß levels in rat wound chambers. *J. Surg. Res.*, 42:622-628, 1987.
12. **Gospodarowicz, D.; Neufeld, G.;** and **Schweigerer, L.:** Fibroblast growth factor: Structural and biological properties. *J. Cell. Physiol. [Suppl.]*, 5:15-26, 1987.
13. **Heino, J.; Ignotz, R.A.; Hemler, M.E.; Crouse, C.;** and **Massague, J.:** Regulation of cell adhesion receptors by transforming growth factor-ß: Concomitant regulation of integrins that share a common ß1 subunit. *J. Biol. Chem.*, 264:380-388, 1989.
14. **Ignotz, R.A.; Endo, T.;** and **Massague, J.:** Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-ß. *J. Biol. Chem.*, 262:6443-6446, 1987.
15. **Ignotz, R.A.,** and **Massague, J.:** Cell adhesion protein receptors as targets for transforming growth factor-ß action. *Cell*, 51:189-197, 1987.
16. **Janat, M.F.; Argraves, W.S.;** and **Liau, G.:** Regulation of vascular smooth muscle cell integrin expression by transforming growth factor ß1 and by platelet-derived growth factor-BB. *J. Cell. Physiol.*, 151:588-595, 1992.
17. **Joyce, M.E.; Roberts, A.B.; Sporn, M.B.;** and **Bolander, M.E.:** Transforming growth factor-ß and the initiation of chondrogenesis and osteogenesis in the rat femur. *J. Cell. Biol.*, 110:2195-2207, 1990.
18. **Joyce, M.E.; Pruitt, D.L.;** and **Manske, P.R.:** Synthesis and in situ localization of TGF-ß1, TGF-ß2, PDGF-A, PDGF-B and bFGF during flexor tendon healing. *Trans. Orthop. Res. Soc.*, 17:680, 1992.
19. **Lee, J.; Chamberlin, T.; Schreck, P.J.;** and **Amiel, D.:** Second Combined Orthop. Res. Soc. Mtg., San Diego, CA, 1995.

20. **Lee, J.; Green, M.H.;** and **Amiel, D.:** Synergistic effect of growth factors on cell outgrowth from explants of rabbit anterior cruciate and medial collateral ligaments. *J. Orthop. Res.,* 13:435-441, 1995.
21. **Letson, A.K.,** and **Dahners, L.E.:** Influence of growth factor combinations on ligament healing. *Trans. Orthop. Res. Soc.,* 19:17, 1994.
22. **Lynch, S.E.; Colvin, R.B.;** and **Antoniades, H.N.:** Growth factors in wound healing: Single and synergistic effects on partial thickness porcine skin wounds. *J. Clin. Invest.,* 84:640-646, 1989.
23. **Pierce, G.F.; Tarpley, J.E.; Tseng, J.; Bready, J.; Chang, D.; Kenney, W.C.; Rudolph, R.; Robson, M.C.; Van de Berg, J.;** and **Reid, P.:** Detection of platelet-derived growth factor PDGF-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDGF in chronic nonhealing wounds. *J. Clin. Invest.,* 96(3):1336-1350, 1995.
24. **Robson, M.C.; Phillips, L.G.; Thomason, A.; Robson, L.E.;** and **Pierce, G.F.:** Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. *Lancet,* 339(8784):23-25, 1992.
25. **Schreck, P.J.; Kitabayashi, L.R.; Amiel, D.; Akeson, W.H.;** and **Woods, V.L., Jr.:** Integrin display increases in the wounded rabbit medial collateral ligament but not the wounded anterior cruciate ligament. *J. Orthop. Res.,* 13:174-183, 1995.
26. **Seppa, H.; Grotendorst, G.; Seppa, S.; Schiffmann, E.;** and **Martin, G.R.:** Platelet-derived growth factor is chemotactic for fibroblasts. *J. Cell Biol.,* 92:584-588, 1982.
27. **Shimokado, K.; Raines, E.W.; Madtes, D.K.; Barrett, T.B.; Benditt, E.P.;** and **Ross, R.A.:** A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell,* 43:277-286, 1985.
28. **Siegbahn, A.: Hammacher, A.; Westermark, B.;** and **Heldin, C.H.:** Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. *J. Clin. Invest.,* 85:916-920, 1990.
29. **Thompson, N.L.; Flanders, K.C.; Smith, J.M.; Ellingsworth, L.R.; Roberts, A.B.;** and **Sporn, M.B.:** Expression of transforming growth factor-ß1 in specific cells and tissues of adult and neonatal mice. *J. Cell Biol.,* 108:661-669, 1988.
30. **Tzeng, D.Y.; Deuel, T.F.; Huang, J.S.;** and **Baehner, R.L.:** Platelet-derived growth factor promotes human peripheral monocyte activation. *Blood,* 66:179-183, 1985.
31. **Wahl, S.M.; McCartney-Francis, N.; Allen, J.B.; Dougherty, E.B.;** and **Dougherty, S.F.:** Macrophage production of TGF-ß and regulation by TGF-ß. *Ann. New York Acad. Sci.,* 593:188-195, 1990.
32. **Wallace, C.D.,** and **Amiel, D.:** Vascular assessment of the periarticular structures of the rabbit knee. *J. Orthop. Res.,* 9:787-791, 1991.
33. **Weiss, J.A.; Beck, C.L.; Levine, R.E.;** and **Greenwald, R.M.:** Effects of platelet-derived growth factor on early medial collateral ligament healing. *Trans. Orthop. Res. Soc.,* 20:159, 1995.
34. **Wiig, M.E.; Amiel, D.; Van de Berg, J.; Kitabayashi, L.; Harwood, F.L.;** and **Arfors, K.E.:** The early effect of high molecular weight hyaluronan (hyaluronic acid) on anterior cruciate ligament healing: An experimental study in rabbits. *J. Orthop. Res.,* 8:425-434, 1990.
35. **Wiig, M.E.; Amiel, D.; Ivarsson, M.; Nagineni, C.; Wallace, C.D.;** and **Arfors, K.E.:** Type I procollagen mRNA levels in normal and early healing of the medial collateral and anterior cruciate ligaments in rabbits: An in situ hybridization study. *J. Orthop. Res.,* 9(3):374-382, 1991.