Platelet-Rich Plasma Enhances the Initial Mobilization of Circulation-Derived Cells for Tendon Healing

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Circulation-derived cells play a crucial role in the healing processes of tissue. In early phases of tendon healing processes, circulation-derived cells temporarily exist in the wounded area to initiate the healing process and decrease in number with time. We assumed that a delay of time-dependent decrease in circulation-derived cells could improve the healing of tendons. In this study, we injected platelet-rich plasma (PRP) containing various kinds of growth factors into the wounded area of the patellar tendon, and compared the effects on activation of circulation-derived cells and enhancement of tendon healing with a control group (no PRP injection). To follow the circulation-derived cells, we used a green fluorescent protein (GFP) chimeric rat expressing GFP in the circulating cells and bone marrow cells. In the PRP group, the numbers of GFP-positive cells and heat-shock protein (HSP47; collagen-specific molecular chaperone)-positive cells were significantly higher than in the control group at 3 and 7 days after injury. At the same time, the immunoreactivity for types I and III collagen was higher in the PRP group than in the control group at early phase of tendon healing. These findings suggest that locally injected PRP is useful as an activator of circulation-derived cells for enhancement of the initial tendon healing process.


A damaged tendon never restores the biological and biomechanical properties of normal tendon completely. The new reparative tissue remains hypercellular early on in the healing process. The collagen fibrils remain thinner, with a reduction in the biomechanical strength of the tendon (Frank et al., 1997). Many investigators have demonstrated the precise origin and secretory profile of reparative cells in the wounded tendon to have a clear understanding of the role of these cells in the healing process (Lundborg, 1976; Matthews and Richards, 1976; Lee et al., 2003). The data from these studies are very valuable, because they may enable researchers to select a target for gene and cytokine therapy. However, the elucidation of the mechanism of the tendon healing process remains insufficient.

Although in the inflammation phase of the healing process of tendon, macrophages play a central role because of their capacity to produce inflammatory cytokines and growth factors as well as the phagocytosis, the site of origin of the macrophages is still controversial. It was previously reported that the adjacent fibroblasts differentiated into macrophage-type cells and migrated into the wounded area and that this balance between macrophage-type cells and fibroblasts in vivo may be under the influence of local soluble factors (Gelberman et al., 1988). In the case of skin wound healing, it was determined by labeling the marrow cells that macrophages were derived from the marrow (Ross, 1968). In a series of studies, it was demonstrated that the principal phagocytic cells in healing wounds arrive via the blood (Volkman and Gowans, 1965; Ross, 1968). Regarding tendons, there is no established mechanism to explain the existence of macrophages in the wounded area of tendon derived from the circulation.

We have been focusing on circulation-derived cells in tendon healing. Blood flow is pivotal to the regeneration of connective tissues. However, the blood flow to the tendon is only about one-third of that to the muscles (Benjamin and Ralphs, 1997). The importance of preserving the tendon’s segmental blood supply has been stressed because tendons that were permanently denied their segmental vascularity underwent necrosis and replacement with undifferentiated scar tissue (Peacock, 1964). It has been reported that fibroblast-like cells migrating through the blood flow contribute to the formation of scars and may play an important role in normal skin wound repair (Bucala et al., 1994; Chesney and Bucala, 1997). Furthermore, pluripotent mesenchymal stem cells that can differentiate into cell lineages of various mesenchymal tissues including bone, cartilage, fat, tendon, ligament and muscle (Pittenger et al., 1999), exist not only in bone marrow but also in the circulation (Kuznetsov et al., 2001). The circulation is essential for tendon healing, because the circulation contains not only macrophages but also mesenchymal cells that have the potential for differentiation into reparative fibroblasts or tenocytes.

We recently reported that circulation-derived cells temporarily exist in the wounded area in the early phase of the healing process of tendons (Kajikawa et al., 2007). Their proportion in the wounded areas decreased with time. We also suggested the possibility that circulation-derived cells appear in...
the wounded area to initiate the healing process and enhance the healing of the entire tendon through their migration. We thought that one of the reasons for the low healing ability of wounded tendon is the time-dependent decrease in the number of circulation-derived cells in the wounded area. If we could activate the circulation-derived cells and prevent the time-dependent decrease of them by some growth factors or cytokines, the wounded tendon would heal more normally.

In this study, as a factor to activate circulation-derived cells in the wounded area, we focused on platelet-rich plasma (PRP). PRP is an autologous concentration of platelets in a small volume of plasma, and contains several kinds of autologous growth factors. PRP is a new application of tissue engineering and a developing area for clinicians and researchers. It has recently been investigated for the regeneration of bone (Marx et al., 1998; Roldan et al., 2004; Lucarelli et al., 2005), cartilage (Akedo et al., 2006; Nagae et al., 2007), and ligaments (Smith et al., 2006). PRP contains several growth factors, including platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), fibroblastic growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-I (IGF-I), and epidermal growth factor (EGF).

We hypothesized that the time-dependent decrease in the number of circulation-derived cells in the wounded area would be one of the reasons for the low healing ability of wounded tendon. Activation of circulation-derived cells in the wounded area of tendon with many kinds of growth factors contained in PRP would prevent the time-dependent decrease of them and would enhance the tendon healing to restore the properties of normal tendon.

In the present study, to follow the circulation-derived cells, we used a GFP bone marrow chimeric model that we previously developed in which the GFP marker is expressed only in the bone marrow and circulating cells (Kajikawa et al., 2007).

The objective of this study was to evaluate the effect of PRP on tendon healing as an activator of circulation-derived cells with a GFP bone marrow chimeric rat model.

Materials and Methods

Transgenic animals

Female GFP transgenic rats (Japan SLC, Inc., Hamamatsu, Japan) and Sprague-Dawley (SD) rats, genetically identical except for the GFP transgene, were used. GFP is a 27-kDa protein, originally discovered in the jellyfish Aequorea Victoria. In GFP transgenic rats, this marker is expressed in all cells except those of the hair and erythrocytes, with GFP signals found in the cytoplasm and nucleus. Cells that differentiate and divide from GFP-positive cells also express GFP. GFP theoretically is not rejected in the rat (Okabe et al., 1997; Ito et al., 2001; Kobayashi et al., 2005; Oshima et al., 2005). Green fluorescence is emitted as long as the cells remain alive and can be detected under a fluorescence microscope using excitation light without staining, washing, protease treatment, blocking or incubation. Animals were kept in the animal facility of our institute in accordance with the policies and procedures detailed in the guide for the Care and Use of Laboratory Animals of Kyoto Prefectural University of Medicine. The research protocol was reviewed and approved by the Ethics Committee of the university.

Generation of bone marrow chimeric rats

For the generation of bone marrow chimeric rats, bone marrow transplantation was performed as described previously (Ito et al., 2001; Kajikawa et al., 2007). Briefly, before marrow harvest, recipient rats (7-week-old SD rats, n = 60) were irradiated with 10 Gy from a (137) Cs source (Gammarcell 40 Exactor; Nordion International Inc., Kanata, Ontario, Canada) under deep anesthesia. Bone marrow cells were obtained aseptically from the tibias, femurs and humeri of donor rats (7-week-old GFP transgenic rats, n = 20). Marrow cavities were flushed with phosphate buffered saline (PBS) using 25-gauge needles. Single cell suspensions were prepared by repeatedly pipetting and passed through 70 μm nylon mesh twice to remove particulate matter. Cells were counted using a hemocytometer and resuspended at 1.0 × 10^6 cells/ml. The irradiated rats were injected intravenously with 1.0 × 10^6 bone marrow cells using 1 ml syringes with 25-gauge needles. After the transplantation, 500 mg/L of erythropoietin, 1.1 g/L of neomycin sulfate and 30 mg/L of amphotericin B (Nacalai Tesque Inc., Kyoto, Japan) dissolved in acidic water was administered orally for 2 weeks. The peripheral blood cells were examined by flow cytometry to determine the GFP chimeric rate at 14 days after the transplantation. After washing and hemolyzis, samples were suspended in PBS containing propidium iodide (PI) (Sigma–Aldrich Corp., St. Louis, MO) to identify and gate out dead cells. Cell suspensions were analyzed using a FACS caliber (Becton Dickinson, Franklin Lakes, NJ) with excitation at 488 nm and fluorescence detection at 530 nm. All flow cytometric data were analyzed with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). In addition, at the time of sacrifice, the degree of chimerism was analyzed again to examine whether stable chimerism was demonstrated or not.

PRP preparation

PRP was prepared as described previously (Nagae et al., 2007). Twelve-week-old SD rats (n = 3) were sacrificed to make PRP. Twenty milliliters of fresh blood was obtained from each rat using a syringe containing 2.0 ml of acid citrate dextrose-A solution to prevent coagulation. A total of 60 ml of whole blood was centrifuged (KN70, Kyoto, Japan) at 500 g for 10 min. Subsequently, only the plasma fraction was collected and further centrifuged at 3,000 rpm (1,000g) for 10 min to precipitate the platelets. The precipitated platelets at the bottom of the centrifuge tube were collected with 300 μl of the supernatant (platelet-poor plasma) to yield PRP. A platelet count was conducted in whole blood, PRP, and platelet-poor plasma (PPP) (all measurements were performed by FALCO, Japan). The PRP that we made was stored at −80°C until it was injected into the wounded areas of tendons.

To confirm the concentrations of growth factors in the PRP, we measured the concentrations of TGF-β1 and PDGF-BB in peripheral whole blood, PRP, and PPP by enzyme-linked immunosorbent assay (ELISA) using a TGF-β1 and PDGF-BB ELISA Kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer’s instructions.

Tendon injury model and local injection of PRP

At 14 days after bone marrow transplantation, lateral partial perpendicular wounds were made in the middle of the bilateral patellar tendons of bone marrow chimeric rats as previously performed (Kajikawa et al., 2007). At the same time, 20 μl of PRP was injected into the wounded area of the right patellar tendon (PRP group), and the left patellar tendon remained uninjected (control group). Using this tendon injury model, we can observe the bilateral knees with same GFP chimerism and can examine the localized response of PRP. The wounded area was covered with fascia and epitenon that were tightly sutured using 7-0 nylon. At 14 days after transplantation (before injury) and 3, 7, 14, 28, and 56 days after injury, the bilateral knees were harvested and histology and GFP signals were examined (n = 5, in each time point).

Tissue preparation

The harvested knees from all rats were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 72 h at 4°C. The solution was gradually replaced with 20% sucrose for 24 h at 4°C. Tissue blocks were quick-frozen in OCT compound and serial coronal sections were made at a thickness of 14 μm on a cryostat (CM3050 S, Leica; Nussloch, Germany). The sections were...
mounted on slide glasses coated with 3-aminopropyltriethoxysilane (Nacalai tesque, Inc.) and some of them were stained with HE for light microscopic examination.

**Immunohistochemistry for type I, III collagen, HSP47, macrophage, and BrdU.**

The frozen sections were dried at room temperature. After several washes with PBS, they were incubated with 10% normal goat serum in PBS for 10 min under microwave irradiation (M-77, Azumaya Co. Ltd., Tokyo, Japan) to prevent nonspecific staining. To examine collagen synthesis, the sections were incubated with rabbit anti-rabbit type I, III collagen polyclonal antibody (1:100, Biogenesis, UK; Balbin et al., 2001) for 12 h at 4°C. Alexa 546 goat anti-rabbit IgG (1:1,000, Molecular Probes, Eugene, OR) was applied as a second antibody.

To examine the character of circulation-derived cells, the mouse anti-rat heat-shock protein (HSP) 47 monoclonal antibody (1:1,000, Stressgen, BC, Canada) and the mouse anti-rat macrophage/dendritic cell monoclonal antibody (1:250, TransGenInc., Hyogo, Japan) were applied for 12 h at 4°C. HSP 47 (collagen-specific molecular chaperone) is used as a marker of the cells that can produce collagens (Masuda et al., 1998). Alexa 546 goat anti-mouse IgG (1:1,000, Molecular Probes) was applied as a second antibody.

To monitor DNA replication in circulation-derived cells, BrdU immunohistochemical staining was performed as described previously (Hirakawa et al., 2003). BrdU (10 mg/kg body wt; Sigma) in 2.5 ml of PBS was injected intraperitoneally into each rat 1 h before they were sacrificed. Frozen sections were incubated with a mouse anti-BrdU monoclonal antibody (1:100, Amersham Pharmacia, Piscataway, NJ) and then with Alexa 546 goat anti-mouse IgG (1:1,000, Molecular Probes). We performed an additional procedure using PI in order to distinguish GFP-positive cells from negative cells. All nuclei were counterstained with PI (2 μg/ml) for 10 min after BrdU immunohistochemical staining and the sections were covered with gelatine.

**Immunoelectron microscopy for GFP-positive cells**

To examine the structure of the circulation-derived cells, we performed immunoelectron microscopy for GFP-positive cells in the wounded tendon. Particularly, we examined whether the circulation-derived macrophages were founded in the wounded area or not. In previous study, we have revealed that there were macrophages in the wounded area (Kajikawa et al., 2007). Therefore, we used the PRP and control groups, all images were coded and evaluated without knowledge of the experimental group, and the codes were not broken until the analysis was complete. The images were converted into black and white, processed using the same software package. The optical density (OD) of the immunoreactive area was measured and normalized to the OD of the unwounded normal patellar tendon.

**Statistical analysis**

Statistical analyses were performed using the Statcel program. Significant differences (P < 0.05) for each analysis between PRP and control groups at each time point were examined using the Mann-Whitney U-test.

**Results**

**Evaluation of the GFP bone marrow chimeric model**

The GFP-chimeric rate of the GFP bone marrow chimeric model in the peripheral blood was 35.4 ± 11.5% (mean ± SD) at 14 days after transplantation. In a previous study, we confirmed that the GFP-chimeric rate in bone marrow was nearly equal to that of the peripheral blood. Furthermore, we showed that the GFP signal was only found in cells in the circulation, including bone marrow cells, in this model (Kajikawa et al., 2007). The GFP-chimeric rate of all bone marrow chimeric rats at the time
of sacrifice was higher than that at the time of wounding (74.2 ± 18.2%).

Concentration of platelets and growth factors
The blood cell count of whole blood and PRP was conducted. The purified PRP contained about 8.8 times the number of platelets in the whole blood (59.3 ± 10^7/μl in the whole blood, and 523.8 ± 10^7/μl in the purified PRP). TGF-β and PDGF-BB concentrations were greater in PRP compared to all other blood products. The TGF-β1 concentration was 10.8-fold greater in PRP compared to whole blood (5.8 × 10^2 ng/ml in the whole blood, and 62.3 × 10^2 ng/ml in purified PRP). PDGF-BB concentration was 7.7-fold greater in PRP compared to whole blood (2.9 ng/ml in the whole blood, and 22.2 ng/ml in purified PRP). Few growth factors were found in PPP.

Light microscopic histological evaluation
Microscopically, at 3 days after injury, much of the damage was repaired with fibrous tissue and inflammatory cells. Fibroblast-like cells filled the wounded area but the cell density was still low. In contrast, in the PRP group, the cell density was higher in the wounded area and in the border area between the wounded area and the tendon proper than in the control group. At 7 days after injury, the wounded area changed from cellular to fibrous with many fibroblast-like cells. The number of vessels decreased. There was no marked difference between groups at this time point (data not shown). From 28 to 56 days after injury, the fibrous tissue including fibroblast-like cells became dense and the alignment of collagen bundles became partially regular and longitudinal with time. The border between the wounded area and the tendon proper became unclear.

Appearance of GFP-positive cells in the wounded tendon
On laser scanning microscopic examination at 3 days after injury, many GFP-positive circulation-derived cells were present in the wounded area (Fig. 1B,G). The density of GFP-positive cells was significantly higher in the PRP group than in the control group (Fig. 1L). GFP-positive cells had particularly accumulated at the edge of the wound in the PRP group (Fig. 1G). The density was still significantly higher in the PRP group than in the control group at 7 days after injury (Fig. 1L) while the density of GFP-positive cells decreased in both groups (Fig. 1C,H). From 14 days to 56 days after injury, there were few GFP-positive cells in the wounded area in either group (Fig. 1D–I). At these time points, there was no significant difference in the density of GFP-positive cells between the control and PRP groups (Fig. 1L).

Cell proliferation rate
BrDU immunohistochemical staining was performed to examine the proliferative activity of GFP-positive cells in the wounded area. At 3 days after injury in the control group, 25.3 ± 6.3% of GFP-positive cells were proliferating (shown in white in Fig. 2A). In the PRP group, the proliferation rate was twofold higher than in the control group (56.0 ± 18.5%; Fig. 2A,D,G). At 7 days, proliferative activity decreased in both groups (Fig. 2B,E). However, in the PRP group, the proliferation rate was still twofold higher than in the control group (38.5 ± 16.3% to 17.3 ± 3.1%; Fig. 2B,E,G). There were few proliferating GFP-positive cells at 14 days (Fig. 2C,F).

Immunohistochemistry of Hsp47
To evaluate the character of GFP-positive cells in the wounded area, immunostaining for Hsp47 was conducted. The coexpression of Hsp47 could not be found in the cytoplasm of GFP-positive cells (Fig. 1G, yellow-framed inset). The number of Hsp47-positive cells increased and peaked at 7 days after injury in both groups. After this period, the number of Hsp47-positive cells decreased with time (Fig. 1B–K). However, the number of Hsp47-positive cells was still higher than normal level (Fig. 1A) at 56 days after injury (Fig. 1F,K). The number of Hsp47-positive cells was significantly higher in the PRP group than in the control group until 7 days after injury (Fig. 1M).

Immunohistochemistry of macrophages
For the further characterization of GFP-positive cells, immunostaining for macrophages was conducted. Most GFP-positive cells showed immunoreactivity for RM-4 a specific membrane protein in endosomes of macrophages (Iyonaga et al., 1997; Fig. 3A–F). The proportion of macrophages to total GFP-positive cells was approximately 50–60% at 3 and 7 days after the injury in both groups (Fig. 3G). There was no significant difference between groups or time points (Fig. 3G). However, the total number of macrophages was significantly higher in the PRP group than in the control group at 3 and 7 days after injury (Fig. 3H).

Immunoelectron microscopy for GFP-positive cells
There were many cells in the wounded area of tendon (Fig. 4A) and the edge of the tendon (Fig. 4B) with the deposition of the crystal-like reaction-products (TMB-DAB) (pointed with dotted arrows) indicating the presence of GFP. Most of the GFP-immunoreactive cells had a macrophage-like appearance. They had abundant lysosomes in the cytoplasm (arrow) and pseudopodia (arrow head in Fig. 4A,B). The dotted line indicates the edge of the wounded area.

Immunohistochemistry of Type I, III collagens
To evaluate the effect of PRP on collagen synthesis after injection, immunostaining for type I and III collagens was conducted. In both groups, the immunoreactivity of type I collagen increased with time (Fig. 5A–K). The immunoreactivity of type I collagen was significantly higher in the PRP group than in the control group at 3 days after injury. The immunoreactivity of type III collagen remained irregular (Fig. 5F,K). Regarding type III collagen, the level of immunoreactivity peaked at different time points between the groups (Fig. 5L–W,Y). In the control group, the immunoreactivity reached a peak 28 days after injury. On the other hand, the PRP group reached a peak 14 days (Fig. 5Y). After these time points, the immunoreactivity decreased and returned to almost normal levels at 56 days. Until 14 days after injury, the immunoreactivity in the PRP group was significantly higher than that in control group (Fig. 5Y).

Discussion
The contributions of circulating cells to the healing of tissue have been examined in several tissues such as intestine (Hayakawa et al., 2003), kidneys (Ito et al., 2001; Imai and Ito, 2002; Duffield et al., 2005), bone (Taguchi et al., 2005), and skin (Fathike et al., 2004) using GFP bone marrow chimeric animals. The contribution of circulation-derived fibroblast-like cells to healing in the skin, which involves the formation of a scar and is similar to that in the tendons, has also been demonstrated (Bucala et al., 1994; Chesney and Bucala, 1997; Badiavas et al., 2003). Recently, collagen-secreting cells have been reported in the peripheral blood (Quan et al., 2004). Accordingly, circulation-derived cells could contain not only inflammatory cells that can secrete many cytokines and growth factors, but also fibroblast-like cells that synthesize matrix. In our previous study, we reported that circulation-derived cells temporarily remain in the wounded area in the early phase of the healing process of the tendon and their numbers decrease with time (Kajikawa et al., 2007). We suggested that a time-dependent
decrease in the number of circulation-derived cells in the wounded area could cause impaired healing of the tendon.

PRP has recently been used therapeutically as a source of numerous growth factors released from platelet α-granules during regeneration of several tissues. Regarding the musculoskeletal system, PRP has been investigated for regeneration of bone (Marx et al., 1998; Roldan et al., 2004; Lucarelli et al., 2005), cartilage (Akeda et al., 2006; Nagae et al., 2007), ligament (Murray et al., 2006; Smith et al., 2006; Murray et al., 2007a,b), and tendon (Schnabel et al., 2007). Safety is one of the advantages of using PRP for tissue regeneration, since there is no need for concern about transmissible diseases or immune reactions because PRP is an autologous material. Many studies have tried to define the roles played by growth factors in tendon and ligament regeneration and to determine appropriate strategies for the use of growth factors in tissue engineering of these structures in vitro and in vivo. In vitro, it has been reported that PDGF-AB significantly increased cell proliferation, and TGF-β1 significantly increased collagen and proteoglycan synthesis in canine anterior cruciate ligament cell
cultures (DesRosiers et al., 1996). On the other hand, it has been shown that individual growth factors did not increase cell numbers or cell migration out of ligament tissue explants, although combinations of growth factors did increase cell migration (Amiel et al., 1995). Other advantages of PRP include the convenience of preparation and efficacy due to the synergistic effects of various endogenous growth factors. In vivo, it has been reported that PDGF-BB and TGF-β1 applied individually were found to increase the structural properties of the healing ligament. These effects were seen by 2 weeks after injury and were still present at 6 weeks after injury (Hildebrand et al., 1998; Woo et al., 1999). It was also determined that application of PDGF-BB within 24 h of injury led to greater improvements in the structural properties of ligaments compared with application more than 48 h after injury (Batten et al., 1996). Accordingly, in the present study, we studied the effects of local single application of PRP just after injury and examined whether PRP had an effect on activation of circulation-derived cells in tendon healing.

The number of circulation-derived cells that infiltrated into the wounded area was significantly increased in the PRP group, and the cells were more proliferative than in control group at
3 and 7 days after injury. From these results, we confirmed the value of PRP as an activator of circulation-derived cells in the early phase of tendon healing. Chemotaxis of circulation-derived cells might have been induced in the PRP group. It has been previously reported that PDGF, EGF, HGF, BMP-2, and IL-1 have positive effects on fibroblast migration (Hannafin et al., 1999). However, chemotaxis was not clearly observed in this study. In contrast, the cell proliferation of circulation-derived cells was twofold higher in the PRP group than in the control group at 3 and 7 days after injury (Fig. 2G). Therefore, the proliferation of recruited circulation-derived cells may be induced more intensely in the PRP group than in the control group. At 14 days after injury, the number of circulation-derived cells markedly decreased in both groups (Fig. 1L). Probably, circulation-derived cells were replaced by tendon-derived cells. Consequently, the survivability of circulation-derived cells could not be changed by a local single application of PRP.

There is still no specific marker for reparative fibroblast-like cells in the wounded tendon. These cells are sometimes called tenoblasts and regarded as an activated form of tenocytes in the case of intrinsic healing of tendon injuries (Chuen et al., 2004). In the present study, we examined HSP47 immunoreactivity to characterize the circulation-derived cells. HSP47 is a collagen-specific molecular chaperone that assists in collagen synthesis and production (Masuda et al., 1998; Nagata, 1998). As shown in this study, circulation-derived cells of wounded area did not express HSP47 in their cytoplasm. This finding indicates that circulation-derived cells did not produce collagen. In other words, the cells that produced collagen may be derived from adjacent tissues without circulation. The number of HSP47-positive cells was greater in the PRP group when compared to the control group at 3 and 7 days after injury. It seems quite likely that the growth factors contained in PRP induced the activation of collagen synthesis in the wounded area. On the other hand, at 3 and 7 days after injury, the circulation-derived cells were mostly immunoreactive for an anti-macrophage antibody. There was no significant difference in the proportion of macrophage-type cells to circulation-derived cells between groups at 3 and 7 days after injury (Fig. 3H). Therefore, PRP seemed not to have an activity of cytokines such as MCP-1 (Yamamoto et al., 2000) that specifically induce the migration of macrophages or monocytes to wounded areas. However, these findings demonstrate for the first time that circulation-derived macrophages exist in the wounded area of tendon. At 3 and 7 days after injury, the density of macrophage-type cells was significantly higher in the PRP group than in the control group. It can be inferred from these data that the proliferation of macrophages was induced by growth factors present in PRP.

In the present study, we demonstrated that collagen synthesis was enhanced in the PRP group during the early period (Fig. 5X,Y). In the PRP group, the immunoreactivity of type III collagen peaked at 14 days and returned to close to the normal level at 28 days after injury (Fig. 5Y). On the other hand, in the control group, the immunoreactivity of type III collagen peaked at 28 days and continued to approach the normal level with time. Locally injected PRP could change the peak time of type III collagen synthesis. The immunoreactivity of type I collagen continued to increase with time in both groups. The trend observed for the HSP47-positive cell count (Fig. 1M) was similar to that observed for the immunoreactivity of type III collagen (Fig. 5Y), but not type I collagen (Fig. 5X). This result showed that the wounded area is primarily occupied by type III collagen after tendon injury.

Immunoelectron microscopic study clearly revealed that there were many macrophage-type cells derived from the circulation in the wounded area at 3 days after injury. Intriguingly, the macrophage-type cells at the edge of the wound had many pseudopodia directed toward the tendon proper, as if the cells were migrating into the tendon proper from the wounded area (Fig. 4B). We previously reported that circulation-derived cells were found in the tendon proper far from the edge of the wound rather than in the wounded area at 7 days after the tendon injury (Kajikawa et al., 2007). These data might suggest the participation of circulation-derived macrophages in the remodeling of the entire tendon through migration.

Fig. 4. Immunoelectron microscopy for GFP-positive cells in the wounded tendon of bone marrow chimeric rats at 3 days after injury. There were many cells in the wounded area of tendon (A) and the edge of the tendon (B) with deposits of the crystal-like reaction-products (TMB-DAB) (pointed with dotted arrows) in the cytoplasm that indicated the presence of GFP. Most of the immunoreactive cells had a macrophage-like appearance. They had abundant lysosomes in the cytoplasm (arrow) and pseudopodia (arrow head). Dotted line indicates the edge of the wounded area.
fibroblasts. In this way, we believe that PRP may have a synergistic bimodal activation effect on fibroblasts.

In conclusion, the bone marrow chimeric model generated using GFP transgenic rats enabled us to follow circulation-derived cells and to elucidate their spatiotemporal dynamics during tendon healing. Locally injected PRP enhanced the contribution of circulation-derived cells to tendon healing in the early phase of the healing process. Circulation-derived cells were essential for tendon healing, but they seemed not to have ability to produce collagen directly. The viability of circulation-derived cells could not be maintained long time after injury. This is the limitation of the study. To improve the tendon healing in a longer term, we should try to get the sustained actions of growth factors in vivo. Repeat application of PRP or any drug delivery systems (DDS) may be practical for clinical application from the perspective of safety and invasiveness. Further investigation of PRP is needed to provide insights into a new clinical therapeutic approach to promote human tendon healing.

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