

Supplementary Appendix 1

MATERIALS AND METHODS

PRP Preparation

PRP was prepared by a modification of the method of Landesberg et al.²⁵ Fifteen mice were anaesthetized with chloroform and blood was drawn using a 1 mL syringe containing 100 μ L of 3.2% sodium citrate, an anticoagulant, via cardiac puncture. In the preparation of Pure platelet rich plasma(p-prp), a volume of \approx 0.7 mL of whole blood was collected from each mouse in 1.5 mL microcentrifuge tubes that contained 100 μ L of 3.2% sodium citrate. Whole blood was centrifuged at different centrifugal forces ranging from 100 to 200 g (100, 150, 200 g) for 6 minutes, 10 minutes, 15 minutes, and 20 minutes (n = 15) in a table top centrifuge.* After the formation of three layers (a bottom layer composed of red blood cells; an upper layer composed of plasma, platelets, and some WBCs; and an intermediate layer, or buffy coat, composed mostly of WBCs), the upper layer was collected with a pipette. This collection was performed carefully to avoid disturbing the bottom layer of red blood cells and the buffy coat layer. Depending on the centrifugal force of the spin, the collected volume ranged from 40 to 200 μ L. The upper half of the plasma volume, PPP, was removed. The remaining volume of P-PRP was analyzed for platelets and WBCs. The P-PRP was characterized by measuring the platelet concentration gradient prior to PPP removal. The collected sample was then transferred to a 1.5 mL microcentrifuge tube with 100 μ L of 3.2% sodium citrate. After the sample was adequately mixed, a blood cell count was performed with a hemocytometer (neubauer chamber). This preparation method yields an average platelet count of 0.5 to

1.0×10^6 platelets/ μL . The activation of the platelets was carried out by adding 10% CaCl_2 to the PRP at a 1:4 ratio.

Quantification PDGF-AB Release by ELISA

The supernatant concentrations of PDGF-AB, -AA, and -BB released from the alginate and PPP beads were determined by ELISA.[†] The corresponding concentrations of growth factors remaining in the beads were also measured. To extract the factors from the hydrogel and plasma, the samples were first dissolved in 55 mM of sodium citrate, as the competitive binding of sodium citrate for Ca^{2+} leads to dissociation of the matrix. The amount of PDGF-AB was measured following the manufacturer's protocol. Briefly, the samples were incubated for 90 minutes at 37°C on a plate coated with monoclonal antibody. Following washing, 0.1 mL of biotinylated anti-mouse PDGF-AB antibody working solution was added and the samples were incubated at room temperature for 1 hour. After buffer washes, an avidin-biotin-peroxidase complex solution was added. Then, 90 μL of prepared (3,3',5,5'-Tetramethylbenzidine)TMB color developing agent was added and the reaction was terminated after 20 minutes. The absorbance was determined at 450 nm.[‡] A standard curve was generated and used to determine sample PDGF concentrations (pg/mL). The amount of growth factor was calculated based on total sample volume and dilution factor.

Preparation and Culture of MSCs

MSCs was prepared as previously described²⁷. Wild-type C57BL/6J mice, aged 6 to 8 weeks, were euthanized by CO₂. After removal of the skin and muscle tissue, the mouse femurs and tibias were dissected in a sterile hood. The metaphyses from both the ends of the femur and tibia were resected and the bone marrow was collected by flushing the diaphysis with PBS. After separating the bone marrow by density gradient centrifugation[§] at 400 g for 20 minutes, it was then washed twice with Dulbecco PBS supplemented with 2 mM EDTA (DPBS-E), in order to obtain the bone mononuclear cells (BMSCs). BMSCs were then cultured in complete media (i.e., α -minimum essential medium^{||} supplemented with 10% fetal bovine serum,[¶] 2 mmol/L L-glutamine, and 100 U/mL penicillin). Non-adherent cells were removed with fresh complete media at day 3, and then the media was changed every other day. After 5 days, the adherent cells were released by incubating the cells with 1×TrypLE™ (Trypsin)[#] for 3 minutes. The cells were then reseeded onto a tissue culture plate for subsequent passages as described.²⁷

BMP2 Gene Transfer

BMP2 adenovirus was generated and titered as previously described²⁷. For the transfection of the MSCs, the Ad-*BMP2* with serum-free media was added to MSCs. Serum was added to a concentration of 5% after 4 hours, and cells were cultured for an additional 24 hours. Cells were then transferred to OS media (i.e., α -minimum essential medium, supplemented with 10% fetal bovine serum, 2 mmol/L L -glutamine, and 100

U/mL penicillin supplemented with 50 µg/ml ascorbic acid, 10^{-8} M dexamethasone and 10 mM sodium β-glycerol-phosphate) and were fed every 2 days.

ALP Activity Assay

First, the effect of PRP on MSCs alone or in conjunction with BMP2 was tested. P-PRP was administered alone or in doses to the cells and the ALP activity was assayed. The ALP effect of the varying concentrations of PRP on MSC/BMP2 cells was determined as well. Next, the ALP activity of the alginate beads containing PRP was tested on MSCs and MSCs in conjunction with BMP2. Quantitative analysis of ALP activity was determined by colorimetric assay of enzyme activity using an ALP assay kit** following the manufacturer's instructions as described previously²². Briefly, cells were added to an alkaline buffer solution (1.5 M, pH10.3) containing 10 mM p-nitrophenyl phosphate as a substrate. NaOH solution (3 M) was used as stop solution, and optical density was determined at 405 nm. The protein concentration of the cell lysate was measured with a bicinchoninic acid protein assay kit^{††} and the ALP activity was then expressed as OD_{405} per mg protein.

Alizarin Red Assay

The alizarin red activity of the alginate beads containing PRP was tested on MSCs and MSCs in conjunction with BMP2. Briefly, the MSCs were infected with BMP2 for 24 hours in an osteogenic media and were then exposed to the alginate beads containing

PRP. Alizarin red S staining and calcium mineral content quantitation were performed as described previously.²⁸ In brief, the cells were stained for 10 minutes with 40 mM alizarin red S solution (pH 4.2) at room temperature. The cells were rinsed five times with water followed by a 15-minute wash with PBS to reduce nonspecific alizarin red S stain. Stained cultures were scanned, followed by a quantitative destaining procedure using 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0), for 15 minutes at room temperature, and the alizarin red S concentrations were determined by absorbance measurement at 540 nm on a multiplate reader[‡] using an alizarin red S standard curve in the same solution.

Hemocompatibility Test

The percentage hemolysis was calculated by the following equation:

$$\text{Hemolysis (\%)} = \frac{[A(\text{sample of alginate hydrogel}) - A(-)\text{control}] / (A(+)\text{control} - A(-)\text{control}) \times 100}$$

Where, A is absorbance spectrophotometric value. A(sample of alginate hydrogel) is absorbance of tested alginate hydrogel sample, A(-)control is absorbance of tube without alginate hydrogel sample which contains Acid citrate dextrose(ACD) solution and 1 mL PBS, and A(+)control is absorbance of tube without alginate hydrogel sample which contains 3.2% sodium citrate solution and 1 mL distilled water.

Footnotes

* Rotina 380 R, Hettich, Beverly, MA

† Boster, Pleasanton, CA.

‡ SpectraFluor Plus, Tecan, Männedorf, Switzerland.

§ Histopaque-1083, Sigma, St. Louis, MO.

|| Sigma-Aldrich, St. Louis, MO.

¶ Gibco, Thermo Fisher Scientific, Waltham, MA.

Invitrogen, Carlsbad, CA

** Cat. No. 245 to 325-0, Sigma Aldrich.

†† Thermo Fisher Scientific, Waltham, MA.

‡‡ Victor 3 V, Perkin Elmer, Waltham, MA