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Platelet-rich plasma

Growth factor enhancement for bone grafts

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Platelet-rich plasma is an autologous source of platelet-derived growth factor and transforming growth factor beta that is obtained by sequestering and concentrating platelets by gradient density centrifugation. This technique produced a concentration of human platelets of 338% and identified platelet-derived growth factor and transforming growth factor beta within them. Monoclonal antibody assessment of cancellous cellular marrow grafts demonstrated cells that were capable of responding to the growth factors by bearing cell membrane receptors. The additional amounts of these growth factors obtained by adding platelet-rich plasma to grafts evidenced a radiographic maturation rate 1.62 to 2.16 times that of grafts without platelet-rich plasma. As assessed by histomorphometry, there was also a greater bone density in grafts in which platelet-rich plasma was added ($74.0\% \pm 11\%$) than in grafts in which platelet-rich plasma was not added ($55.1\% \pm 8\%$; $p = 0.005$). (*Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998;85:638-46)

In 1994 Tayapongsak et al.¹ introduced the novel idea of adding autologous fibrin adhesive (AFA) to cancellous bone during mandibular continuity reconstructions. They identified earlier radiographic bone consolidation in 33 cases; they attributed this to enhanced osteoconduction afforded to the osteocompetent cells in the graft by virtue of the fibrin network developed by AFA. They also reported the remarkable adhesive advantage of binding cancellous marrow particles during graft placement. Tayapongsak et al.¹ produced their AFA in a blood laboratory setting, separating one unit of whole blood into the red blood cell component and the plasma fraction for use

over the following 2 to 3 weeks as a cryoprecipitate. This was then thawed over a 24-hour period to yield a final "fibrinogen-rich concentrate" of 10 to 15 ml.

Since the early 1990s we have been exploring the parallel but more specific sequestration and concentration of autologous platelets in plasma (platelet-rich plasma [PRP]) and studying the growth factors contained within platelets in relation to their biologic enhancement of continuity bone grafts to the mandible. The first purpose of this article is to introduce our studies of PRP; we present data documenting that PRP increases platelet concentration when placed into grafts, showing the presence of at least three growth factors (platelet-derived growth factor [PDGF], transforming growth factor beta 1 [TGF- β_1] and transforming growth factor beta 2 [TGF- β_2]), and indicating that cancellous marrow cells have receptors for these growth factors. The second purpose of the article is to explore the potential of PRP to increase the rate of bone formation in a graft and enhance the density of the bone formed at 6 months. The third purpose of the article is to present a model of bone graft bone regeneration illustrating the mechanism by which PRP may enhance bone regeneration both in rate and amount.

MATERIAL AND METHODS

Eighty-eight elective cancellous cellular marrow bone graft reconstructions of mandibular continuity defects 5

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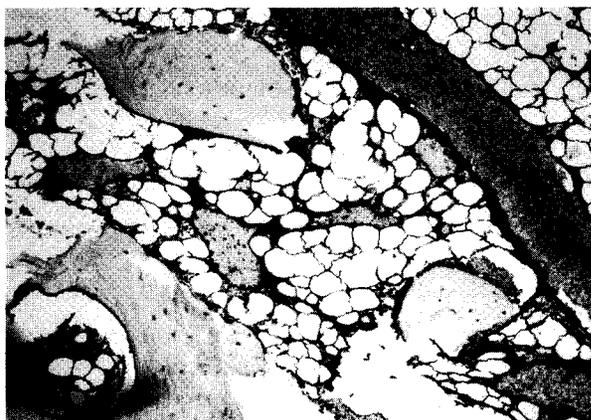


Fig. 1. TGF- β_1 monoclonal antibody staining shows cancellous marrow grafts to possess cells bearing receptors for TGF- β_1 growth factors; they are more concentrated around vessels (perivascular loci) and on endosteal osteoblasts. TGF- β RI (V-22) epitope correspond to amino acids 158-179 (Santa Cruz Biotechnology stains, original magnification

cm or greater arising from benign and malignant tumor extirpations without radiotherapy were randomized into two groups. One group received cancellous cellular marrow grafts without added PRP. The second group received grafts with PRP added during the bone-milling phase of graft preparation and applied topically after bone placement into the defect. For each graft the posterior ilium was used as a donor site.

The PRP was obtained by means of an Electro Medics 500 gradient density cell separator (Medtronic) used in the operating room simultaneously with bone graft harvesting. This cell separator withdraws 400 to 450 ml of autologous whole blood through a central venous catheter placed during surgery. With a centrifuge speed of 5600 RPM, whole blood is drawn at a rate of 50 ml/min. As it withdraws the blood the separator adds citrate phosphate dextrose (CPD) at a ratio of 1 ml of CPD to 5 ml of blood to achieve anticoagulation. The blood is then centrifuged into its three basic components; red blood cells, PRP (sometimes referred to as "buffy coat"), and platelet-poor plasma (PPP). Because of differential densities, the red blood cell layer forms at the lowest level, the PRP layer in the middle, and the PPP layer at the top. The cell separator incrementally separates each layer, from the less dense to the more dense; therefore it separates PPP first (about 200 ml) and PRP second (about 70 ml), leaving the residual red blood cells (about 180 ml). Once the PPP is collected, the centrifuge speed is lowered to 2400 RPM to allow for a precise separation of the PRP from the red blood cells. In fact, both our experience and testing by Reeder et al.⁴ have shown

that the platelets most recently synthesized, and therefore of greatest activity, are larger and mix with the upper 1 mm of red blood cells, so that this layer is included in the PRP product. This imparts a red tint to the PRP, which would otherwise be straw colored. The red blood cells and PPP are returned to the patient from their collection bags through either the central venous catheter or a peripheral venous access.

This procedure takes approximately 20 to 30 minutes. However, it is accomplished simultaneously with either the bone harvesting procedure or preparation of the recipient tissues, and therefore it does not add to operating room time. The Medtronic cell separator is in the armamentaria of most operating rooms that are also used for major orthopedic and cardiovascular surgery; there are thus no additional expenses except those associated with disposable catheters, a central venous line, and an internal centrifuge bowl, which together cost approximately \$300.00.

Samples of PRP and venous blood were submitted for machine platelet counts and a smear with Giemsa staining for a manual count. Two additional PRP smears were stained with monoclonal antibody stains (Santa Cruz Biotechnology, Santa Cruz, Calif.). One was stained for PDGF and the other for TGF- β . A sample of the autogenous graft material was placed in formalin, processed with a slow formic acid decalcification, and stained with monoclonal antibodies to identify PDGF receptors (PDGF $_r$) and TGF- β receptors (TGF- β_r).

The PRP application requires initiating the coagulation process with a mixture of 10 ml of 10% calcium chloride mixed with 10,000 units of topical bovine thrombin (Gentrac). The protocol for PRP application requires the use of an individual 10-ml syringe for each mix. Each mix draws, in order, 6 ml of PRP, 1 ml of the calcium chloride/thrombin mix, and 1 ml of air to act as a mixing bubble. The syringe is agitated for 6 to 10 seconds to initiate clotting. The PRP, now a gel, is added to the graft in several mixes. If several mixes are used, a sterile new syringe is required at each mix. The addition of a small amount of calcium chloride and thrombin from a reused syringe can coagulate the remainder of the PRP in its container. Once the PRP is added to the graft the fibrin formation binds the otherwise loose cancellous cellular marrow together to assist the surgeon in sculpting the graft. The fibrin network established in the graft is thought to assist the osteoconduction component of bone regeneration.¹

The bone grafts were allowed to consolidate and mature for 6 months. Panoramic radiographs were taken at the 2-, 4-, and 6-month intervals. The unlabeled panoramic films were assessed by two investigators (S.R.S. and R.E.M.) as to the age of the graft at

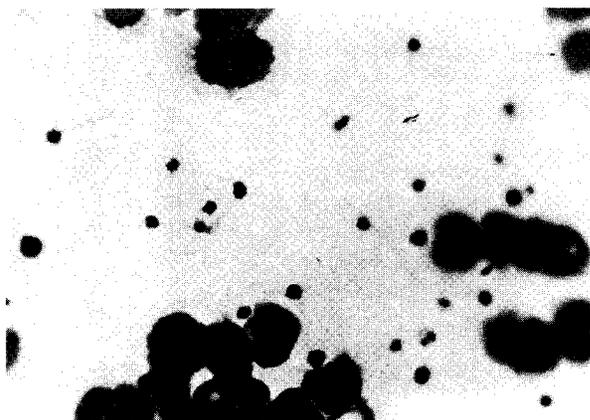


Fig. 2. Normal platelet density in peripheral blood smear (Giemsa stain, original magnification $\times 10$).

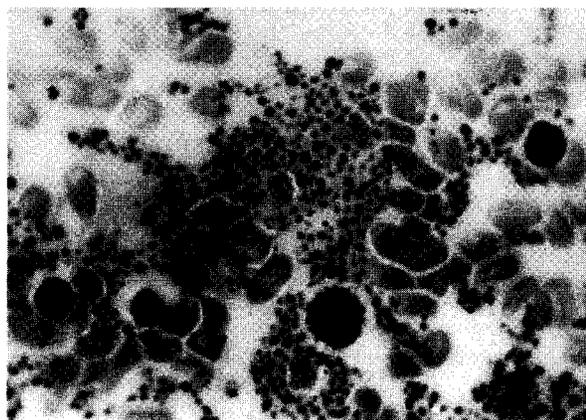


Fig. 3. Obvious concentration of platelets seen on PRP smear indicates transplantation of additional platelets into graft. Cytoplasmic granules (alpha granules) are known location of PDGF, TGF- b_1 , and TGF- b_2 , among other growth factors.

Table 1. Platelet counts: 338% increase

Baseline platelet count	PRP platelet count
232,000	785,000
(111,000-523,000)	(595,000-1,100,000)

each interval. The ratio of assessed graft maturity to actual graft maturity gave a numeric index of graft maturity (graft maturity index [GMI]). At least one osseointegrated implant was placed into each graft at the 6-month interval. The placement of implant fixtures through use of a /3i implant (Implant Innovations, Inc.) with a diameter of 4.0 mm allowed a core bone specimen 2.9 mm in diameter to be processed for histomorphometry and for monoclonal antibody staining for PDGF $_r$ and TGF- b_r . Histomorphometry was accomplished with a semiautomatic computer image system (SMI Unicomp, Atlanta, Ga.). This system projects the histologic image onto a video screen. Random areas were traced on a digitizing pad to calculate area of mineralized bone matrix versus total area of view. The area of mineralized bone matrix was recorded as trabecular bone area (TBA) versus marrow space area. For purposes of comparison and control, 10 resection specimens of the midbody of the mandible were assessed with the same histomorphometric technique, and a TBA was calculated for each.

RESULTS

PRP monoclonal antibody study

The platelets sequestered by the centrifugation process showed an intense uptake of both PDGF and TGF- b monoclonal antibodies in all slides, thus confirming the presence and retention of these growth factors in the PRP preparation.



Fig. 4. Functioning cancellous marrow graft where PRP was not used is well consolidated at 6 months.

Bone graft harvest material monoclonal antibody study

All slides of harvested cancellous cellular marrow showed cell populations that tested positive for receptors to PDGF and TGF- b . It was observed that most of these cell populations were centered about blood vessels in a perivascular sheet. Lesser numbers were observed on the trabeculae of the cancellous bone's endosteal surface and randomly dispersed between fat cells in the marrow. (Fig. 1). These results identified the presence of marrow stem cells and osteoprogenitor cells within human cancellous marrow capable of responding to the increased PDGF and TGF- b in the PRP preparation.

Platelet count study

Platelet counts done on each patient yielded a mean platelet count value of 232,000, with a range of 111,000 to 523,000. The PRP mean platelet count was 785,000, with a range of 595,000 to 1,100,000. These values confirmed the platelet

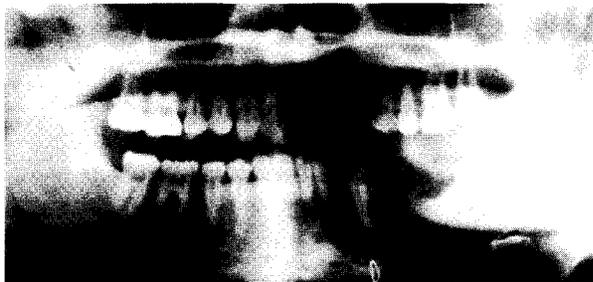


Fig. 5. Functioning cancellous marrow graft where PRP was used shows an enhanced maturity and bone consolidation at 6 months.



Fig. 6. TGF-b_{1r} monoclonal antibody staining shows cell producing TGF-b, presumably to maintain bone formation in graft as an autocrine stimulation. TGF-b_{1r} (v) epitome corresponds to amino acids 352-377 (Santa Cruz Biotechnology

sequestration ability of the process and quantified the concentration as 338% of baseline platelet counts (Table I; Figs. 2 and 3).

Assessment of radiographic graft maturity

The results of the panoramic radiographic assessment are illustrated in Table II. At 2 and 4 months the grafts without PRP growth factor additions were assessed at or just below their actual maturity; at 6 months they were assessed at or just ahead of the actual graft maturity. The grafts with PRP growth factor additions were consistently assessed either at or at slightly more than twice their actual maturity, with ratios of 2.16 at 2 months, 1.88 at 4 months, and 1.62 at 6 months. Each comparison of the average graft maturity index values of PRP-added grafts with those of the grafts without PRP was assessed by means of a Student *t* test. The *p* value for each comparison was 0.001 (Figs. 4 and 5).



Fig. 7. Trabecular bone area of human posterior mandible. Mean trabecular bone area is 38.9% ± 6%.

Table II. Graft maturity index

Time (mo)	Grafts	Graft + PRP	P
2	0.92	2.16	0.001
4	0.88	1.88	0.001
6	1.06	1.62	0.001

Table III. Histomorphometric findings at 6 months

	TBA	P
Native mandible (10)	38.9% ± 6%	-
Bone grafts (44)	55.1% ± 8%	0.005
Bone grafts with PRP (44)	74.0% ± 11%	0.005

Six-month graft assessment with monoclonal antibodies

Processed core bone specimens of each graft type at 6 months demonstrated a continued production of TGF-b. Monoclonal antibodies identified TGF-b but not PDGF by marrow stem cells and endosteal osteoblasts. The TGF-b-positive cells were noted to be concentrated on the trabecular bone endosteal surface, on the periosteal surface, and within active marrow stem cells. Only rare cells stained positive for PDGF and were thus interpreted as nonreactive (Fig. 6).

Six-month graft assessment with histomorphometry

The results of the histomorphometric study, which are illustrated in Table III, indicated that bone grafts in general produce a trabecular bone area greater than that of native posterior mandible (55.1% ± 8% vs 38.9% ± 6%; *p* = 0.005). This was not unexpected and has been reported earlier.^{2,3} However, bone grafts with growth factors added by means of PRP demonstrated even greater trabecular bone density than did bone

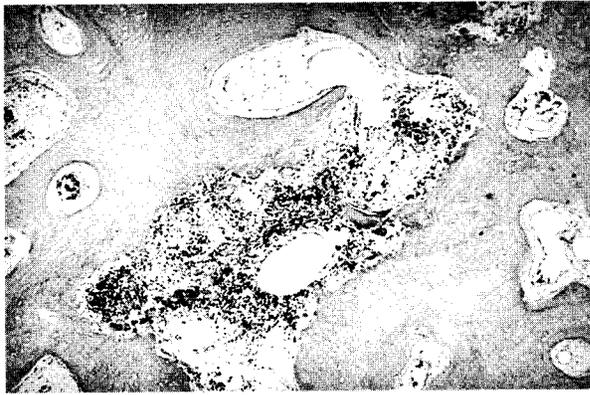


Fig. 8. Trabecular bone area of non-PRP-enhanced cancellous cellular marrow graft at 6 months. Mean trabecular bone area is $55.1\% \pm 8\%$. Note that bone is partially woven bone (immature phase I bone) and partially lamellar bone (mature phase II bone).

grafts without PRP ($74.0\% \pm 11\%$ vs $55.1\% \pm 8\%$; $p = 0.005$; Figs. 7, 8, and 9).

The results in these studies suggested that PRP addition accelerated the rate of bone formation and the degree of bone formation in a bone graft through at least the first 6 months. The data indicated that increased numbers of platelets containing the documented growth factors PDGF and TGF- β , as well as other growth factors that have been identified in the alpha granules of platelets,^{4,6} can be technically sequestered, concentrated, and added to bone grafts along with the fibrin network originally identified by Tayapongsak et al.¹ Cell separator technology allows the specific sequestration and harvesting of only the platelet- and leukocyte-rich layer, avoiding the concentrated but mixed, and therefore diluted, PRP and PPP combined fractions of earlier studies.^{1,4} Furthermore these studies showed that cancellous marrow grafts contain cells bearing PDGF and TGF- β receptors as the probable targets of PRP, as well as being intimately involved in the bone regeneration process. These cells, referred to as stem cells by Caplan,⁷ were found in three locations in cancellous marrow. The perivascular location of marrow stem cells has been suggested by many authors,⁸⁻¹⁰ and it accounted for the greatest number of cells with such receptors. The endosteal location probably represents osteoblasts or preosteoblasts, which are known to be activated by PDGF and TGF- β . The more finely dispersed interstitial cells probably represent diffuse stem cells occupying locations of structural opportunity within the marrow space.



Fig. 9. Trabecular bone area of PRP-enhanced cancellous marrow graft at 6 months. Mean trabecular bone area is $74.0\% \pm 11\%$. Note that bone is all lamellar (phase II) bone, indicative of advanced maturity.

DISCUSSION

Nature of PDGF and TGF- β growth factors

PDGF. A glycoprotein, PDGF has a molecular weight of approximately 30 kd.¹² Although it was first described in the alpha granules of platelets, it is also synthesized and secreted by other cells, such as macrophages and endothelium.¹³ It seems to be the first growth factor present in a wound, and it initiates connective tissue healing, including bone regeneration and repair. In humans, it exists mostly as a heterodimer of two chains—termed *A* and *B* chains—of about equal size and molecular weight (approximately 14 kd to 17 kd).^{13,14} In lesser quantities, A-A and B-B homodimers exist in human beings with the same activity.¹³ The reason for three distinct dimeric forms remains unclear, but differential binding by various receptor cells, such as endothelium, fibroblasts, macrophages, and marrow stem cells, has been suggested.^{13,15} It is known to emerge from degranulating platelets at the time of injury. Its mechanism is to activate cell membrane receptors on target cells, which in turn are thought to develop high-energy phosphate bonds on internal cytoplasmic signal proteins; the bonds then activate the signal proteins to initiate a specific activity within the target cell.¹⁵ The most important specific activities of PDGF include mitogenesis (increase in the cell populations of healing cells), angiogenesis (endothelial mitoses into functioning capillaries), and macrophage activation (debridement of the wound site and a second-phase source of growth factors for continued repair and bone regeneration). There are approximately 0.06 ng of PDGF per one million platelets, a fact that underscores this molecule's great potency.¹⁶ Stated in other terms, there are 6×10^{-17} g of PDGF, or about 1200 molecules of PDGF, in every individual platelet.^{16,17}

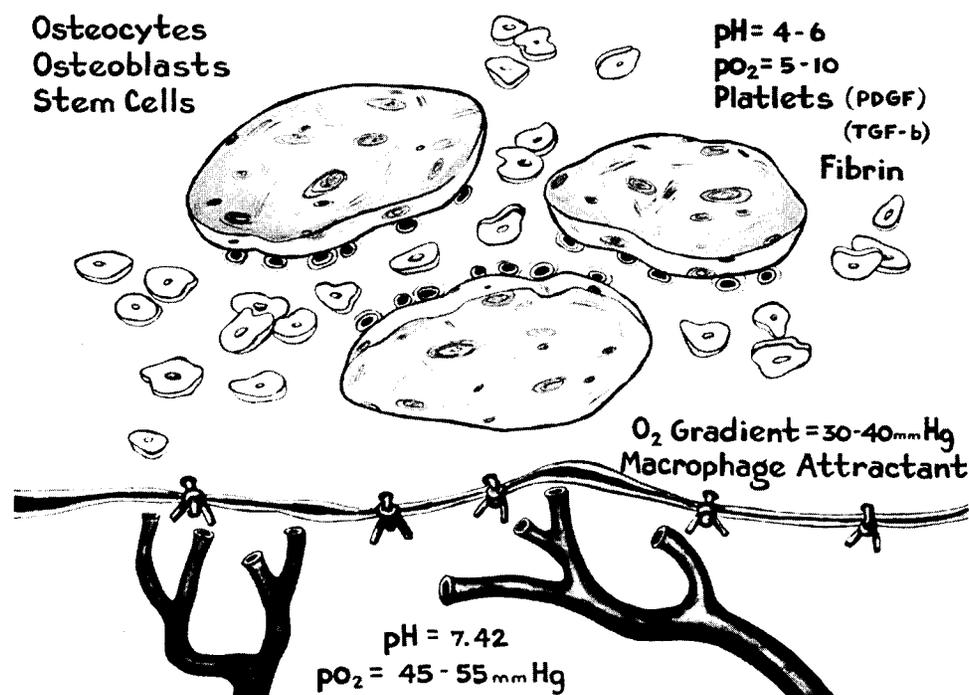


Fig. 10. Basic cells, biochemistry, and growth factors associated inside and outside wound space of cancellous marrow graft.

Therefore a threefold or greater concentration of platelets, as was measured in PRP, can be expected to have a profound effect on wound healing enhancement and bone regeneration.

TGF- β . The term *transforming growth factor beta* is applied to the superfamily of growth and differentiating factors of which the bone morphogenetic protein (BMP) family, containing at least 13 described BMPs, is a member.¹⁸ The TGF- β s referred to and studied in this article are the TGF- β_1 and TGF- β_2 proteins, which are the more protean and generic growth factors involved with general connective tissue repair and bone regeneration.^{5,6} TGF- β_1 and TGF- β_2 are proteins that have molecular weights of approximately 25 kd.^{5,6} Like PDGF, they are synthesized and found in platelets and macrophages, as well as in some other cell types. When released by platelet degranulation or actively secreted by macrophages, they act as paracrine growth factors (ie, growth factors secreted by one cell exerting its effect on an adjacent second cell), affecting mainly fibroblasts, marrow stem cells, and the preosteoblasts. However, each of these target cells has the ability to synthesize and secrete its own TGF- β proteins to act on adjacent cells in a paracrine fashion or act on itself as an autocrine growth factor (ie, a growth factor which is secreted by a cell and acts on its own cell membrane to continue its activity).¹⁹ TGF- β s therefore represent a mechanism for sustaining a

long-term healing and bone regeneration module and even evolve into a bone remodeling factor over time. The most important functions of TGF- β_1 and TGF- β_2 seem to be the chemotaxis and mitogenesis of osteoblast precursors, and they also have the ability to stimulate osteoblast deposition of the collagen matrix of wound healing and of bone.²⁰ In addition, TGF- β s inhibit osteoclast formation and bone resorption, thus favoring bone formation over resorption by two different mechanisms.²¹

A model of bone graft regeneration

From our previous work and from a new knowledge of growth factor influences, we can propose a reasonable model for the bone regeneration observed in cancellous cellular marrow grafts. This model can also illustrate out where PDGF and TGF- β growth factors, at least, influence bone regeneration normally, and how increased quantities of each factor through PRP produced the faster rate of bone formation and the greater quantity of bone seen in this study.

A cancellous cellular marrow graft, whether for a mandibular continuity defect, an alveolar cleft, or a sinus lift surgery, is placed into a dead space filled with clotted blood. The wound dead space is hypoxic (pO_2 , 5-10 mm Hg) and acidotic (pH, 4-6) and contains platelets, leukocytes, red blood cells, and fibrin in a complex clot adjacent to the transferred osteocytes, endosteal osteoblasts,

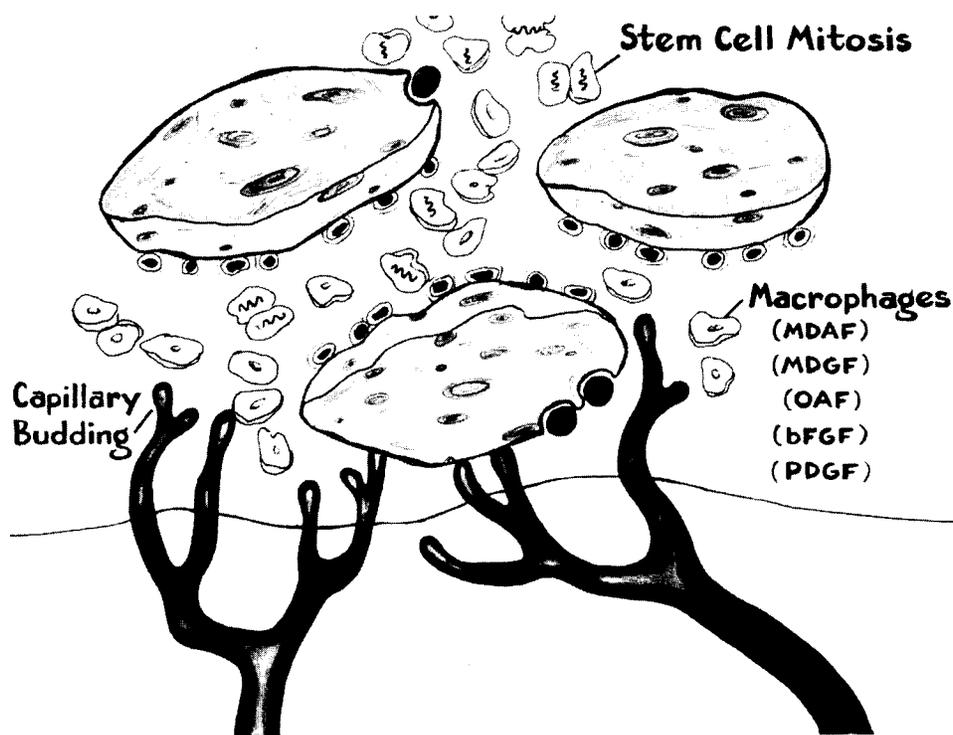


Fig. 11. By day 3 capillary ingrowth begins in response to PDGF and TGF-b. Stem cells and endosteal osteoblasts mitose in response to these same growth factors to create a cell population capable of producing functional quantity of new bone. Macrophage becomes main growth factor elaborating cell, inasmuch as platelets have completely degranulated by now.

and marrow stem cells^{2,22,23} (Fig. 10). The marrow stem cells, which are the primary bone-regenerating cells, normally exist in very small numbers (approximately 1 per 400,000 structural cells in a 50-year-old human being).^{7,10} Just outside the surgeon's periosteal level closure, the tissue is physiologically normal.²³ The tissue is normoxic (pO_2 , 45-55 mm Hg) and at physiologic pH (pH, 7.42), and it contains a population of structural cells, healing-capable stem cells (also in very small numbers), and cut capillaries with clots and exposed endothelial cells. This complex environment—simplified in our model—is the product of millions of years of evolution; it starts, maintains, and promotes mature bone repair related to injury and can be used by surgeons today to regenerate bone through bone grafting.

The initiation of bone regeneration starts with the release of PDGF and TGF-b from the degranulation of platelets in the graft. The PDGF stimulates mitogenesis of the marrow stem cells and endosteal osteoblasts transferred in the graft to increase their numbers by several orders of magnitude. It also begins an angiogenesis of capillary budding into the graft by inducing endothelial cell mitosis. The TGF-b initially activates fibroblasts and preosteoblasts to mitose and increase their numbers, as

well as promoting their differentiation toward mature functioning osteoblasts. Continued TGF-b secretion influences the osteoblasts to lay down bone matrix and the fibroblast to lay down collagen matrix to support capillary ingrowth. These activities begin immediately on wound closure. By the third day capillaries can be seen to penetrate the graft. Complete capillary permeation of the graft is seen by day 14 to day 17² (Figs. 11 and 12).

This initial flurry of cellular activity, though it is also the result of some other growth factors, is primarily the direct result of PDGF and TGF-b. Its evolutionary purpose is simply organism energy efficiency. That is, most body cells are differentiated structural or functional cells. It would be energy-foolish and actually impossible for an organism's energy economy to maintain a large population of cells for healing and no other purpose. Instead, mammalian evolution proceeded to maintain only minutely small numbers of healing-capable stem cells (1/100,000 in a teenager, 1/250,000 in a person 35 years of age, 1/400,000 in a person 50 years of age and 1/1,200,000 in a person 80 years of age)¹⁰; growth factors are relied on to rapidly increase the numbers of these cells and promote their activity during a time of injury.

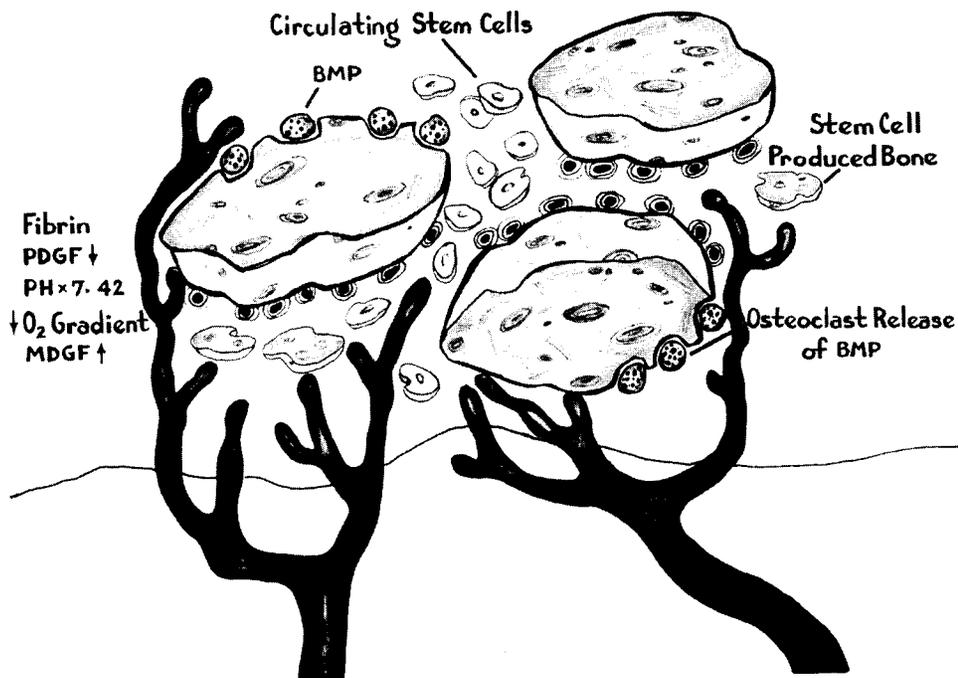


Fig. 12. By day 14 capillary ingrowth is nearly complete. Bone-forming cells are now sustaining themselves with their own autocrine production of TGF- β . With physiologic normalization of wound by capillary perfusion, macrophage function begins to dissipate.

The life span of a platelet in a wound and the period of the direct influence of its growth factors is less than 5 days. The extension of healing and bone regeneration activity is accomplished by two mechanisms. The first is the increase and activation of marrow stem cells into osteoblasts, which secrete TGF- β themselves. The second and more dominant mechanism seems to be the chemotaxis and activation of macrophages that replace the platelets as the primary source of growth factors after the third day.^{20,24} The macrophage is attracted to the graft by the actions of PDGF and by an oxygen gradient between the graft dead space and the adjacent normal tissue that is greater than 20 mm Hg.^{23,25} In fact, the graft's inherent hypoxia of 5 to 10 mm Hg establishes an oxygen gradient of 30 to 40 mm Hg adjacent to the normoxic tissues, which are at 45 to 55 mm Hg.

As PDGF fades in influence, macrophage-derived growth and angiogenic factors take over. However, macrophage-derived growth factors and angiogenic factors may actually be identical to PDGF—only synthesized by macrophages instead of by platelets. The marrow stem cells will secrete TGF- β to continue a self-stimulation of bone formation as an autocrine response; the identification of continued TGF- β activity arising from marrow cells in our study confirms this (Fig. 6). By 4 weeks, the

revascularized graft eliminates the oxygen gradient needed to maintain macrophage activity. Thus the macrophage leaves the area, no longer required by a graft that is now self-sustaining even though immature, with woven osteoid bone rather than mature lamellar bone.^{23,25}

The actual maturation of the graft from a disorganized woven bone into a mature lamellar bone with haversian systems involves the third and final growth factor group in this model, which was not part of this study and is not contained in PRP. It is bone morphogenetic protein (BMP). As bone matrix is formed and then mineralized by osteoblasts, BMP is laid down within the bone matrix.²⁶ This acid-insoluble protein is then released by the osteoclastic resorption of normal bone remodeling, which progresses at a rate of 0.7% per day in normal bone but may occur as rapidly as 5% to 8% per day in a maturing bone graft.^{2,27} The released BMP links bone resorption to new bone formation by acting on adjacent stem cells to increase their numbers and differentiate into functioning osteoblasts that actively secrete bone matrix. Thus the graft cycle from a cellular transplant that is placed into a complex biochemical environment progresses to a mature functional bone that is self-maintaining through the normal resorption-remodeling cycle.

CONCLUSIONS

Today's understanding of bone science recognizes the pivotal role of growth factors in clinical bone grafting success. This article elucidates the mechanism of action and the points of influence that the fundamental growth factors PDGF and TGF- β exert on bone regeneration. The amplification of PDGF and TGF- β through the technique of platelet sequestration and concentration into a platelet-rich plasma is seen as an available and practical tool for enhancing the rate of bone formation and the final quantity of bone formed. The fact that PRP is an autologous preparation introduced at the time of surgery eliminates concerns about disease transmission and immunogenic reactions, which are associated with allogeneic or xenogeneic preparation, and about the possibility of mislabeling a sample, which might occur through a laboratory system.

In this study it was shown that PRP indeed contains a concentration of platelets and a concentration of growth factors. The graft material was shown to contain cells responsive to these growth factors. Finally, our study presented evidence that these growth factor additions to bone grafts produced a quantifiably enhanced result in comparison with grafts performed without its use.

The authors wish to emphasize that PDGF and TGF- β are not the only growth factors that influence bone regeneration and are not the only growth factors contained in PRP. Although it is an admittedly oversimplified model that focuses on the growth factors available to surgeons, the mechanism of action presented in this article is a good working model with which surgeons can understand and plan strategies for growth factor uses.

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