Nonactivated versus Thrombin-Activated Platelets on Wound Healing and Fibroblast-to-Myofibroblast Differentiation In Vivo and In Vitro

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Background: Platelet preparations for tissue healing are usually preactivated before application to deliver concentrated growth factors. In this study, the authors investigated the differences between nonactivated and thrombin-activated platelets in wound healing.
Methods: The healing effects (i.e., wound closure, myofibroblast formation, and angiogenesis) of nonactivated and thrombin-activated platelets were compared in experimental wounds in diabetic (db/db) animals. In vitro, fibroblast phenotype and function were tested in response to platelets and activated platelets. No treatment served as a negative control.
Results: Wounds treated with platelets reached 90 percent closure after 15 days, faster than activated platelets (26 days), and with higher levels of myofibroblasts and angiogenesis. In vitro, platelets enhanced cell migration and induced twofold higher myofibroblast differentiation and contraction compared with activated platelets.
Conclusions: Platelets stimulate wound healing more efficiently compared with activated platelets by enhancing fibroblast differentiation and contractile function. Similar levels of growth factors may induce different biological effects when delivered "on demand" rather than in an initial bolus. (*Plast. Reconstr. Surg.* 129: 46e, 2012.)

With the increasing prevalence of diabetes and obesity over the past few decades, a concurrent surge in chronic wounds and complex tissue defects has been registered.[1] Among the different treatments that have been developed to improve healing, topical application of autologous platelets offered the advantage of promptly providing high doses of growth factors without adding significant costs.

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Platelets have been the object of clinical and experimental wound healing studies for more than 30 years.[2] Recent discoveries in basic science revealed that platelets not only play a key role in hemostasis but also participate actively in multiple biological events such as tissue regeneration, cell proliferation, and immunologic defense.[3] During the early phases in wound healing, platelets participate in initiating repair by forming a provisional fibrin scaffold that allows cell migration into the tissue defects. In later phases, platelets support the recruitment, differentiation, and cross-talk of cells by releasing several bioactive soluble factors.[4,5] Most likely, during physiologic wound healing, platelets participate in these events in a time-controlled fashion.

In clinical practice, surgeons commonly use activated platelets by pretreatment with thrombin

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and/or calcium to induce the immediate and full release of soluble factors. It has been shown that on thrombin activation, platelets release most of their content, inducing hundreds-fold increases in growth factor concentrations in plasma over nonactivated controls.[6,7] However, once activated, platelets lose the ability to specifically interact with the environment.[8,9] For example, the structural and functional integrity of platelets is required to promote angiogenesis, whereas platelet-derived growth factors such as vascular endothelial growth factor (VEGF) alone are less efficient.[10] Treatment with growth factors in high doses has consistently met with only limited success in clinical applications.[11]

We hypothesize that nonactivated platelets are more efficient for stimulating wound healing than thrombin-preactivated ones. We propose that platelets may be used as a cell therapy rather than exploited only for their growth factor content in wound healing. To explore this hypothesis, we compared the effects of activated versus nonactivated platelets in vivo in a diabetic wound healing model and in vitro on fibroblasts.

MATERIALS AND METHODS

Preparation of Platelets

Single-donor human blood was collected into 1:10 volume/volume sodium citrate and placed on a Nutator Mixer (BD Diagnostics, Franklin Lakes, N.J.) until processed. Platelets in plasma were obtained by centrifugation of the citrated blood at 180 *g* for 20 minutes at room temperature, followed by collection of the upper phase. The platelet concentration was then adjusted to 3×10^5 cells/μl in the same plasma. Platelets were stored at room temperature (22°C) on an Eberbach shaker (Eberbach Corp., Ann Arbor, Mich.) until used. In blinded experiments, platelets were applied either nonactivated or after activation with 100 IU thrombin per milliliter immediately before application.

In Vivo Wound Model

We used 8- to 12-week-old homozygous genetically diabetic db/db male mice (strain C57BL/KsJ-Lepr^{db}) as an accepted model for impaired wound healing.[12] Animals were used under an approved animal protocol in an accredited facility. The day before surgery, hair was clipped and depilated (Nair; Church & Dwight Co., Princeton, N.J.). On the day of the surgery, animals were weighed and anesthetized with 60 mg/kg pentobarbital (Nembutal; Lundbeck, Inc., Deerfield, Ill.). A dorsal 1.0-cm^2 area of skin, including the panniculus carnosus, was excised. Wounds were then photographed and individually sealed with semiocclusive polyurethane dressings (Tegaderm; 3M, St. Paul, Minn.) that were kept in place between dressing changes. Injection of 100 μl of platelet preparations using a 30-gauge needle through the dressing into randomly selected wounds followed (n = 12 per treatment group). Preparations of activated or nonactivated platelets were administered once on the day of surgery or wounds were left untreated in the control group.

Wound Closure Analysis

To macroscopically assess wound closure, digital photographs were captured two times per week and compared with initial photographs by two independent treatment-blinded observers using planimetric methods (ImageJ; National Institutes of Health, Bethesda, Md.). Wound closure was quantified as a percentage of the original wound area and expressed as wound contraction and reepithelialization. The sum of contracted, reepithelialized, and open wound areas equals 100 percent of the original wound size, as described previously.[13]

For histologic and immunohistochemical analysis, six animals per group were killed 7 days after wounding, and excised wounds were fixed in 4% buffered formaldehyde. Central wound cross-sections were embedded in paraffin, sectioned, and stained according to routine hematoxylin and eosin protocols. Panoramic cross-sectional digital images of each wound were prepared using Adobe Photoshop (Adobe Systems, Inc., San Jose, Calif.). Sections were analyzed with digital planimetry (ImageJ) by two independent treatment-blinded observers.

Antibodies and Microscopy

For immunofluorescence, we used primary antibodies against the myofibroblast marker α-smooth muscle actin (mouse IgG2a, αSM-1, a gift from Dr. G. Gabbiani, University of Geneva, Geneva, Switzerland).[14] For cell culture experiments, primary antibodies were added after cell fixation and permeabilization, and probed with Alexa Fluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Molecular Probes, Invitrogen, Basel, Switzerland). DNA was probed with 4′6-diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, Mo.) and F-actin with Phalloidin-Alexa 488 (Molecular Probes). Phase-contrast and epifluorescence microscopy was

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performed using oil immersion objectives (Plan-Neofluar 40×/1.2 Ph3, Plan-Neofluar 63×/1.4 Ph3; Carl Zeiss AG, Feldbach, Switzerland) mounted on an inverted microscope (Axiovert 135; Carl Zeiss) and a digital charge-coupled device camera (Hamamatsu C4742-95-12ERG; Bucher Biotec AG, Basel, Switzerland). Images were acquired with Openlab 3.1.2 software (Improvision, Basel, Switzerland), and assembled with Adobe Photoshop CS3.

For immunohistochemistry of tissues, excised wounds were fixed in 4% buffered formaldehyde. Immunostaining for α-smooth muscle actin was performed on paraffin-embedded transverse sections using biotinylated goat anti-mouse immunoglobulin 2a (Jackson Immunoresearch, West Grove, Pa.) and treatment with streptavidin-biotin-peroxidase (Dako, Glostrup, Denmark). Peroxidase activity was detected with diaminobenzidine (Serva, Heidelberg, Germany) as described previously.[15] Software-aided quantification of α-smooth muscle actin–positive cells and stress fibers was performed as described previously.[15] To evaluate α-smooth muscle actin expression as an indicator of the presence of myofibroblasts, vessels were excluded manually from the image to retain only the area of interest.

For all other stainings, paraffin-embedded sections were rehydrated and treated with 40 μg/ml proteinase K (Roche Diagnostics Corp., Indianapolis, Ind.) for 25 minutes at 37°C as described previously.[16] Primary antibodies against platelet endothelial cell adhesion molecule-1 (Pharmingen, San Jose, Calif.) were incubated at 4°C overnight. Platelet endothelial cell adhesion molecule-1 signal was intensified using the tyramide amplification system (Perkin-Elmer, Boston, Mass.). The total number of blood vessels (positive for platelet endothelial cell adhesion molecule-1) was counted using 40× magnification for three images (one from the center and two from the margins of the wound) for each sample. Six samples were used for each condition and epitope.

Cell Culture and Cell Contraction Assays

Primary human dermal fibroblasts, obtained from three different healthy donors, were grown from dermal explants and used between passages 2 and 5 as described previously.[17] Cells were seeded onto collagen-coated (collagen type I, 10 μg/ml; Sigma) glass coverslips at 5000 cells/cm^2. Experiments were performed with fibroblasts from at least three different human samples from discarded tissues according to an approved ethical protocol of our hospital. Fibroblast cultures were treated on the first day with 3×10^5 activated or nonactivated platelets per milliliter of media and compared with control medium (Dulbecco's Modified Eagle Medium; Gibco-Invitrogen Basel, Switzerland) with 20% fetal calf serum (HyClone; Thermo-Fisher Scientific, Waltham, Mass.) for 1 to 5 days. Platelet preparations were added onto the collagen-coated surface before cells and left to incubate 15 minutes with the fibroblasts before addition of media.

We observed that fibroblasts formed membrane extensions shortly after contact with platelets that delimitated vessel-like structures. The number of cells contributing to confluent structures was counted per high-power field in three representative areas. Wrinkling silicone substrates (Excellness Biotech SA, Lausanne, Switzerland) were used to identify individual contractile cells.[18] Substrates were used with a stiffness of 10 kPa to restrict wrinkle formation to highly contractile α-smooth muscle actin–positive cells. Substrates were rendered cell-adhesive with collagen type I (10 μg/ml; Sigma), and stiffness was controlled by atomic force microscopy (NanoWizard II; JPK Instruments, Berlin, Germany).[19] As another contraction test, fibroblasts were grown in attached three-dimensional collagen lattices.[15] Populations were initiated with 0.25 to 10.0×10^5 cells/ml collagen type I (1.0 mg/ml; Sigma) adapted to obtain similar cell numbers at the time of contraction measurement after 24 hours of growth.

In Vitro Wound Healing Assay

To characterize the interactions between platelet preparations and fibroblasts in an in vitro wound healing assay, modified fibroblast-populated collagen gels (1.75×10^5 cells/ml) were prepared with a circular hole in the center to simulate a tissue defect. The hole was created by placing a 5-mm-diameter tube in the middle of a 35-mm culture dish while adding 400 μl of collagen solution in acetic acid. The tube was removed after gels were polymerized under neutralization with sodium hydroxide. Activated or nonactivated platelets (100 μl) were then added to fill the hole and were left to incubate before the addition of media for 15 minutes. Fibroblast growth into the tissue defect was assessed morphologically every day using contrast-phase microscopy for 21 days.

Statistical Analysis

All values are expressed as mean ± SD. One-way analysis of variance and ad hoc least signif-

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icant difference variance analysis were used to determine the significance of differences between treatment modes. Multivariate analysis was performed using Statistica version 7.0 (StatSoft, Inc., Tulsa, Okla.). A value of $p < 0.05$ was considered significant.

RESULTS

Application of Platelets Accelerates Healing of Diabetic Mouse Wounds

To test the differences between activated and nonactivated platelets on experimental wound healing, we generated full-thickness skin defects on the dorsa of diabetic mice. Human platelets were used to obtain single donor samples with standardized levels of activation and cytokines. As published previously, single topical doses of human platelets applied to mouse wounds did not induce any aberrant immunologic response.[8] Single topical application of nonactivated platelets improved wound contraction after 7 days (40 percent; $p < 0.05$) compared with treatment with activated platelets (25 percent) and untreated wounds (15 percent) (Fig. 1, *left*). Both activated and nonactivated platelets induced similar levels of reepithelialization (15 percent and 8 percent,

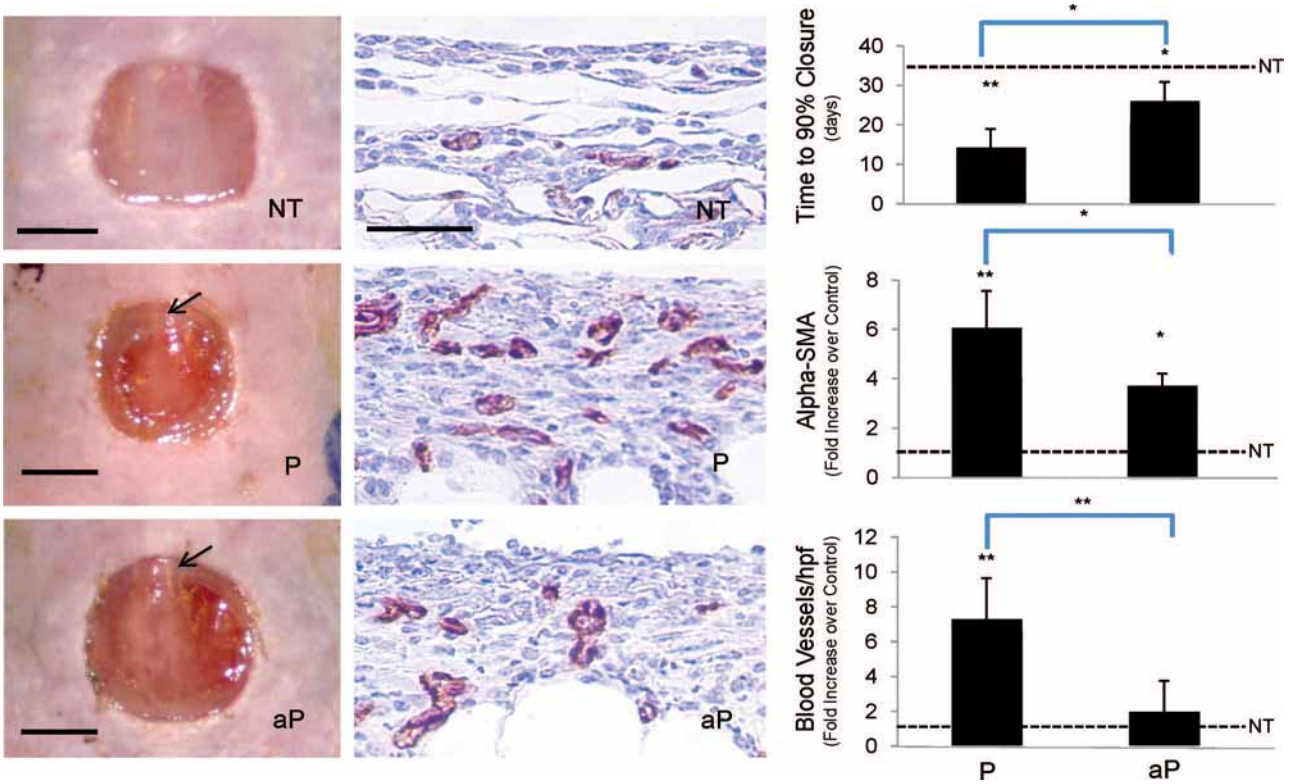

Fig. 1. Diabetic mouse full-thickness wound healing. (*Left*) Appearance of full-thickness wounds 7 days after wounding either healing spontaneously (*NT*), or treated with nonactivated platelets (*P*) or activated platelets (*aP*). In the early phase of healing, nonactivated platelets stimulated wound contraction (40 percent, $p < 0.05$) compared with activated platelets (25 percent) and untreated wounds (15 percent). Reepithelialization (*arrows*) was similarly induced by both nonactivated and activated platelets (15 percent and 8 percent, respectively) compared with nontreated wounds ($p < 0.05$), where only scarce signs of reepithelialization were noted. *Scale bar* = 0.5 cm. (*Center*) Platelet endothelial cell adhesion molecule-1 expression in healing wounds on day 7: increased levels of angiogenesis were found in platelet-treated wounds compared with nontreated and activated platelet treatment. *Scale bar* = 50 μm. (*Above, right*) In the later phase of healing, nonactivated platelet treatment induced faster wound closure, reaching 90 percent wound closure within 15 days from wounding, whereas activated platelet–treated wounds needed 26 days ($p < 0.01$ compared with platelets). Untreated wounds needed more than 35 days to reach 90 percent wound closure. (*Center, right*) α-Smooth muscle actin expression in granulation tissues on day 7 was augmented in both activated (3.8-fold) and nonactivated platelet (6-fold) treated wounds compared with control wounds. Platelets induced a 1.5-fold higher α-smooth muscle actin expression in wounds over activated platelet treatment ($p < 0.05$) (*center, below*). (*Below, right*) Blood vessel density in 7-day-old granulation tissue was 3.7-fold higher after platelet treatment than after treatment with activated platelets and 7.3-fold higher than untreated wounds. *$p < 0.05$, **$p < 0.01$, compared with untreated or activated platelet-treated wounds.

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respectively) (Fig. 1, *left*), whereas untreated wounds ($p < 0.05$) showed only scarce signs of epithelium. In the later phases of wound healing, platelets significantly accelerated wound closure compared with activated platelets and untreated wounds. Nonactivated platelet-treated wounds reached 90 percent wound closure 1.7 times faster (day 15) compared with wounds treated with activated platelets (26 days; $p < 0.05$) (Fig. 1, *above*, *right*) and 2.3 times faster than untreated wounds (35 days; $p < 0.01$) (Fig. 1, *above*, *right*). Wounds treated with activated platelets reached 90 percent wound closure 1.3 times faster than untreated wounds ($p < 0.05$) (Fig. 1, *above*, *right*).

Nonactivated Platelets Induced Higher Expression of α-Smooth Muscle Actin and Angiogenesis in Healing Wounds than Activated Platelets

To test whether enhanced wound contraction is related to higher occurrence of contractile myofibroblasts, we assessed expression of α-smooth muscle actin in cross-sections of wounds on day 7. Wounds treated with activated and nonactivated platelets both exhibited increased levels of α-smooth muscle actin expression (6-fold, $p < 0.001$; and 3.8-fold, $p < 0.05$, respectively) compared with untreated wounds 7 days after wounding (Fig. 1, *center*, *right*). Platelets significantly stimulated α-smooth muscle actin expression by 1.5-fold compared with activated platelets (Fig. 1, *center*, *right*) ($p < 0.05$).

Blood vessel density was assessed by immunolocalization of platelet endothelial cell adhesion molecule-1 in the granulation tissue on day 7. The highest density (37.5 blood vessels per microscopic field) was measured in 7-day-old wounds treated with nonactivated platelets ($p < 0.05$ compared with activated platelets) (Fig. 1, *center*, *center*, and *below*, *right*). Angiogenesis was 7.3-fold higher compared with untreated wounds ($p < 0.001$, 5.1 blood vessels per image field) and 3.7-fold higher compared with activated platelet wounds (10 blood vessels per image field, $p < 0.05$) (Fig. 1, *center*, and *below*, *right*).

Nonactivated Platelets Induce an Areolar Organization of Fibroblasts and Stimulate Expression of α-Smooth Muscle Actin In Vitro

To understand the role of platelets in fibroblast-to-myofibroblast differentiation in wound healing, human dermal fibroblasts were cultured on two-dimensional collagen-coated glass coverslips in control media and in the presence of activated or nonactivated platelets. Comparable numbers of fibroblasts were quantified in control media and platelet treatments (106 and 88 cells per image field at 10× magnification, respectively), which were found to be significantly higher ($p < 0.05$) compared with activated platelet treatment (42 cells per image field).

In control media, fibroblasts grew separated and tended to become confluent over a period of 5 days with random spatial distribution. In the presence of activated platelets, fibroblasts developed nonorganized dendritic appendages starting from 24 hours (Fig. 2, *above*), whereas nonactivated platelet treatment induced a more organized blood vessel–like pattern, formed by the interdigitation of the dendritic appendages of neighboring cells (Fig. 2, *above*). Notably, already 24 hours after platelet treatment, the formation of long dendritic, syncytial structures interconnecting adjacent fibroblasts was noted (Fig. 2, *above*, *left*, and *right*). These blood vessel–like structures were found to be increased 10-fold compared with control and activated platelet conditions ($p < 0.01$).

The level of myofibroblast differentiation in culture was quantified by immunofluorescence staining for α-smooth muscle actin and cell counting. After 5 days of culture, nonactivated platelets most efficiently induced myofibroblast differentiation, with up to 57.3 percent α-smooth muscle actin–positive cells compared with control media (21.5 percent) and activated platelet treatment (29.6 percent; $p < 0.01$) (Fig. 2, *left*, *center*). We further assessed the contractile function of fibroblasts by testing their ability to generate wrinkles on the surface of soft silicone culture substrates 24 hours after treatment. Platelets induced a higher amount of wrinkling and contractile cells (35 percent; $p < 0.05$) compared with control media (20 percent) or activated platelets (10 percent) (Fig. 2, *below*, *left*, *arrows*).

Nonactivated Platelets Stimulate Fibroblast Migration into In Vitro Extracellular Matrix Defects

To finally test the effect of platelets on the capacity of fibroblasts to fill a "tissue defect," we prepared fibroblast-populated, donut-shaped collagen gels (Fig. 3, *left*). Activated or nonactivated platelets were then added to the fibroblast- and extracellular matrix–free central space. Activated platelets formed a clot (Fig. 3, *center*) where virtually no fibroblasts were found for up to 21 days of culture (not shown). In contrast, when nonactivated platelets were added, fibroblasts actively

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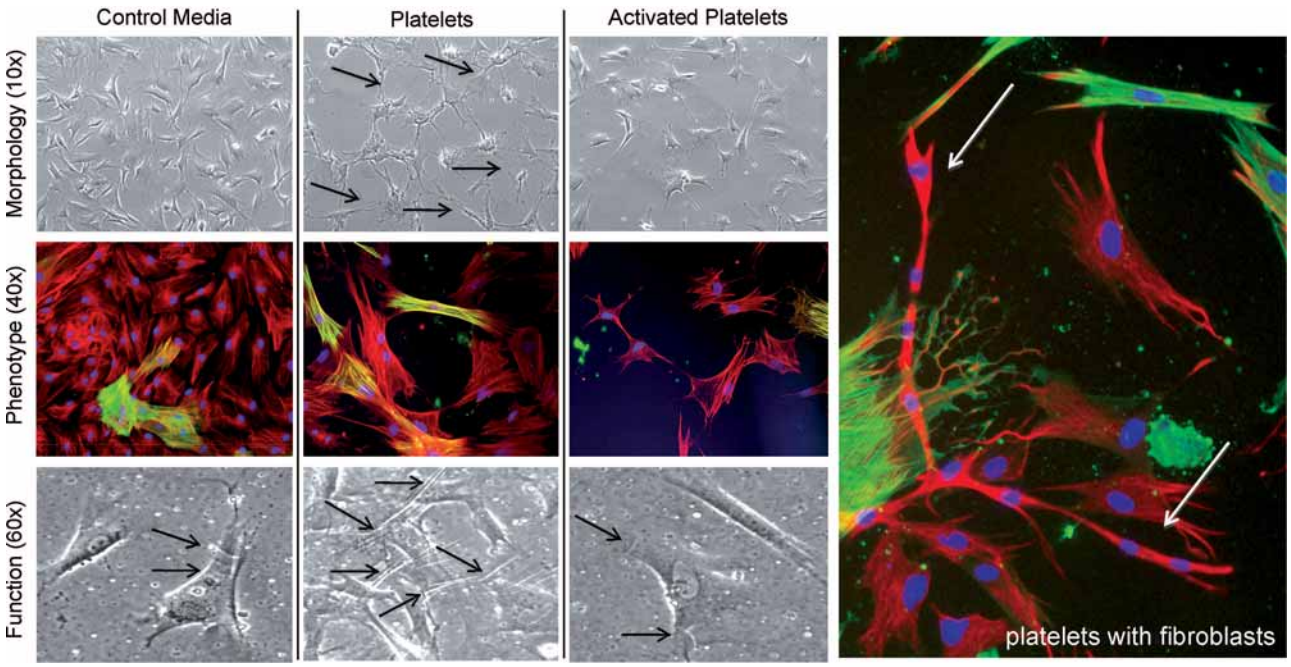

Fig. 2. Effects of platelet treatment on fibroblast cultures. Microscopic images show cultured human fibroblasts in three different experimental conditions: the *left column* shows fibroblasts in control medium, the *second column* shows fibroblasts treated with nonactivated platelets, and the *third column* shows fibroblasts treated with activated platelets. Three parameters are presented: fibroblast morphology, myofibroblast phenotype, and contractile function. (*Above*, *left*) Image demonstrating morphology: 24 hours after seeding, fibroblasts showed a characteristic distribution when cultured with nonactivated platelets, forming extracellular areolar blood vessel–like structures. Cells cultured in control media or activated platelets showed the usual pattern of cell adherence with random orientation. (*Right*) High magnification (60×) image of platelet–fibroblast co-cultures after 5 days immunostained with α-smooth muscle actin (*green*), vimentin (*red*), and nuclei (*blue*). Dendritic structures of fibroblasts are marked with *white arrows*. The connection of these structures formed a tubular-like architecture. (*Second row of small images, across*) Images demonstrating phenotype: fluorescence microscopic images show α-smooth muscle actin–stained myofibroblast (*green*) and cytoskeletal staining (F-actin, *red*) and their respective nuclear staining (4′6-diamidino-2-phenylindole, *blue*) after 5 days of culture. Nonactivated platelets induced increased levels of α-smooth muscle actin (57.3 percent, $p < 0.01$) compared with control media (21.5 percent) and activated platelets (29.6 percent). (*Below*) Images demonstrating function: the contractile activity of human dermal fibroblasts was assessed by the observation of soft substrate wrinkling induced by cells in cell cultures treated by control media, platelets, and activated platelets. Wrinkling was augmented after platelet (35 ± 3 percent, $p < 0.05$) compared with activated platelet treatment (10 ± 5 percent) and cells in control medium (20 ± 7 percent). *Arrows* indicate wrinkling of the substrate by contractile cells.

migrated into the center (Fig. 3, *right*) from the first day of culture. Fibroblasts that migrated into the center after platelet treatment survived up to 21 days after seeding as tested by light microscopy (not shown).

DISCUSSION

Platelets, commonly preactivated, are widely used for nonhealing wounds as a cost-effective source of growth factors.[4] In this study, we first compared the effect of activated versus nonactivated platelets in multiple experimental settings. Our in vivo and in vitro results show that thrombin activation causes partial loss of the platelets' healing properties. Platelets promoted better healing than activated platelets by stimulating (1) more efficient wound contraction and faster wound closure, (2) fibroblast migration and differentiation into contractile myofibroblasts, and (3) formation of capillary-like structures by fibroblasts in vitro, which corresponded to enhanced angiogenesis in vivo.

Blood vessels provide the infrastructure for nutrition and cell delivery to the wound. Angiogenesis occurs by the sprouting of endothelial cells into the new forming tissue. It is known that dermal fibroblasts regulate blood vessel formation through the expression of matrix metalloproteinases (e.g., membrane-type matrix metalloproteinase 1) or other cytokines and growth factors (e.g.,

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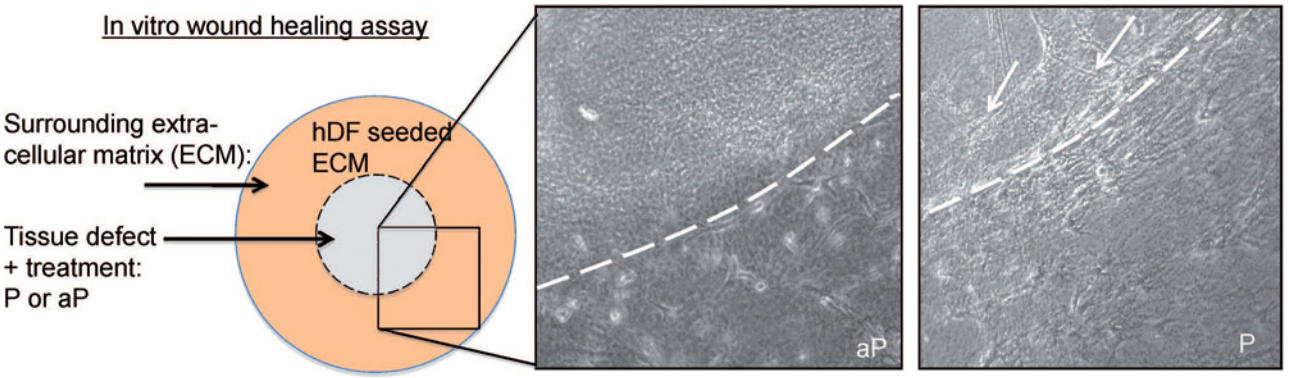

Fig. 3. In vitro three-dimensional tissue defect assay. (*Left*) The center of a donut-shaped fibroblast-populated collagen gel was filled with either nonactivated or activated platelets. Light microscopic photographs of the in vitro wound healing assay on day 7 with (*center*) activated platelets (*aP*) or (*right*) nonactivated platelets (*P*) are shown. Platelets allowed fibroblast migration into the tissue defect (*arrows*). When thrombin was added to platelets (*aP*), a clot formed in the center, which prevented the migration of cells up to day 7 (no cells beyond the wound margin). The *dotted line* indicates the margin between the original collagen defect and fibroblast-seeded center. Fibroblasts that migrated into the platelet-filled center exhibited typical long dendritic extensions and areolar distribution.

tissue inhibitor of matrix metalloproteinase 1 and VEGF-C) in tumors and healing wounds.[20–22] In this study, we observe for the first time in vitro that interactions of platelets with fibroblasts, as early as 24 hours after culture, generate capillary-like structures in the absence of endothelial cells. It is possible that these structures formed by fibroblasts in the maturing extracellular matrix are the first event that is necessary to physically guide endothelial cells to form new blood vessels. This view is supported by a sentinel function of fibroblastic cells played during tumor vascularization.[23] Interestingly, these dendritic structures were characteristically α-smooth muscle actin–negative, possibly to prevent the obliteration of the intravascular space by contraction before endothelial cell migration, in the early phases of angiogenesis. More studies are required to further investigate the importance of fibroblasts and platelets as primary "angioarchitects" during normal wound healing.

It is becoming increasingly evident that platelets have a function in wound healing that goes beyond their role in hemostasis. Recent fundamental work showed that antiangiogenic factors such as endostatin and proangiogenic factors such as VEGF are both stored in platelets but released independently and antagonistically on specific receptor interaction (proteinase activated receptor-4 for endostatin and proteinase activated receptor-1 for VEGF).[24,25] Thrombin (factor II), the

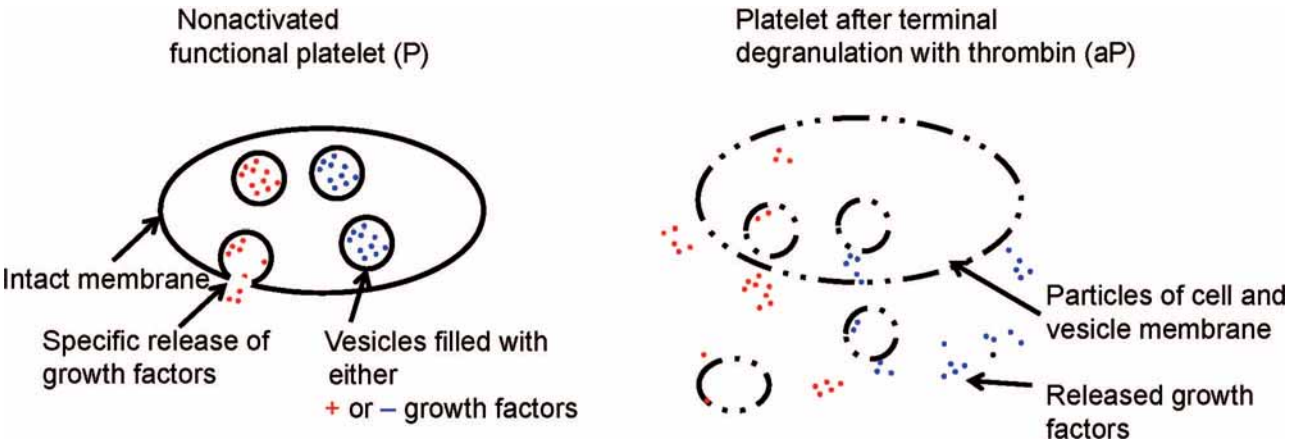

Fig. 4. Schematic depiction of the effect of physiologic and unspecific activation of platelets on wound healing. (*Left*) Nonactivated platelets represent a drug delivery system controlled by specific release of proangiogenic and antiangiogenic factors. (*Right*) Activated platelets release high doses of both stimulatory and inhibitory factors, with counterregulatory effect.

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most commonly used factor to preactivate platelets for wound healing, activates both receptors nonspecifically, leading to a potential contemporary release of stimulatory and inhibitory growth factors at once.[9,26] Because the growth factor content was standardized between activated and nonactivated platelets (same quantity from single-donor samples), the differences in the effect may be attributed to the way the growth factors were presented to the cells (Fig. 4). Although activated platelets delivered all the growth factors (proangiogenic and antiangiogenic) in a concentrated bolus, nonactivated platelets likely released growth factors more specifically, on receptor interactions.[6,27] The possibility of harvesting platelet drug delivery ability for wound healing is a fascinating hypothesis that deserves to be further supported by additional mechanistic studies.

In the effort to best harvest the properties of platelet therapy for wound healing, activation is not the only variable that should be studied. Several other aspects should be considered, such as the activation of platelet aggregation (calcium) or the protocol for platelet preparation (centrifugation and final concentration) and topical application (alone or with associated specific dressing).

CONCLUSIONS

Our results support the use of nonactivated physiologic platelets for wound healing, to best activate fibroblast differentiation and migration, promote angiogenesis, and ultimately stimulate healing. Future studies will concentrate on the characterization of growth factor delivery by nonactivated platelets during wound healing.

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