Thrombin-activated platelets induce proliferation of human skin fibroblasts by stimulating autocrine production of insulin-like growth factor-1

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ABSTRACT **Platelet components have found successful clinical utilization to initiate or to accelerate tissue-repair mechanisms. However, the molecular pathways by which platelet factors contribute to tissue regeneration have not been fully elucidated. We have studied the effect of thrombin-activated platelets (TAPs) on cell growth *in vivo* and in cultured cell systems. Application of TAPs to ulcerative skin lesions of diabetic patients induced local activation of ERK1/2 and Akt/PKB. Moreover, when applied to cultured human skin fibroblasts, TAPs promoted cell growth and DNA synthesis and activated platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF)-1 receptor tyrosine kinases. PDGF was released by TAPs and rapidly achieved a plateau. At variance, the release of IGF-1 was mainly provided by the TAPs-stimulated fibroblasts and progressively increased up to 48 h. The PDGF-R blocker Ag1296 reduced the activation of Akt/PKB and, at a lesser extent, of ERK1/2. Conversely, inhibition of IGF-1 signaling by Ag1024 and expression of a dominant-negative IGF-1R mutant selectively reduced the stimulation of ERK1/2 by TAPs and fibroblast-released factors, with minor changes of Akt/PKB activity. Thus, platelet factors promote fibroblast growth by acutely activating Akt/PKB and ERK1/2. Sustained activation of ERK1/2, however, requires autocrine production of IGF-1 by TAPs-stimulated fibroblasts.—Giacco, F., Perruolo, G., D'Agostino, E., Fratellanza, G., Perna, E., Misso, S., Saldalamacchia, G., Oriente, F., Fiory, F., Miele, C., Formisano, S., Beguinot, F., Formisano, P. Thrombin-activated platelets induce proliferation of human skin fibroblasts by stimulating autocrine production of insulin-like growth factor-1. *FASEB J.* 20, E1763–E1772 (2006)**

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WOUND HEALING REQUIRES a concerted interplay of numerous cell types, modulated by soluble factors, extracellular matrix (ECM), and blood elements (1, 2). The initial phase of the healing is represented by the hemostatic process triggered by the activation of the clotting cascade. Platelets then appear to be major players in tissue repair for their mechanical and releasing properties (3). Alterations in platelet number or function are indeed associated to abnormal repair mechanisms (4).

Since their first appearance in clinical studies (5), autologous platelet factors have been considered as useful therapeutic tools to accelerate tissue repair and regeneration. More recently, a blood component generated by thrombin-activated platelets (TAPs) and usually identified as platelet gel (PG) or platelet releasate (PR) has become available (6). Current therapeutic application of PG includes a number of pathological conditions requiring bony and soft tissue reconstruction (7–9). In fact, beside the important hemostatic functions, the release of cytokine and growth factors by activated platelets is apparently crucial in increasing the probability of healing and in reducing the healing time (10). Among platelet-released factors, several growth factors (including PDGF-PDGF, vascular endothelial growth factor (VEGF)-VEGF and IGF-IGF-1) initiate their cellular action by binding to and activating specific receptor tyrosine kinases (RTKs). Following tyrosine auto- and transphosphorylation, these early events selectively induce the formation of multimolecular complexes of proteins involved in the regulation of cell survival and proliferation, including the pleiotropic kinases of the Akt/protein kinase B (PKB) and of the extracellular signals-regulated kinase (ERK) families (11, 12).

Attention has been given to the clinical utilization of individual growth factors, such as PDGF (13). The overall experience with recombinant PDGF in wound

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healing has not been completely successful (1, 14–16). This is not surprising, however, given the consideration that wound healing is the outcome of an intricate network of circulating and tissue elements. The complexity is linked, at least in part, to the combination of the released growth factors, the timing of their release by platelets, and the cell-specific response to individual growth factors or combinations of them. These issues need to be explored further to achieve a better understanding of the therapeutic potential of TAPs. Indeed, despite the large utilization of platelet components as therapeutic tools in pathologies requiring tissue repair, the detailed molecular events underlying this beneficial effect are poorly defined.

In this paper, we have analyzed *in vivo* and in cultured cell models the molecular mechanisms responsible for the growth-promoting action of TAPs. We have shown that PDGF and IGF-1 play a concerted role on cell survival and proliferation of human fibroblasts. While PDGF is sufficient to promote Akt/PKB activation, TAPs-induced fibroblast secretion of IGF-1 is required for the sustained activation of ERK1/2 and the completion of the proliferation program.

MATERIALS AND METHODS

Materials

Media, sera, and antibiotics for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit polyclonal ERK 1/2 antibodies and rabbit polyclonal Akt antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies toward the phosphorylated forms of the Akt/PKB and ERK 1/2 were from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal IGF-1R and phospho-tyrosine antibodies and rabbit polyclonal PDGF-R antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). Western blotting, enhanced chemiluminescence (ECL) reagents and radiochemicals were from Amersham Biosciences (Piscataway, NJ, USA). Electrophoresis reagents were from Bio-Rad (Milan, Italy). All other reagents were from Sigma (St. Louis, MO, USA).

Cell culture and cell growth

Shoulder skin fibroblasts were obtained by punch biopsy and cultures established as described previously (17). The cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum in a 5% CO_2–95% air-humidified atmosphere. Cultures were used for experimental procedures between the 8th and 15th passage, and, for each individual experiment, the cells were maintained in culture for an equal number of generations. Parental NIH-3T3 cells and those over-expressing IGF1R or IGF1R-DK have been kindly provided by Dr. D. LeRoith and cultured as described previously (18).

Platelet-rich plasma and platelet gel

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP; <3000 plts/μl) were obtained from healthy donor volunteers (for *in vitro* experiments) and from diabetic patients undergoing autologous platelet gel (PG) treatment for ulcers of the lower extremities. Pertinent clinical features of the patients and criteria for the assignment to the study group treated with standard care plus PG have been previously reported (19). The preparation procedures for platelets were performed according to standardized methods (20, 21). For PG preparations, autologous thrombin (0.1 NIH unit/ml final concentration) and calcium gluconate (10 mg/ml final concentration) were added to PRP or to PPP (in control experiments) for 5 min at room temperature to allow clot (PG) formation. For the treatment of the patients, the volumes were dictated by the size of the lesions and the applications were repeated twice a week. For *in vitro* experiments, 1 cm^2 aliquots of the PG clot were applied to cells plated in 100 mm diameter dishes.

Thymidine incorporation

Cells (10^5/plate) were seeded in 1 ml of DMEM supplemented with 10% FBS in 6-well plates. After incubation for 24 h at 37°C, the medium was removed and replaced with DMEM containing 0.25% BSA and no serum. After additional 24 h, the medium was removed again and replaced with DMEM, 0.25% BSA, and platelet gel. Incubation was prolonged for additional 16 h, and the incubation media were replaced with the same media supplemented with [^{3}H]thymidine (500 nCi/ml). After 1-h incubation, media were removed and cells were washed three times with ice-cold PBS. The cell monolayers were solubilized in 1 ml of SDS (0.1%) solution for 30 min at 37°C. An equal vol of 20% trichloroacetic acid was added to the detergent extract, and radioactivity in the trichloroacetic acid precipitate was measured by liquid scintillation counting after solubilization of the pellet in 1 N NaOH (0.3 ml).

Western blot

For Western blotting, the cells were solubilized in lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 4 mM EDTA; 10 mM Na_4PO_7; 2 mM Na_3VO_4; 100 mM NaF; 10% glycerol; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 100 μg of aprotinin/ml; 1 mM leupeptin) for 60 min at 4°C. Cell lysates were clarified at 5000 *g* for 15 min. Solubilized proteins were then separated by SDS-PAGE and transferred onto 0.45 μm-pore-size Immobilon-P membranes (Millipore, Bedford, MA, USA). On incubation with the primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions.

PDGF and IGF-1 determination

PDGF and IGF-1 levels in the extracellular media were measured by Human Quantikine Elisa kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

ERK1/2 activity

ERK1/2 activity was assayed as described previously (22). Briefly, cell lysates (200 μg of protein/assay) were immunoprecipitated with ERK 1/2 antibodies and then incubated with protein A-Sepharose for 2 h. Immobilized ERK 1/2 was washed three times with ice-cold TAT buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 10 mM EDTA; 10 mM $Na_4P_2O_7$; 2 mM Na_3VO_4; 10% glycerol; 1% Triton X-100), twice more with HNTGVa buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 2 mM Na_3VO_4; 10% glycerol; 1% Triton X-100) and then resuspended in HNTGVa supplemented with 60 mM magnesium acetate, 30 μM ATP, 6 mM dithiothreitol, 1 μg/ml myelin basic protein, and 0.5 μCi of [γ-^{32}P]ATP. On incubation for 30 min at 25°C, reaction mixtures were spotted on

phosphocellulose discs and washed three times with 1% (v/v) phosphoric acid and once more with ethanol. Disc-bound radioactivity was quantitated by liquid scintillation counting.

Akt/PKB activity

Akt/PKB activity was assayed *in vitro* as previously reported (23). Briefly, the cells were solubilized in lysis buffer and lysates were clarified by centrifugation at 5000 *g* for 20 min. The lysates (200 μg) were immunoprecipitated with Akt/PKB antibodies. The precipitates were incubated in a kinase reaction mixture containing 20 mM HEPES, pH 7.2; 10 mM $MgCl_2$; 10 mM $MnCl_2$; 1 mM dithiothreitol; 5 mM ATP; 0.2 mM EGTA; 1 mM protein kinase inhibitor; 10 μCi of [γ-^{32}P]ATP; and recombinant GSK-3 as substrate. Phosphorylation reactions were prolonged for 10 min, stopped by cooling on ice, and spotted on phosphocellulose disk papers. Disks were washed with 1% H_3PO_4, and disk-bound radioactivity was quantified by liquid scintillation counting.

RESULTS

In vivo activation of Akt/PKB and ERK1/2 by thrombin-activated platelets

Thrombin-activated platelets are commonly used in the treatment of ulcerative skin lesions and in other processes requiring wound healing and tissue regeneration (3, 6). Seven consecutive diabetic patients, with grade II/III ulcers at the lower extremities according to Wagner (24), were treated with local applications of TAPs. Pertinent clinical data of participants have been previously described (19). After 2 weeks of treatment, the ulceration size was reduced by ~40%. To elucidate the molecular mechanisms by which TAPs induce cell growth, the activation of Akt/PKB and ERK1/2, key mediators of survival and proliferation signals, has been investigated *in vivo*. Specimens derived from peri-lesional biopsies, taken before (day 0) and after (weeks 1 and 2) repeated topic applications of TAPs, were solubilized and probed with antibodies against phosho-Ser-473-Akt/PKB and phospho-Thr (202)/Tyr (204)-ERK1/2 (**Fig. 1**). Both Akt/PKB (Fig. 1*A*) and ERK1/2 phosphorylation (Fig. 1*B*) were increased on 1 wk of treatment, by 3- and 2.5-fold, respectively. At week 2, ERK1/2 phosphorylation levels were significantly higher than those detected at week 1 ($P<0.05$). No change of Akt/PKB and ERK1/2 total levels was observed, however. At variance, Akt/PKB activation at week 2 was similar to that obtained at week 1 and still higher than that at day 0. No significant changes of Akt/PKB and ERK1/2 phosphorylation were observed in bioptic samples obtained by lesions from patients treated with standard care (data not shown). Thus, topic application of TAPs increases local activation of Akt/PKB and ERK1/2, enzymes involved in growth-related events.

In vitro effect of thrombin-activated platelets on human fibroblasts cell growth

To analyze the molecular details of TAPs-induced cell growth, increasing concentrations of human platelets

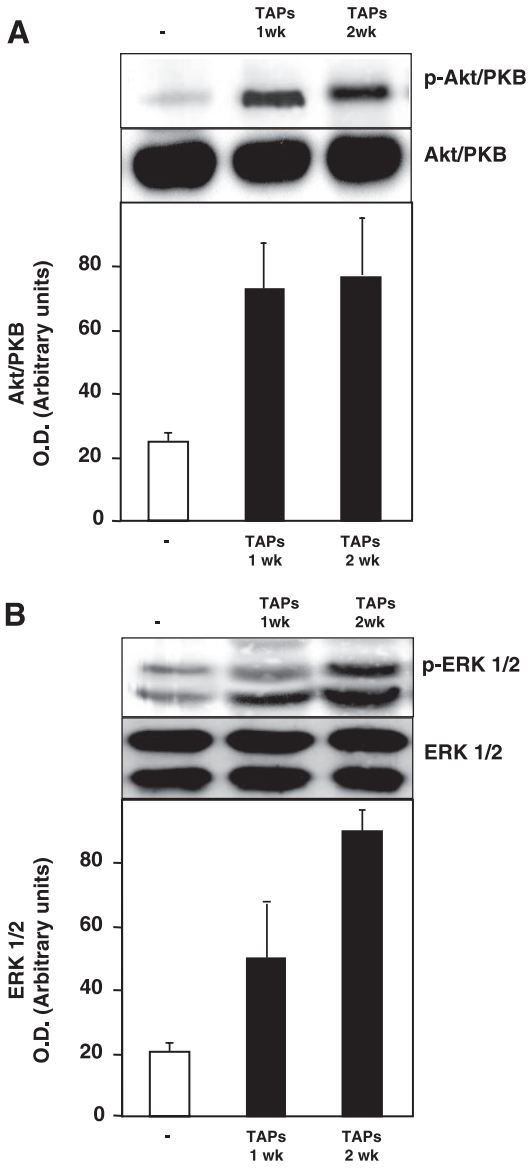

Figure 1. Effect of TAPs on ERK 1/2 and Akt/PKB in peri-lesional skin biopsies. PG was applied twice a week to skin lesions of 7 consecutive diabetic patients, diagnosed with grade II/III ulcers. Peri-lesional skin biopsies were taken at indicated time. The specimens were solubilized and immunoblotted with specific antiphospho antibodies (Akt/PKB, panel *A* and ERK1/2, panel *B*). The bar graphs represent means ± SD of the densitometric values of the bands obtained by four independent experiments (n=7). The autoradiographs in the upper insets show representative experiments.

(from 0.2×10^9 to 1×10^9) were activated with thrombin and applied in gel form (1 cm^2) to the surface of dishes containing cultured human fibroblasts (1×10^5) derived from skin biopsies of healthy volunteers. The application of TAPs to the dish was sufficient to induce cell growth in the absence of serum in a concentration-dependent fashion (**Fig. 2*A***). When treated with 1×10^9 TAPs, the cells became confluent after 4 d. Fibroblasts cultured in serum-free media detached from the plate starting from day 2. In contrast, TAPs addition progressively increased the number of cells. Indeed, the growth curves were comparable to those achieved

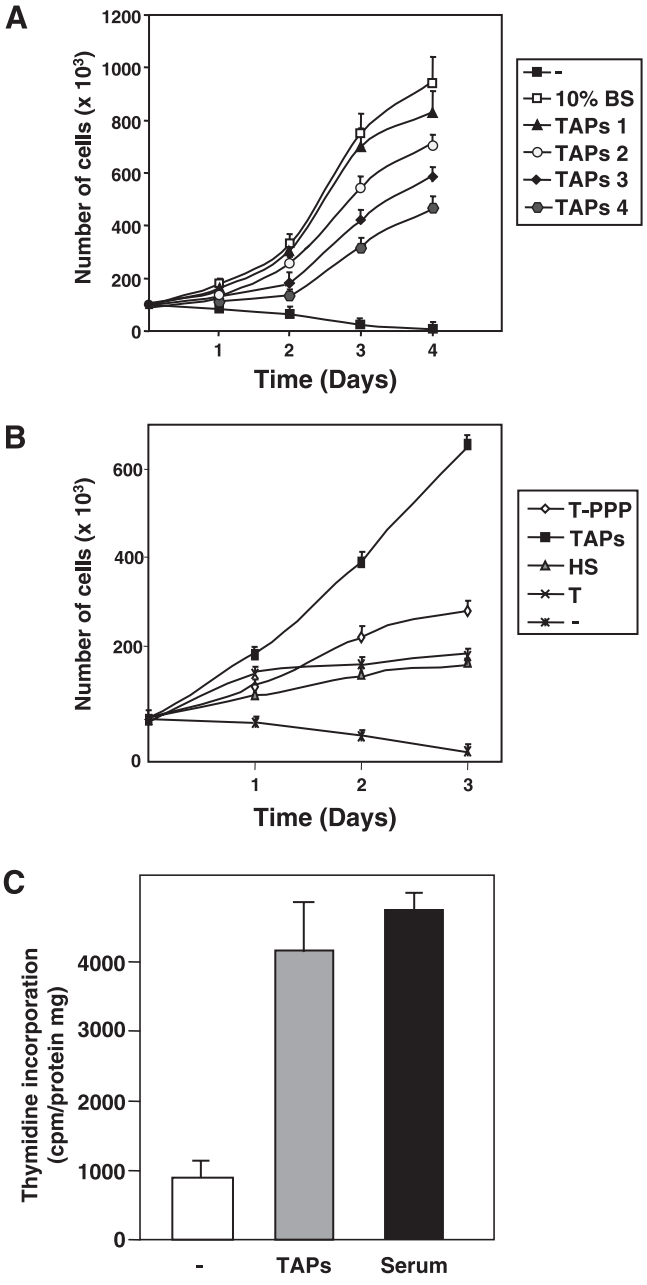

Figure 2. Effect of TAPs on cell growth in cultured human fibroblasts. *A*) Cells (10^5) were plated and supplied with complete medium (10% BS) or serum-free media or were serum-free (–). Where indicated, a 1 cm^2 aliquot of TAPs (TAPs $1=1.0\times10^9$; TAPs $2=0.75\times10^9$; TAPs $3=0.5\times10^9$; TAPs $4=0.2\times10^9$) obtained as described in Materials and Methods, has been added to the serum-free medium. At the indicated times, the cells were trypsinized and counted. Data represent means ± SD of six independent experiments in duplicate. *B)* Alternatively, equal amounts of cells were placed in serum-free media (–) and supplied with thrombin-treated platelets' poor plasma (T-PPP), thrombin-activated platelets (TAPs; 1.0×10^9), 10% human serum, and thrombin (T; 0.5 NIH U), as indicated. At the indicated times, the cells were trypsinized and counted. Data represent means ± SD of three independent experiments in duplicate. *C)* Six-well plates were seeded with 10^5 cells/plate in 1 ml complete medium. After incubation for 24 h at 37°C, the medium was removed and replaced with DMEM containing 0.25% BSA and no serum. After an additional 24 h, the medium was removed again and replaced with complete DMEM or with DMEM 0.25% albumin with or without TAPs. Incubation was prolonged for an additional 16 h, and the incubation media were replaced with the same media supplemented with [^{3}H]thymidine (500 nCi/ml). After 1 h, media were removed and thymidine incorporation was measured as described in Materials and Methods. The bar graph represents means ± SD of three independent experiments in triplicate.

in the presence of 10% FBS (Fig. 2*A*). Alternatively, cells were exposed to equal volumes of thrombin-treated platelets' poor plasma (T-PPP), serum (10% final concentration), or thrombin (0.1 NIH U/ml) derived from the same platelets donors (Fig. 2*B*). In each of those conditions, however, human fibroblasts featured a significantly slower growth profile, compared to that stimulated by TAPs.

TAPs-treated cells also displayed a 4-fold increase in thymidine incorporation into DNA, as compared to untreated cells ($P<0.001$), further indicating a growth-promoting action of TAPs (Fig. 2*C*).

In vitro effect of thrombin-activated platelets on Akt/PKB and ERK1/2 activation

Based on immunoblot with phospho-specific antibodies and on kinase activity assays, the application of TAPs to cultured human fibroblasts enhanced phosphorylation and activity of both Akt/PKB and ERK1/2 (**Fig. 3**). Akt/PKB phosphorylation peaked at 15 min, then remaining stable up to 48 h (Fig. 3*A*). TAPs-induced phosphorylation of ERK 1/2 was equally rapid but, at variance with Akt/PKB, further increased in a time-dependent manner (Fig. 3*B*). Comparable results were obtained by measuring Akt/PKB and ERK1/2 activities (Fig. 3*C*).

To address whether the activation of Akt/PKB and/or ERK1/2 was required for the proliferative effect, TAPs-induced DNA synthesis was measured in the presence of LY294002 (an inhibitor of the PI3K-PKB/Akt pathway) and PD98059 (an inhibitor of the MEK-ERK1/2 pathway). Thymidine incorporation was reduced by 40% and 50%, respectively, by the pretreatment of the fibroblasts with 100 μM LY294002 and 50 μM PD98059 (**Fig. 4**), indicating that those molecular pathways are involved in TAPs-induced cell growth.

Activation of tyrosine kinase receptors by thrombin-activated platelets

Both Akt/PKB and ERK1/2 are often engaged by mechanisms requiring tyrosine kinase signaling (10, 11). Therefore, we analyzed the pattern of tyrosine phosphorylated proteins in human fibroblasts in the absence or in the presence of TAPs (**Fig. 5*A***, left panel). Interestingly, TAPs treatment of the cultured cells rapidly (15 min) increased tyrosine phosphorylation of 185 kDa species, which remained stable up to 48 h. In addition, phosphorylation of another major 95 kDa band was detected. Tyrosine phosphorylation of the

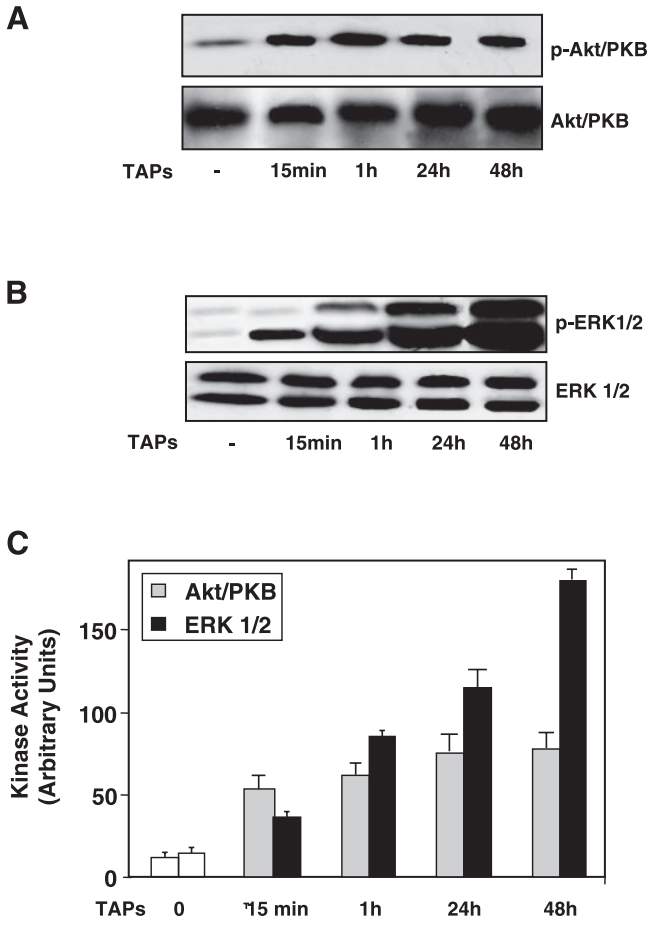

Figure 3. Effect of TAPs on ERK1/2 and Akt/PKB activation in human fibroblasts. Cells were treated with thrombin-activated platelets for the indicated time and were solubilized. Western blot with antiphospospecific ERK1/2 (*A*, upper panel) and Akt/PKB (*B*, upper panel) antibodies was performed. As control, a Western blot with anti total ERK1/2 and Akt/PKB was also performed (*A* and *B*, lower panels). Autoradiographs representative of four independent experiments are shown. The activity of both kinases was measured at the indicated time (*C*) as described in Materials and Methods. The bar graph represents the mean ± SD of three independent experiments in triplicate.

latter molecular species progressively increased in a time-dependent manner.

Platelets are a major source of growth factors, which are *bona fide* activators of tyrosine kinase receptors (TKR) (3). We then tested the effect of specific TKR inhibitors (tyrphostins) on TAPs-induced tyrosine phosphorylation and cell growth. A PDGF-R inhibitor, AG1296 (10 μM) reduced the PDGF-R 185 kDa and the IGF-1-R β-subunit 95 kDa species by 70% and 50%, respectively (Fig. 5*A*, right panel). At variance, AG1024 (10 μM), an IGF-1-R inhibitor, selectively decreased IGF-1-R β-subunit tyrosine phosphorylation. The identity of the bands was confirmed by immunoblot with PDGF-R and IGF-1-R β-subunit antibodies (data not shown). In addition, AG1296 and AG1024 reduced thymidine incorporation by 50% and 60%, respectively (Fig. 5*B*). By contrast, no effect was achieved when the cells were pretreated with 10 μM SU1498 (a VEGF-R inhibitor).

Akt/PKB and ERK1/2 activities were also measured following the pretreatment of the cells with tyrphostins (**Fig. 6**). Akt/PKB activity was reduced by >80% by AG1296 PDGF receptor kinase inhibitor, both on 15 min and on 24 h TAPs addition. No significant change was observed following AG1024 and SU1498 pretreatment (Fig. 6*A*). AG1296 only slightly reduced (~20%) the acute (15 min) activation of ERK/12. By contrast, the AG1024 inhibitor decreased by ~50% the activity of ERK1/2 induced by 15 min incubation with TAPs (Fig. 6*B*). Moreover, TAPs-mediated long-term (24 h) stimulation of ERK1/2 activity was reduced by 50% and 75%, by AG1296 and AG1024, respectively. No effect on ERK1/2 was achieved on SU1498 pretreatment of the fibroblasts (Fig. 6*B*).

Release of growth factors by platelets and fibroblasts

Because data were consistent with the hypothesis of a major involvement of PDGF and IGF-1 receptors on fibroblast growth and transduction of mitogenic signals, the concentration of these growth factors has been determined in media exposed to TAPs and to human fibroblasts, either alone or in combination (**Fig. 7**). TAPs were first applied to culture dishes with or without fibroblast monolayer; media were then removed from the culture dishes, centrifuged to remove platelets and/or cellular debris, and analyzed for growth factors content. In TAPs-derived media, PDGF-AB concentration raised in 15 min and remained stable up to 48 h (Fig. 7*A*). Almost no PDGF was detectable in media from cultured fibroblasts. PDGF levels in media from fibroblasts coincubated with TAPs

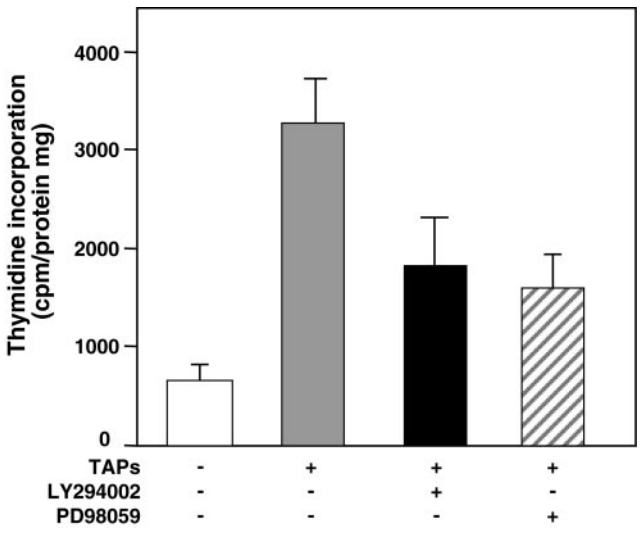

Figure 4. Role of ERK1/2 and Akt/PKB in PG-induced thymidine incorporation. Thymidine incorporation experiments were performed, as described previously in the absence or presence of 50 μM PD98059 and or 100 μM LY294002. The bar graph represents means ± SD of three independent experiments in triplicate.

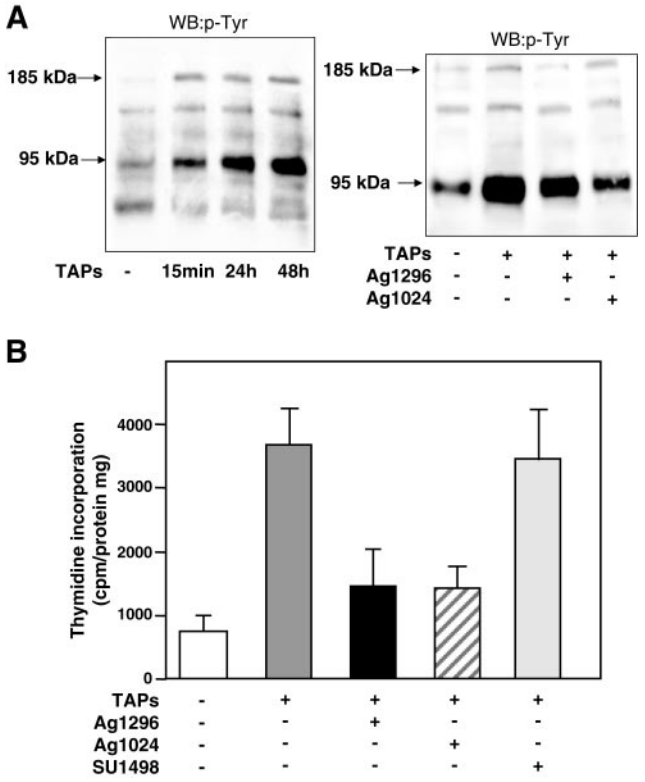

Figure 5. Effect of TAPs on receptor tyrosine kinases. *A,* left panel) Thrombin-activated platelets (TAPs) were applied to fibroblasts for the indicated times. Cells were solubilized, and Western blot with antiphosphotyrosine antibodies was performed. The experiment has been repeated six times with comparable results. The autoradiograph shows one representative experiment. *A,* right panel) TAPs were applied to fibroblasts for 24 h, in the absence or in the presence of AG1296 and AG1024. Cells were solubilized, and Western blot with antiphosphotyrosine antibodies was performed. The experiment has been repeated three times with comparable results. The autoradiograph shows one representative experiment. *B)* Thymidine incorporation experiments were performed as described previously in the absence or in the presence of specific AG1296, AG1024, or SU1498. The bar graph represents means ± SD of three independent experiments in triplicate.

were slightly lower than those determined in media from TAPs alone, and the difference was significant ($P<0.05$) at 24 h and 48 h, presumably due to the consumption of the growth factor.

IGF-1 was also detectable in TAPs media, and, at very low levels, in media derived from fibroblasts alone (Fig. 7*B*). No time-dependent changes were observed in these media, however. Different from PDGF, coincubation of fibroblasts with TAPs led to a progressive increase of IGF-1 release (the difference was significant starting from 6 h).

Effect of conditioned media on cell growth and receptor kinase signaling

Human fibroblasts were incubated for 15 min with media that have been preexposed for 24 h to either TAPs alone, or to cultured fibroblasts alone or to TAPs-stimulated fibroblasts prior to analyze protein tyrosine phosphorylation (**Fig. 8*A***). No significant change was detected on incubation of the cells with conditioned media from fibroblasts alone compared with cells incubated with serum-free media. However, tyrosine phosphorylation levels of the 185 kDa and the 95 kDa species were increased by conditioned media from TAPs and from TAPs-stimulated fibroblasts. While the phosphorylation of the 95 kDa band was increased by TAPs-conditioned media, a further 3-fold enhancement was achieved by stimulation with conditioned media from TAPs-stimulated fibroblasts. This finding is consistent with the IGF-1 levels detected in the conditioned media (see also Fig. 7*B*). Also, in parallel with PDGF levels, tyrosine phosphorylation of the 185 kDa

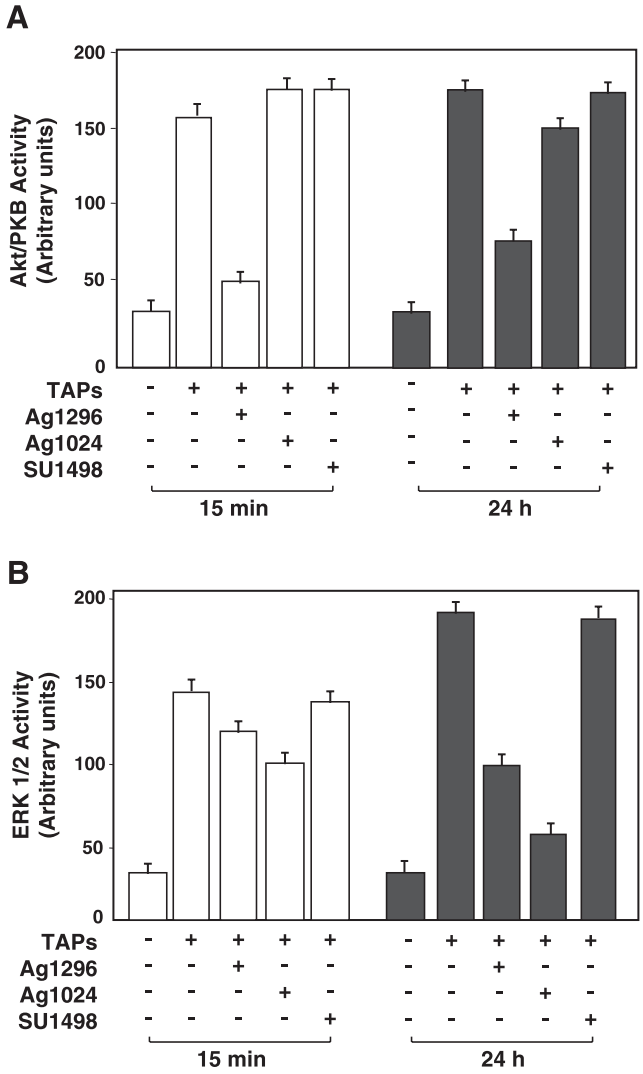

Figure 6. Effect of RTK inhibitors on ERK1/2 and Akt/PKB kinase activity. Cultured human fibroblasts were incubated for the indicated times with thrombin-activated platelets (TAPs) in the absence or in the presence of AG1296, AG1024, or SU1498. The activity of Akt/PKB (*A*) and ERK1/2 (*B*) was measured in specific immunoprecipitates as described in Materials and Methods. The bar graphs represent the means ± SD of three independent experiments in triplicate.

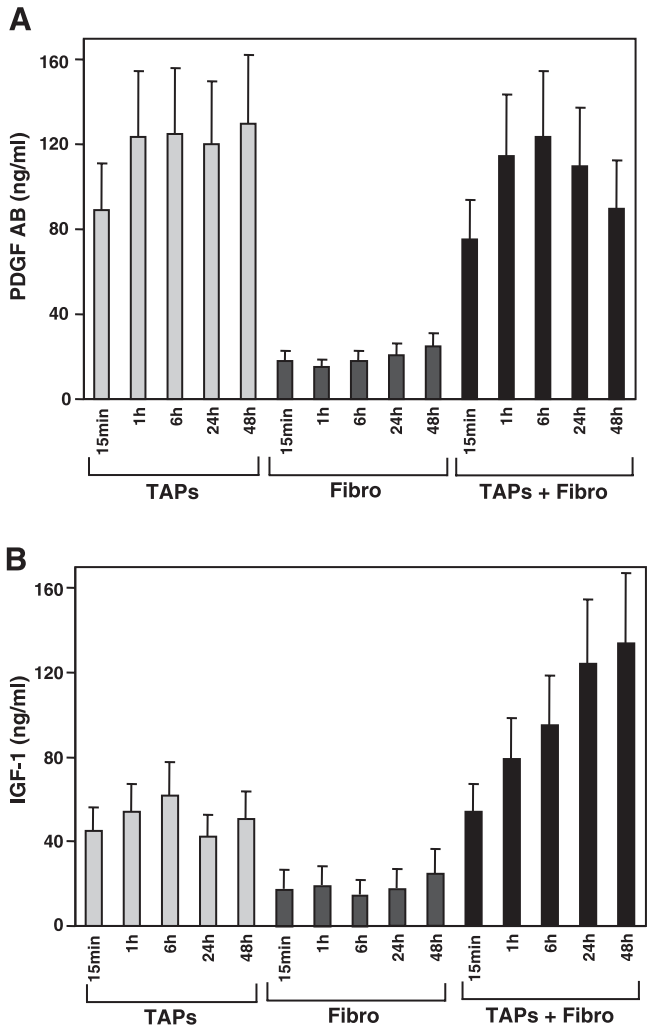

Figure 7. PDGF and IGF-1 release by thrombin-activated platelets and fibroblasts. Media were collected from thrombin-activated platelets, fibroblasts, and TAPs-stimulated fibroblasts at the indicated times. The concentration of PDGF-AB (*A*) and IGF-1 (*B*) was determined by ELISA as described in Materials and Methods. The bar graphs represent the means ± SD of three independent experiments in triplicate.

protein was slightly decreased when the cells were incubated with conditioned media derived from TAPs-stimulated fibroblasts, compared with TAPs alone. The 185 kDa and the 95 kDa bands comigrated, respectively, with the full-length PDGF-R (Fig. 8*B*) and the IGF-1-R β subunit (Fig. 8*C*).

In addition, TAPs-conditioned media increased by 4-fold both Akt/PKB (**Fig. 9*A***) and ERK1/2 activity (Fig. 9*B*). No effect was detectable with conditioned media derived from fibroblasts alone. Conditional media derived from TAPs-stimulated fibroblasts increased Akt/PKB activation by 4-fold similar to that achieved in the presence of TAPs-alone conditioned media—and ERK1/2 by 6-fold, which further enhanced the effect of TAPs alone.

To further assess whether TAPs-stimulated fibroblast-released factors were responsible for the activation of Akt/PKB and ERK1/2, human fibroblasts were pretreated with AG1296 or AG1024 before stimulation with conditioned media. AG1296 inhibited by >80% Akt/PKB (Fig. 9*A*) and by only 20% ERK1/2 (Fig. 9*B*) activation induced by conditioned media derived from both TAPs and TAPs-stimulated fibroblasts. By contrast, the IGF-1-R inhibitor AG1024 had no significant effect on Akt/PKB activity but reduced by 25% and 70% ERK1/2 activity that was induced, respectively, by TAPs and TAPs-stimulated fibroblasts conditioned media.

Effect of thrombin-activated platelets on cells expressing a kinase-deficient IGF-1 receptor

To further investigate the relevance of IGF-1 in fibroblast growth, we have applied TAPs to NIH-3T3 cells expressing either high levels of IGF-1 receptor or a kinase-defective mutant lacking important autophosphorylation sites (18). TAPs application had a more pronounced effect in 3T3-IGF1R cells than in parental NIH-3T3 fibroblasts. At variance, TAPs-induced cell growth was ~2-fold decreased in the cells expressing

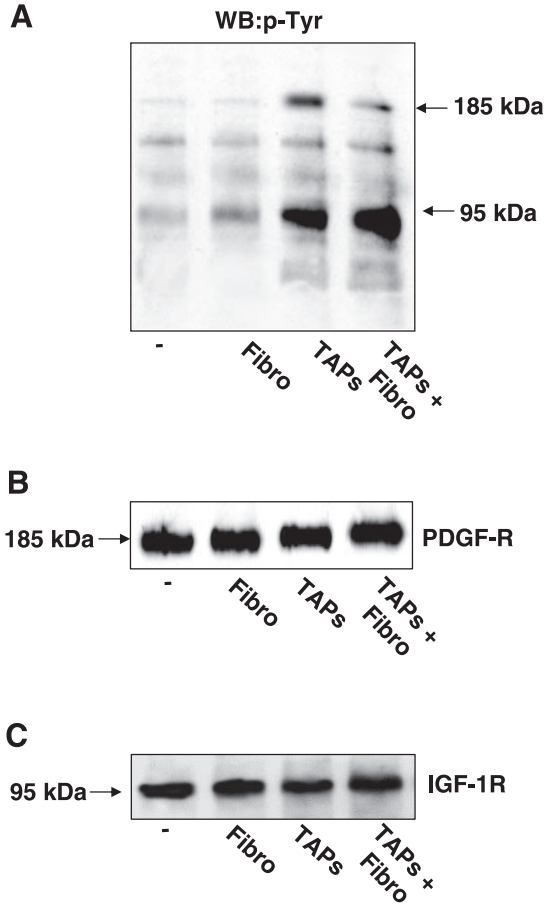

Figure 8. Effect of conditioned media on RTK phosphorylation. Human fibroblasts were incubated for 15 min with media that have been preexposed for 24 h to either thrombin-activated platelets (TAPs), serum-starved cultured fibroblasts (Fibro), or TAPs-stimulated fibroblast (TAPs+Fibro). Phosphotyrosine blotting (*A*) was then performed. Also, the expression of PDGF-R (*B*) and IGF-1 R (*C*) has been controlled with specific antibodies. Autoradiographs representative of four independent experiments are shown.

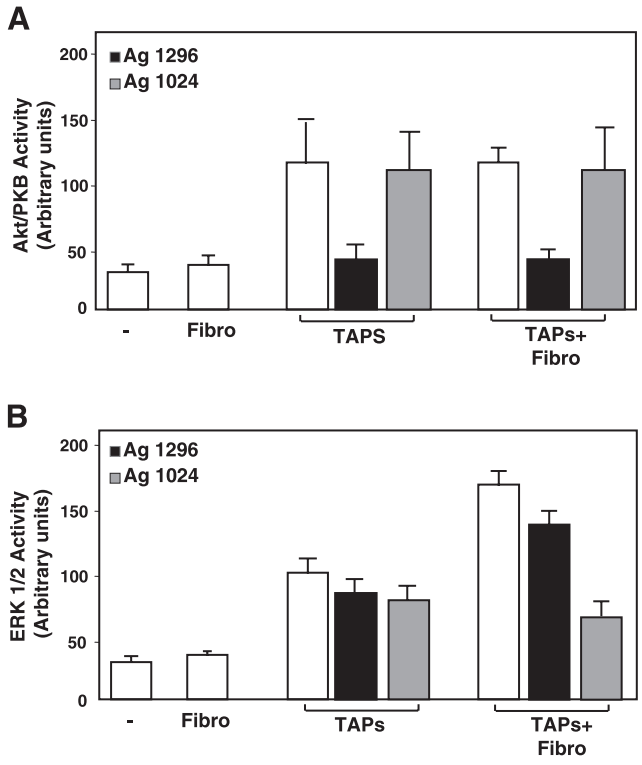

Figure 9. Effect of conditioned media on Akt/PKB and ERK1/2 activation. Human fibroblasts, pretreated or not with AG 1296 or AG1024, were incubated for 15 min with media that have been preexposed for 24 h to either TAPs, serum-starved fibroblasts, or TAPs-stimulated fibroblasts. Then, the activity of Akt/PKB (*A*) and ERK1/2 (*B*) has been analyzed in specific immunoprecipitates as described in Materials and Methods. The bar graphs represent the means ± SD of three independent experiments in triplicate.

the mutant receptor (3T3-IGF1DK) compared with the cells expressing a similar number of wild-type (WT) receptors (**Fig. 10*A***). Moreover, TAPs induced a comparable increase of Akt/PKB activity in parental, IGF1R and IGF1RDK, both on 15 min and on 24 h treatment (Fig. 10*B*). ERK1/2 activation on 15 min treatment with TAPs was also similar in all the cell clones. At variance, on 24 h TAPs treatment, we observed further 1.5- and 2-fold increases of ERK1/2 activity, respectively, in parental and in 3T3-IGF1R cells, but no significant change in 3T3-IGF1DK cells.

DISCUSSION

Since the first report, the application of platelet components has been successfully used in orthopedic and maxillo-facial surgery for bone reconstruction and soft tissue regeneration (3). According to recent recommendations (25–27) and our own investigation (19), treatment with autologous platelets represents an important therapeutic tool for diabetic patients with ulcers at the lower extremities. We sought, therefore, to investigate whether activated platelets could induce a cellular growth response when applied to ulcerative skin lesions of diabetic individuals. We found that the growth-related molecules Akt/PKB and ERK1/2 were activated in the peri-lesional skin following TAPs applications. We have then explored the molecular mechanisms responsible for the beneficial effect of platelets in wound-healing processes. To this end, a method was

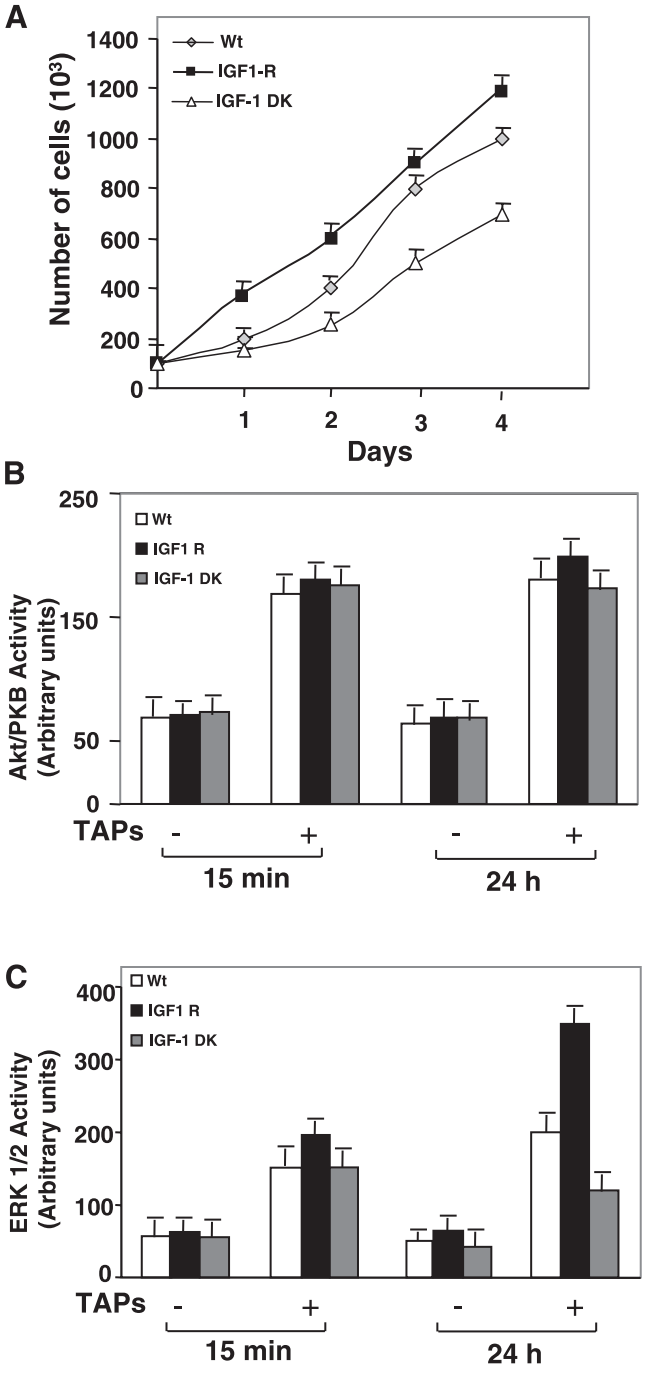

Figure 10. Effect of TAPs on IGF-1R defective cells. Thrombin-activated platelets have been applied to parental NIH-3T3 cells and to clones stably overexpressing wt IGF-1R or IGF-1R-DN. *A)* Cells have been counted as described in the legend to Fig. 2*B, C.* Alternatively, at the indicated times, cells have been solubilized and the activity of Akt/PKB and ERK1/2 determined as described in Materials and Methods. The graphs represent the means ± SD of three independent experiments in triplicate.

devised to analyze the effect of TAPs in cultured cells. Aliquots of TAPs were applied to monolayers of cultured cells as serum substitute. Interestingly, the addition of TAPs to human fibroblasts induced cell growth in a fashion comparable with 10% FBS. This finding is consistent with recent evidence reporting the use of platelet extracts for culturing stem cells of mesenchimal origin (28) and support the possible utilization of human platelet factors as a substitute for animal serum for cell-based therapeutic applications. TAPs effect, however, was cell-specific, as we failed to observe a similar growth induction in human umbilical vein endothelial cells (data not shown). TAPs-induced proliferation was mainly due to factors released following thrombin activation of the platelets. For instance, thrombin itself may induce fibroblast growth (29, 30). However, neither thrombin nor platelet-free plasma was able to fully mimic the effect of TAPs on fibroblast proliferation.

In the fibroblasts, the growth effect was accompanied by the rapid tyrosine phosphorylation of the PDGF and the IGF-1 receptors and by the activation of Akt/PKB and ERK1/2. The presence of platelet-released factors in media incubated with TAPs was sufficient to elicit these effects. In agreement with other reports (9, 31), activated platelets release a wide variety of growth factors and cytokines, including PDGF, IGF-1, and TGF-beta. The absolute levels of PDGF as well as of the other growth factors released by the TAPs showed a certain degree of variability in the different preparations. Nonetheless, the time-dependent variation of PDGF concentration was extremely conserved. In particular, PDGF levels rapidly increased in the media incubated with TAPs. When assayed in the media of TAPs-cocultured fibroblasts, PDGF levels reached an early plateau (15 min) and declined thereafter, possibly because of consumption by the target cells. TAPs also induced a very rapid activation of Akt/PKB, which remained stable up to 48 h. At variance, ERK1/2 activity progressively increased, reaching maximal activation only on 48 h of TAPs exposure. This sustained activation of ERK1/2 was also detected in the bioptical specimens of the ulcerative lesions on treatment with TAPs.

Tyrphostin inhibition of PDGF-R signaling drastically reduced PG-induced cell growth and activation of Akt/PKB but only slightly decreased the early ERK1/2 activation, indicating that PDGF signaling was not the major stimulus for the induction of the latter kinase activity. Thus, the amount of PDGF released by activated platelets preferentially activates the survival factor Akt/PKB but is not sufficient to elicit the total effects.

As in the case of osteoblastic cells (32), other growth factors may play crucial role to elicit cell cycle progression. However, the PDGF receptor blocker Ag1296 significantly reduced late activation of ERK1/2 and DNA synthesis, suggesting that PDGF control is necessary to allow the proliferative response to TAPs.

Interestingly, inhibition of IGF-1 signaling by the specific tyrphostin Ag1024 selectively reduced both early and late TAPs-induced ERK1/2 activation, with minor effect on Akt/PKB. In addition, IGF-1, which is present at relatively low levels in TAPs- conditioned media, time-dependently increased in the supernatant of TAPs-stimulated human fibroblasts. These same conditioned media could also stimulate IGF-1 receptor tyrosine phosphorylation and ERK1/2 activation, when added to recipient fibroblasts, at higher levels compared with conditioned media from isolated fibroblasts or TAPs. These data are consistent with the hypothesis that IGF-1 autocrine production by fibroblast is needed to propagate TAPs proliferative signals. This finding is further supported by the evidence that the effect of TAPs-treated fibroblast conditioned media on ERK1/2 is selectively inhibited by 10 μM Ag1024. At the concentration used the inhibitory effect of this tyrphostin is selectively exerted on the IGF-1 receptor kinase activity (33–35). Moreover, TAPs treatment of NIH-3T3 cells expressing a dominant-negative mutant IGF-1 receptor failed to induce long-term ERK1/2 activation and cell proliferation, while normally stimulating Akt/PKB. TAPs, however, can still stimulate short-term activation of ERK1/2, even in the absence of IGF-1 signaling, indicating that, in these cells, PDGF or other factors may account for this effect.

It is conceivable that platelet factors stimulate production and release of IGF-1 by both human fibroblasts (Fig. 7) and NIH-3T3 cells (data not shown), which may act as an autocrine stimulator of cell proliferation. TAPs-induced IGF-1 release and signaling, however, are only partially inhibited by the treatment with Ag1296, which suggests that platelet factors other than PDGF may be also involved in the elicitation of this effect.

The combined action of PDGF and IGF-1 may be therefore responsible for a large fraction of the growth effect of TAPs on human fibroblasts and implicated in the repair mechanisms involving connective tissue. Accordingly, it has been recently described that the application of recombinant PDGF and IGF-1 ameliorates bone repair and regeneration after test implant insertion in dogs (36). Consistently, Loot *et al.* (37) reported that stimulation with PDGF and IGF-1 leads to a strong proliferative response in human fibroblasts, further supporting the hypothesis that the sequential action of these growth factors may be relevant in the wound-healing process.

Thus, one might argue that activated platelets represents an important source of growth factors to induce the proliferation of human fibroblasts, *in vivo* as well as in cultured systems. The release of PDGF is necessary and sufficient to activate Akt/PKB and to promote cell survival. Autocrine production of IGF-1 by fibroblasts, however, is needed to sustain the prolonged activation of ERK1/2 and to induce the completion of the proliferation program. FJ

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REFERENCES

1. Singer, A. J., and Clark, R. A. F. (1999) Cutaneous wound healing. *N. Engl. J. Med.* **341,** 738–746
2. Diegelmann, R. F., and Evans, M. C., (2004) Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci.* **9,** 283–289
3. Anitua, E., Andia, I., Ardanza, B., Nurden, P., and Nurden, A. T. (2004) Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb. Haemost.* **91,** 4–15
4. Bennett, J. S., and Kolodziej, M. A. (1992) Disorders of platelet function. *Dis. Mon.* **38,** 577–631
5. Knighton, D. R., Ciresi, K. F., Fiegel, V. D., Austin, L. L., and Butler, E. L. (1986) Classification and treatment of chronic nonhealing wounds. Successful treatment with autologous platelet-derived wound healing factors (PDWHF). *Ann. Surg.* **204,** 322–330
6. Marx, R. E., Carlson, E. R., Eichstaedt, R. M., Schimmele, S. R., Strauss, J. E., and Georgeff, K. R. (1998) Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg. Oral Med. Oral. Pathol. Oral Radiol. Endod.* **85,** 638–646
7. Bhanot, S., and Alex, J. C. (2002) Current applications of platelet gels in facial plastic surgery. *Facial Plast. Surg.* **18,** 27–33
8. Marx, R. E. (2004) Platelet-rich plasma: evidence to support its use. *J. Oral Maxillofac. Surg.* **62,** 489–496
9. Borzini, P., and Mazzucco, L. (2005) Tissue regeneration and in loco administration of platelet derivatives: clinical outcome, heterogeneous products, and heterogeneity of the effector mechanisms. *Transfusion* **45,** 1759–1767
10. Pierce, G. F., Mustoe, T. A., Altrock, B. W., Deuel, T. F., and Thomason, A. (1991) Role of platelet-derived growth factor in wound healing. *J. Cell. Biochem.* **45,** 319–326
11. Mitsiades, C. S., Mitsiades, N., and Koutsilieris, M. (2004) The Akt pathway: molecular targets for anti-cancer drug development. *Curr. Cancer Drug Targets* **4,** 235–256
12. Johnson, G. L., and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK and p38 protein kinases. *Science* **298,** 1911–1912
13. Steed, D. L., Goslen, J. B., Holloway, G. A., Malone, J. M., Bunt, T. J., and Webster, M. W. (1992) Randomized prospective double-blind trial in healing chronic diabetic foot ulcers. CT-102 activated platelet supernatant, topical versus placebo. *Diabetes Care.* **15,** 1598–1604
14. Pierce, G. F., and Mustoe, T. A. (1995) Pharmacologic enhancement of wound healing. *Annu. Rev. Med.* **46,** 467–81
15. Bennett, S. P., Griffiths, G. D., Schor, A. M., Leese, G. P., and Schor, S. L. (2003) Growth factors in the treatment of diabetic foot ulcers. *Br. J. Surg.* **90,** 133–146
16. Miller, M. C., and Nanchahal, J. (2005) Advances in the modulation of cutaneous wound healing and scarring. *BioDrugs* **19,** 363–381
17. Sly, W. S., and Grubb, J. (1979) Isolation of fibroblasts from patients. *Methods Enzymol.* **58,** 444–450
18. Kato, H., Faria, T. N., Stannard, B., Roberts, C. T. Jr., and LeRoith, D. (1994) Essential role of tyrosine residues 1131, 1135, and 1136 of the insulin-like growth factor-I (IGF-I) receptor in IGF-I action. *Mol. Endocrinol.* **8,** 40–50
19. Saldalamacchia, G., Lapice, E., Cuomo, V., De Feo, E., D'Agostino, E., Rivellese, A. A., and Vaccaro, O. (2004) A controlled study of the use of autologous platelet gel for the treatment of diabetic foot ulcers. *Nutr. Metab. Cardiovasc. Dis.* **14,** 395–396
20. O'Neill, E. M., Zalewski, W. M., Eaton, L. J., Popovsky, M. A., Pivacek, L. E., Ragno, G., and Valeri, C. R. (2001) Autologous platelet-rich plasma isolated using the Haemonetics Cell Saver 5 and Haemonetics MCS+ for the preparation of platelet gel. *Vox Sang.* **81,** 172–175
21. Zimmermann, R., Jakubietz, R., Jakubietz, M., Strasser, E., Schlegel, A., Wiltfang, J., and Eckstein, R. (2001) Different preparation methods to obtain platelet components as a source of growth factors for local application. *Transfusion* **41,** 1217–1224
22. Oriente, F., Formisano, P., Miele, C., Fiory, F., Maitan, M. A., Vigliotta, G., Trencia, A., Santopietro, S., Caruso, M., Van Obberghen, E., and Beguinot, F. (2001) Insulin receptor substrate-2 phosphorylation is necessary for protein kinase C zeta activation by insulin in L6hIR cells. *J. Biol. Chem.* **276,** 37109–37119
23. Trencia, A., Perfetti, A., Cassese, A., Vigliotta, G., Miele, C., Oriente, F., Santopietro, S., Giacco, F., Condorelli, G., Formisano, P., and Beguinot, F. (2003) Protein kinase B/Akt binds and phosphorylates PED/PEA-15, stabilizing its antiapoptotic action. *Mol. Cell. Biol.* **23,** 4511–4521
24. Wagner, F. W. Jr. (1984) Treatment of diabetic foot. *Compr. Ther.* **10,** 29–38
25. Margolis, D. J., Kantor, J., Santanna, J., Strom, B. L., and Berlin, J. A. (2001) Effectiveness of platelet releasate for the treatment of diabetic neuropathic foot ulcers. *Diabet. Care* **24,** 483–488
26. Eldor, R., Raz, I., Ben Yehuda, A., and Boulton, A. J. (2004) New and experimental approaches to treatment of diabetic foot ulcers: a comprehensive review of emerging treatment strategies. *Diabet. Med.* **21,** 1161–1173
27. Snyder, E. L., and Calhoun, B. C. (2001) Topical platelet growth factor therapy: of lotions and potions. *Transfusion* **41,** 1186–1189
28. Doucet, C., Ernou, I., Zhang, Y., Llense, J. R., Begot, L., Holy, X., and Lataillade, J. J. (2005) Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. *J. Cell. Physiol.* **205,** 228–236
29. Chen, L. B., and Buchanan, J. M. (1975) Mitogenic activity of blood components. I. Thrombin and prothrombin. *Proc. Natl. Acad. Sci. U. S. A.* **72,** 131–135
30. Dawes, K. E., Gray, A. J., and Laurent, G. J. (1993) Thrombin stimulates fibroblast chemotaxis and replication. *Eur. J. Cell Biol.* **61,** 126–130
31. Weibrich, G., Kleis, W. K., Hafner, G., and Hitzler, W. E. (2002) Growth factor levels in platelet-rich plasma and correlations with donor age, sex, and platelet count. *J. Craniomaxillofac. Surg.* **30,** 97–102
32. Chaudhary, L. R., and Hruska, K. A. (2001) The cell survival signal Akt is differentially activated by PDGF-BB, EGF, and FGF-2 in osteoblastic cells. *J. Cell. Biochem.* **81,** 304–311
33. Kovalenko, M., Gazit, A., Bohmer, A., Rorsman, C., Ronnstrand, L., Heldin, C. H., Waltenberger, J., Bohmer, F. D., and Levitzki, A. (1994) Selective platelet-derived growth factor receptor kinase blockers reverse sis-transformation. *Cancer Res.* **54,** 6106–6114
34. Tao, J., Malbon, C. C., and Wang, H. Y. (2001) Insulin stimulates tyrosine phosphorylation and inactivation of protein-tyrosine phosphatase 1B in vivo. *J. Biol. Chem.* **276,** 29520–29525
35. Strawn, L. M., McMahon, G., App, H., Schreck, R., Kuchler, W. R., Longhi, M. P., Hui, T. H., Tang, C., Levitzki, A., and Gazit, A., *et al.* (1996) Flk-1 as a target for tumor growth inhibition. *Cancer Res.* **56,** 3540–3545
36. Stefani, C. M., Machado, M. A., Sallum, E. A., Sallum, A. W., Toledo, S., Nociti, F. H. Jr. (2000) Platelet-derived growth factor/insulin-like growth factor-1 combination and bone regeneration around implants placed into extraction sockets: a histometric study in dogs. *Implant Dent.* **9,**126–131
37. Loot, M. A., Kenter, S. B., Au, F. L., van Galen, W. J., Middelkoop, E., Bos, J. D., and Mekkes, J. R. (2002) Fibroblasts derived from chronic diabetic ulcers differ in their response to stimulation with EGF, IGF-I, bFGF and PDGF-AB compared to controls. *Eur. J. Cell Biol.* **81,** 153–160

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Thrombin-activated platelets induce proliferation of human skin fibroblasts by stimulating autocrine production of insulin-like growth factor-1

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SPECIFIC AIM

This work aims to elucidate the molecular mechanisms elicited by factors released by activated platelets in wound healing processes *in vivo* and in cultured cell systems. In particular, we have investigated the molecular pathways regulating growth-related events induced by thrombin-activated platelets in the healing of ulcerative lesions of diabetic patients and in cultured human fibroblasts.

PRINCIPAL FINDINGS

1. Application of thrombin-activated platelets to ulcerative lesions in diabetic patients induce activation of Akt/PKB and ERK1/2

Thrombin-activated platelets (TAPs) are commonly used in the treatment of ulcerative skin lesions and in other processes requiring wound healing and tissue regeneration. We have found that, when applied to diabetic foot lesions, TAPs induce the activation of Akt/PKB and ERK1/2. This observation derives from Western blot analysis with phospho-specific Akt/PKB and ERK1/2 antibodies of bioptical specimens obtained by seven consecutive patients with II/III grade ulcers, treated with TAPs for al least 1 wk.

2. Thrombin-activated platelets induce proliferation of cultured human fibroblasts

To analyze the molecular details of TAPs-induced cell growth, cultured human fibroblasts have been treated with TAPs as a serum substitute (**Fig. 1**). Application of TAPs to the fibroblasts increased the cell number in a manner comparable to that obtained in the presence of 10% FBS. This increase in cell growth was dependent on platelet number and accompanied by increased DNA synthesis, as assessed by thymidine incorporation experiments.

3. Thrombin-activated platelets activate Akt/PKB and ERK1/2 with different kinetics

Treatment of human fibroblasts with TAPs rapidly increased phosphorylation and activity of both Akt/PKB and ERK1/2. However, while TAPs-induced Akt/PKB activation reached an early plateau, ERK activity progressively increased up to 48 h in the cultured fibroblasts. Interestingly, a raising profile of ERK activation, but not of Akt/PKB, was also displayed *in vivo*, in the protein extracts of the sequential peri-lesional biopsies obtained by TAPs-treated diabetic patients. Moreover, both Akt/PKB and ERK1/2 activity are needed to propagate the growth effect, since treatment of fibroblasts with LY294002 and PD98059, which inhibit PI3K/PKB and MEK/ERK pathways, respectively, drastically reduced thymidine incorporation in response to TAPs.

4. TAPs-induced proliferation of human fibroblasts requires platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF)-1 signaling

Two lines of evidence indicate that PDGF and IGF-1 largely mediate the TAPs effect on cell growth. First, detectable amounts of both PDGF and IGF-1 have been found in the platelet releasates and in media of TAPs-treated fibroblasts and are sufficient to induce the

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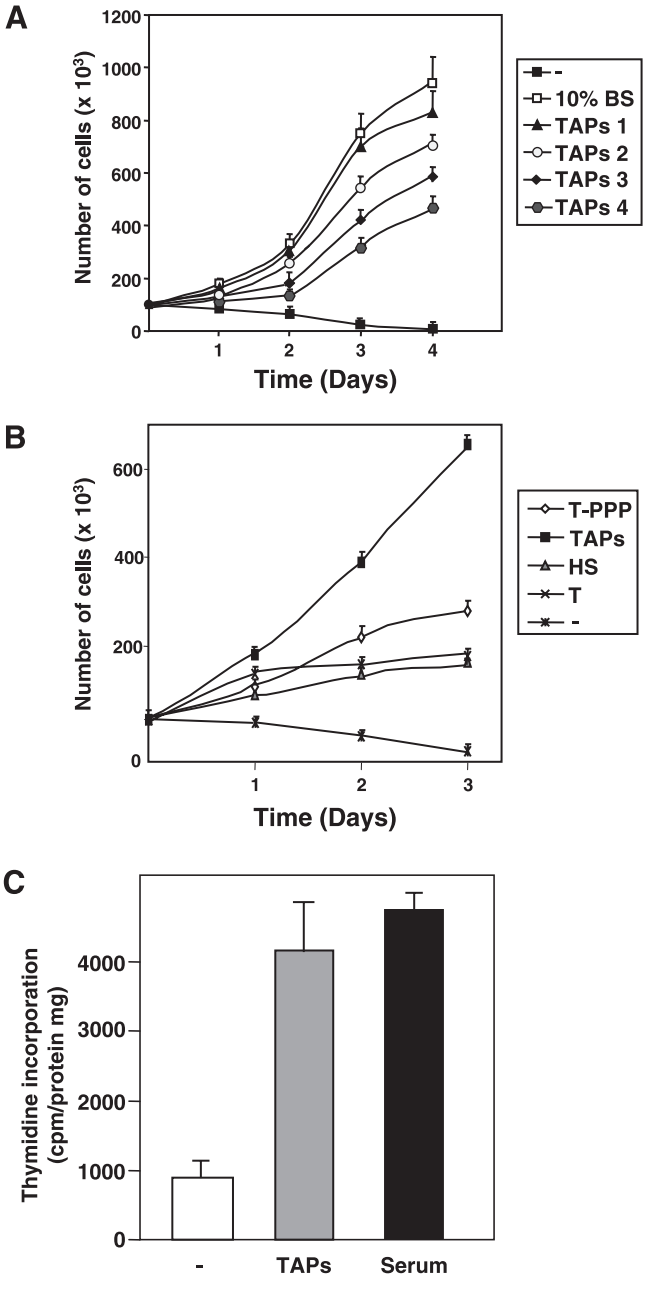

Figure 1. Effect of TAPs on cell growth in cultured human fibroblasts. *A)* Cells (10^5) were plated and supplied with complete medium (10% BS) or serum-free media or were serum-free (–). Where indicated, a 1 cm^2 aliquot of TAPs (TAPs $1=1.0\times10^9$; TAPs $2=0.75\times10^9$; TAPs $3=0.5\times10^9$; TAPs $4=0.2\times10^9$), obtained as described in Materials and Methods, has been added to the serum-free medium. At the indicated times, the cells were trypsinized and counted. Data represent mean ± SD of six independent experiments in duplicate. *B)* Alternatively, equal amounts of cells were placed in serum-free media (–) and were supplied with thrombin-treated platelet-poor plasma (T-PPP), thrombin-activated platelets (TAPs; 1.0×10^9), 10% human serum and thrombin (T; 0.5 NIH U), as indicated. At the indicated times, the cells were trypsinized and counted. Data represent means ± SD of three independent experiments in duplicate. *C)* Six-well plates were seeded with 10^5 cells/plate in 1 ml complete medium. After incubation for 24 h at 37°C, the medium was removed and replaced with Dulbecco's modified Eagle medium (DMEM) containing 0.25% BSA and no serum. After an additional 24 h, the medium was removed again and replaced with complete DMEM, or DMEM 0.25% albumin with or without TAPs. Incubation was prolonged for additional 16 h, and the incubation media replaced with the same media supplemented with [^{3}H]thymidine (500 nCi/ml). After 1 h, media were removed and thymidine incorporation was measured as described in Materials and Methods. The bar graph represents means ± SD of three independent experiments in triplicate.

activation of their own receptors. Second, selective inhibition of PDGF receptor tyrosine kinase activity by AG1296 and of IGF-1 receptor by AG1024, reduced TAPs-stimulated thymidine incorporation.

5. Autocrine production of IGF-1 by human fibroblasts is required for sustained ERK1/2 activation and proliferation

PDGF levels were well detectable in media incubated with TAPs alone. However, PDGF concentration was slightly lower in conditioned media following coincubation with TAPs and fibroblasts. This finding is consistent with PDGF consumption due to the utilization by the targeted fibroblasts. At variance, IGF-1 concentration was relatively low in TAPs alone and progressively increased in TAPs + fibroblasts conditioned media. Treatment of the recipient fibroblasts with the PDGF-R blocker AG1296 slightly inhibited ERK1/2 and strongly reduced Akt/PKB, while AG1024 selectively reduced ERK1/2 activity induced by the TAPs + fibroblast-conditioned media. Moreover, TAPs application onto NIH-3T3 cells overexpressing a dominant-negative mutant of the IGF-1R failed to induce sustained ERK1/2 activation. This was accompanied by reduced TAPs stimulation of cell growth (**Fig. 2**).

CONCLUSIONS AND SIGNIFICANCE

Platelet components have been successfully used in orthopedic and maxillo-facial surgery for bone reconstruction and soft tissue regeneration. According to recent recommendations and our own previous investigation, treatment with autologous platelets also represents an important therapeutic tool for diabetic patients with ulcers at the lower extremities (diabetic foot). We sought therefore to investigate whether activated platelets could induce a cellular growth response when applied to ulcerative skin lesions of diabetic individuals. We found that the growth-related molecules Akt/PKB and ERK1/2 were activated in the peri-lesional skin following TAPs applications. We have then explored the molecular mechanisms responsible for the beneficial effect of platelets in wound healing processes. To this end, a method was devised to analyze the effect of TAPs in cultured cells. Aliquots of TAPs were applied to monolayers of cultured cells as serum substitute. Interestingly, the addition of TAPs to human fibroblasts induced cell growth in a fashion comparable with 10% FBS. This novel observation supports the

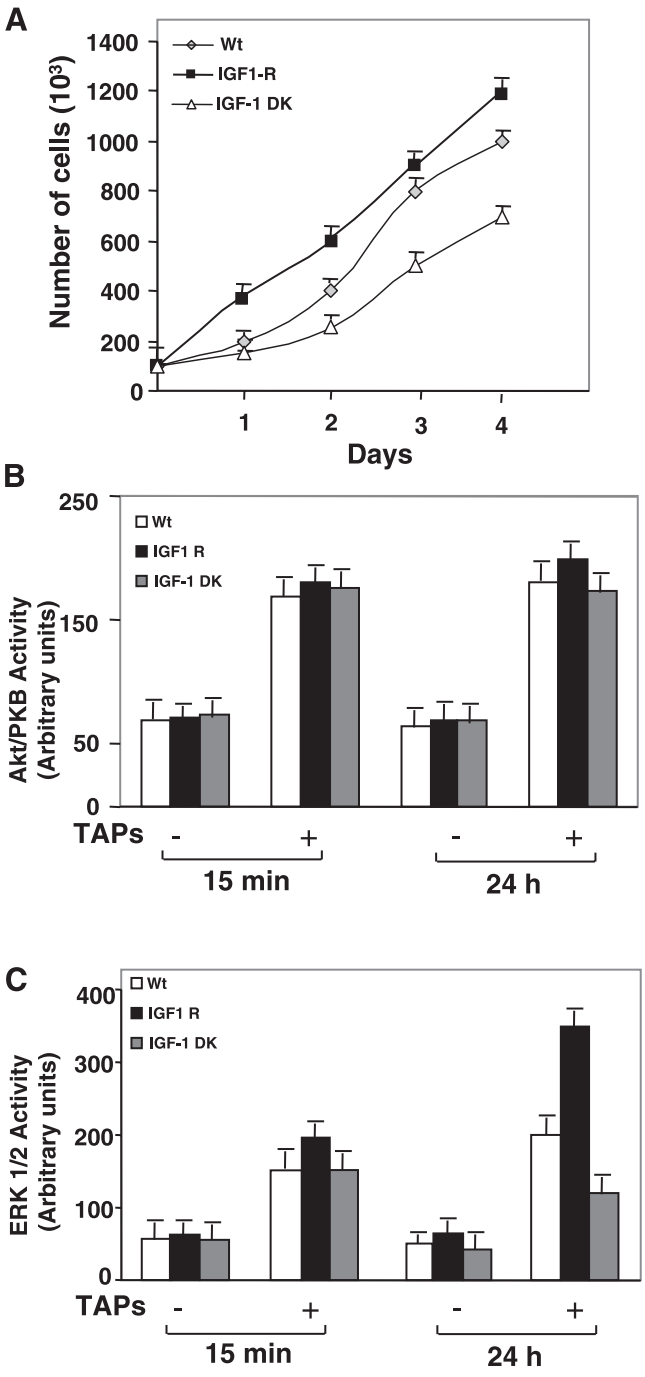

Figure 2. Effect of TAPs on IGF-1R defective cells. Thrombin-activated platelets have been applied to parental NIH-3T3 cells and to clones stably overexpressing wt IGF-1R or IGF-1R-DN. *A)* Cells have been detached and counted. *B, C)* Alternatively, at the indicated times, cells have been solubilized and the activity of Akt/PKB and ERK1/2 determined. The graphs represent the means ± SD of three independent experiments in triplicate.

possible utilization of human platelet factors as a substitute for animal serum for cell-based therapeutic applications. The effect of TAPs, however, was cell-specific, as we failed to observe a similar growth induction in human umbilical vein endothelial cells.

We have also shown that, in the human fibroblasts, the activities of Akt/PKB and ERK1/2 were required to elicit TAPs-stimulated growth effect. However, while the platelet released PDGF preferentially regulates Akt/PKB activity, IGF-1 signaling is necessary for the sustained activation of ERK1/2. Indeed, inhibition of IGF-1 signaling by Ag1024 and expression of a dominant negative IGF-1R mutant selectively reduced the stimulation of ERK1/2 by TAPs and fibroblast-released factors, with minor changes of Akt/PKB activity. Moreover, IGF-1 is detected at levels higher then expected in media coincubated with TAPs and fibroblasts, compared to those incubated with TAPs or fibroblasts alone. These observations are consistent with the hypothesis that platelet factors, including PDGF, can activate Akt/PKB and, at lesser extent, ERK1/2 and induce the release of IGF-1 by the fibroblasts. IGF-1, in turn may participate in an autocrine loop to sustain the activation of ERK1/2, and to elicit DNA synthesis and fibroblast proliferation (Fig. 3). The combined action of PDGF and IGF-1 may be therefore responsible for a large fraction of the growth effect of TAPs on human fibroblasts and implicated in the repair mechanisms involving connective tissue.

Thus, to our knowledge, this is the first report investigating the molecular mechanisms by which the local application of activated platelets can induce growth effects in wound healing processes. Further elucidation of such mechanisms may lead to the identification of novel and more effective therapeutic strategies for the treatment of the diabetic foot as well as of other degenerative disorders requiring tissue reconstruction. FJ

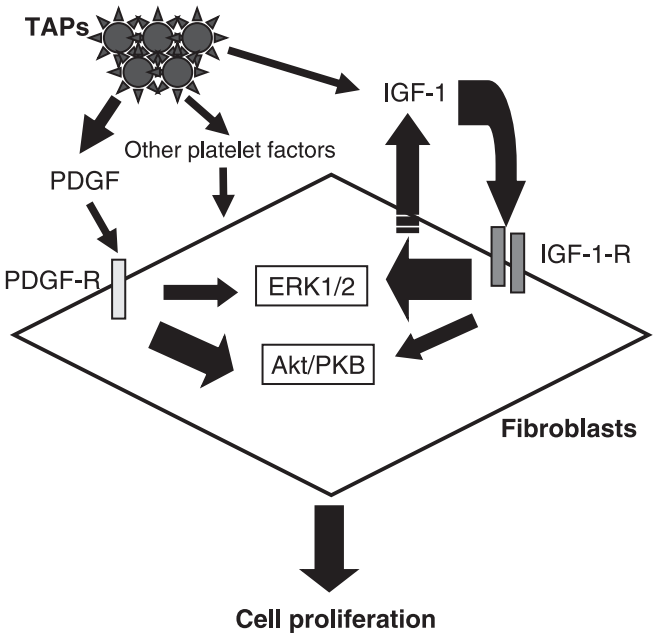

Figure 3. Schematic diagram.