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“Signal transduction mechanisms involved in wound healing”

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INTRODUCTION

Diabetes mellitus (DM) comprises a group of common metabolic disorders that share the phenotype of hyperglycemia. Several distinct types of DM exist and are caused by a complex interaction of genetic and environmental factors, and life-style choices. Depending on the etiology of the DM, factors contributing to hyperglycemia may include reduced insulin secretion, decreased glucose utilization, and increased glucose production. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the individual with diabetes and on the health care system. In the United States, DM is the leading cause of end-stage renal disease (ESRD), nontraumatic lower extremity amputations, and adult blindness. With an increasing incidence worldwide, DM will be a leading cause of morbidity and mortality for the foreseeable future (1).

DM is classified on the basis of the pathogenic process that leads to hyperglycemia. The two broad categories of DM are designated type 1 and type 2. Type 1A DM results from autoimmune beta cell destruction, which leads to insulin deficiency. Individuals with type 1B DM lack immunologic markers indicative of an autoimmune destructive process of the beta cells. However, they develop insulin deficiency by unknown mechanisms and are ketosis prone. Relatively few patients with type 1 DM are in the type 1B idiopathic category. Type 2 DM is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production. Distinct genetic and metabolic defects in insulin action and/or secretion give rise to the common phenotype of hyperglycemia in type 2 DM (1).

TYPE 1 DIABETES MELLITUS

Type 1A DM develops as a result of the synergistic effects of genetic, environmental, and immunologic factors that ultimately destroy the pancreatic beta cells. The temporal development of type 1A DM is shown schematically as a function of beta cell mass. Individuals with a genetic susceptibility have normal beta cell mass at birth but begin to lose beta cells secondary to autoimmune destruction that occurs over months to years. This autoimmune process is thought to be triggered by an infectious or environmental
stimulus and to be sustained by a beta cell–specific molecule. In the majority of individuals, immunologic markers appear after the triggering event but before diabetes becomes clinically overt. Beta cell mass then begins to decline, and insulin secretion becomes progressively impaired, although normal glucose tolerance is maintained. The rate of decline in beta cell mass varies widely among individuals, with some patients progressing rapidly to clinical diabetes and others evolving more slowly. Features of diabetes do not become evident until a majority of beta cells are destroyed (80%) and the individual becomes completely insulin deficient (1).

Genetic susceptibility to type 1A DM involves multiple genes. The concordance of type 1A DM in identical twins ranges between 30 and 70%, indicating that additional modifying factors must be involved in determining whether diabetes develops. The major susceptibility gene for type 1A DM is located in the HLA region on chromosome 6. Polymorphisms in the HLA complex account for 40 to 50% of the genetic risk of developing type 1A DM. Most individuals with type 1A DM have the HLA DR3 and/or DR4 haplotype. Refinements in genotyping of HLA loci have shown that the haplotypes DQA1*0301, DQB1*0302, DQA1*501, and DQB1*0201 are most strongly associated with type 1A DM. The risk of developing type 1A DM is increased tenfold in relatives of individuals with the disease. Nevertheless, most individuals with predisposing haplotypes do not develop diabetes. In addition, most individuals with type 1A DM do not have a first-degree relative with this disorder (1).

**TYPE 2 DIABETES MELLITUS**

Type 2 DM is characterized by three pathophysiologic abnormalities: impaired insulin secretion, peripheral insulin resistance, and excessive hepatic glucose production. Obesity, particularly visceral or central (as evidenced by the hip-waist ratio), is very common in type 2 DM. In the early stages of the disorder, glucose tolerance remains normal, despite insulin resistance, because the pancreatic beta cells compensate by increasing insulin output. As insulin resistance and compensatory hyperinsulinemia progress, the pancreatic islets in certain individuals are unable to sustain the hyperinsulinemic state. Then develops impaired glucose tolerance, characterized by elevations in postprandial glucose,. A further decline in insulin secretion and an increase
in hepatic glucose production lead to overt diabetes with fasting hyperglycemia. Ultimately, beta cell failure may ensue (1).

**METABOLIC ABNORMALITIES**

*Insulin resistance* The decreased ability of insulin to act effectively on peripheral target tissues (especially muscle and liver) is a prominent feature of type 2 DM and results from a combination of genetic susceptibility and obesity. Insulin resistance is relative, however, since supernormal levels of circulating insulin will normalize the plasma glucose. Insulin dose-response curves exhibit a rightward shift, indicating reduced sensitivity, and a reduced maximal response, indicating an overall decrease in maximum glucose utilization (30 to 60% lower than normal individuals). Insulin resistance impairs glucose utilization by insulin-sensitive tissues and increases hepatic glucose output; both effects contribute to the hyperglycemia. Increased hepatic glucose output predominantly accounts for increased fasting plasma glucose levels, whereas decreased peripheral glucose usage results in postprandial hyperglycemia. The precise molecular mechanism of insulin resistance in type 2 DM has not been elucidated. Insulin receptor levels and tyrosine kinase activity in skeletal muscle are reduced, but these alterations are most likely secondary to hyperinsulinemia and are not a primary defect. Therefore, postreceptor defects are believed to play the predominant role in insulin resistance. Another emerging theory proposes that elevated levels of free fatty acids, a common feature of obesity, may contribute to the pathogenesis of type 2 DM. Free fatty acids can impair glucose utilization in skeletal muscle, promote glucose production by the liver, and impair beta cell function (1).

*Impaired insulin secretion* Insulin secretion and sensitivity are interrelated. In type 2 DM, insulin secretion initially increases in response to insulin resistance to maintain normal glucose tolerance. In the beginning, the insulin secretory defect is mild and selectively involves glucose-stimulated insulin secretion. The response to other nonglucose secretagogues, such as arginine, is preserved. Eventually, the insulin secretory defect progresses to a state of grossly inadequate insulin secretion. The reason(s) for the decline in insulin secretory capacity in type 2 DM is unclear. The metabolic environment of diabetes may also negatively impact islet function. For example, chronic hyperglycemia paradoxically impairs islet function (“glucose toxicity”) and leads to a worsening of hyperglycemia. Improvement in glycemic control
is often associated with improved islet function. In addition, elevation of free fatty acid levels ("lipotoxicity") and dietary fat may also worsen islet function.

Increased hepatic glucose production In type 2 DM, insulin resistance in the liver reflects the failure of hyperinsulinemia to suppress gluconeogenesis, which results in fasting hyperglycemia and decreased glycogen storage by the liver in the postprandial state. Increased hepatic glucose production occurs early in the course of diabetes, though likely after the onset of insulin secretory abnormalities and insulin resistance in skeletal muscle.

GENETIC CONSIDERATIONS
Polymorphisms in IRS-1 may be associated with glucose intolerance, raising the possibility that polymorphisms in various postreceptor molecules may combine to create an insulin-resistant state. The pathogenesis of insulin resistance is currently focused on a PI-3-kinase signaling defect, which reduces translocation of GLUT4 to the plasma membrane, among other abnormalities. Of note, not all insulin signal transduction pathways are resistant to the effects of insulin [e.g., those controlling cell growth and differentiation and using the mitogen-activated protein (MAP) kinase pathway].

A common amino acid polymorphism of PPARγ (peroxisome proliferator-activated receptor-γ) has been associated to type 2 diabetes. Individuals homozygotes for Pro12 allele are more insulin resistant and present a risk of development of T2D 1.25 times higher than heterozygotes for alleles Ala12/Pro12.

Modifications of Calpain-10 gene have been also associated to T2D. Affected people have an increase of 3 times in risk of development of T2DM. These modifications affect both beta cell function and insulin function on muscle and adipose tissue.

The Phosphoprotein Enriched in Diabetes/Phosphoprotein Enriched in Astrocytes-15 (PED/PEA-15) is a 15 kDa cytosolic protein widely expressed in different tissues and highly conserved among mammals, whose gene maps on human chromosome 1q21-22 (4). Overexpression of the PED/PEA-15 gene is a common defect in type 2 diabetes. During a study using a differential display technique to identify genes whose expression was altered in type 2 diabetes, it has been demonstrated that both PED/PEA-15 mRNA and protein levels were overexpressed in fibroblasts from type 2 diabetics compared with non-diabetic individuals. Also, skeletal muscle and adipose tissues, two major sites
of insulin resistance in type 2 diabetes, showed the same behaviour (5). Furthermore, a recent study showed that PED/PEA-15 overexpression represents a common abnormality in both T2DM and their First Degree Relatives (FDR) (6). Cells overexpressing PED/PEA-15 showed an impaired insulin-dependent glucose uptake. Transgenic mice overexpressing PED/PEA-15 exhibit mildly elevated random-fed blood glucose levels and become hyperglycemic after glucose loading, indicating that increased expression of this gene is sufficient to impair glucose tolerance. Moreover, transgenic mice become diabetic after administration of high-fat diets, indicating that, in vivo, the overexpression of PED/PEA-15 in conjunction with environmental modifiers may lead to diabetes (7). Thus, these findings identify PED/PEA-15 as a novel gene controlling insulin action contributing, under appropriate environmental conditions, to genetic susceptibility to type 2 diabetes in humans.

**HYPERGLICEMIA AND DIABETIC COMPLICATIONS**

Type 1 DM and type 2 DM share the phenotype of hyperglycemia. Prolonged hyperglycemia is responsible for the onset of diabetic complications. Four main hypotheses about how hyperglycaemia causes diabetic complications have been generated. The four hypotheses based on distinct biochemical abnormalities are: increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux (for further details see: Browblee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 2001 Dec 13;414(6865):813-20).

In human diabetes, the resulting problems are grouped under "microvascular disease" (due to damage to small blood vessels) and "macrovascular disease" (due to damage to the arteries).

The damage to small blood vessels leads to a microangiopathy, which can cause one or more of the following:

- Diabetic retinopathy, growth of friable and poor-quality new blood vessels in the retina as well as macular edema (swelling of the macula), which can lead to severe vision loss or blindness. Retinal damage (from microangiopathy) makes it the most common cause of blindness among non-elderly adults in the US.
• Diabetic neuropathy, abnormal and decreased sensation, usually in a 'glove and stocking' distribution starting with the feet but potentially in other nerves, later often fingers and hands. When combined with damaged blood vessels this can lead to diabetic foot (see below). Other forms of diabetic neuropathy may present as mononeuritis or autonomic neuropathy. Diabetic amyotrophy is muscle weakness due to neuropathy.

• Diabetic nephropathy, damage to the kidney which can lead to chronic renal failure, eventually requiring dialysis. Diabetes mellitus is the most common cause of adult kidney failure worldwide in the developed world.

Macrovascular disease leads to cardiovascular disease, to which accelerated atherosclerosis is a contributor:
  • Coronary artery disease, leading to angina or myocardial infarction ("heart attack")
  • Stroke (mainly the ischemic type)
  • Peripheral vascular disease, which contributes to intermittent claudication (exertion-related leg and foot pain) as well as diabetic foot.
  • Diabetic myonecrosis ('muscle wasting')
  • Diabetic foot, often due to a combination of neuropathy and arterial disease, may cause skin ulcer and infection and, in serious cases, necrosis and gangrene. It is the most common cause of adult amputation, usually of toes and or feet, in the developed world.
**DIABETIC FOOT**

Diabetic foot ulcers (DFUs), a leading cause of amputations, affect 15% of people with diabetes. A series of multiple mechanisms, including decreased cell and growth factor response, lead to diminished peripheral blood flow and decreased local angiogenesis, all of which can contribute to lack of healing in persons with DFUs. Most commonly, patients have neuropathy, which could be causative. When coupled with an impaired ability to fight infection, these patients become largely unable to mount an adequate inflammatory response. Thus, the DFU that may look like a healing wound becomes a portal for infection that can lead to sepsis and require limb amputation. Over 100 known physiologic factors contribute to wound healing deficiencies in individuals with diabetes. These include decreased or impaired growth factor production (8–10), angiogenic response (10, 11), macrophage function (12), collagen accumulation, epidermal barrier function, quantity of granulation tissue (10), keratinocyte and fibroblast migration and proliferation, number of epidermal nerves (13), bone healing, and balance between the accumulation of ECM components and their remodeling by matrix metalloprotease (MMPs) (14). Wound healing occurs as a cellular response to injury and involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. Many growth factors and cytokines released by these cell types are needed to coordinate and maintain healing.

**WOUND HEALING PHYSIOLOGY**

The wound healing process has 3 phases. They are the inflammatory phase, the proliferative phase, and the maturational phase (Figure 1) (15). The inflammatory phase is characterized by hemostasis and inflammation. Collagen exposed during wound formation activates the clotting cascade (both the intrinsic and extrinsic pathways), initiating the inflammatory phase. This initial response helps to limit hemorrhage. After a short period, capillary vasodilatation occurs secondary to local histamine release, and the cells of inflammation are able to migrate to the wound bed. The timeline for cell migration in a normal wound healing process is predictable. Platelets release multiple chemokines, including epidermal growth factor (EGF), fibronectin, fibrinogen, histamine, platelet-derived growth factor (PDGF), serotonin, and von Willebrand factor.
These factors help stabilize the wound through clot formation. These mediators act to control bleeding and limit the extent of injury. The inflammatory phase continues, and more immune response cells migrate to the wound. The second response cell to migrate to the wound, the neutrophil, is responsible for debris scavenging, complement-mediated opsonization of bacteria, and bacteria destruction via oxidative burst mechanisms (ie, superoxide and hydrogen peroxide formation). The neutrophils kill bacteria and decontaminate the wound from foreign debris. The next cells present in the wound are the leukocytes and the macrophages (monocytes). The macrophage, referred to as the orchestrator, is essential for wound healing. Numerous enzymes and cytokines are secreted by the macrophage. These include collagenases, which debride the wound; interleukins and tumor necrosis factor (TNF), which stimulate fibroblasts (produce collagen) and promote angiogenesis; and transforming growth factor (TGF), which stimulates keratinocytes. This step marks the transition into the process of tissue reconstruction, ie, the proliferative phase.

![Figure 1. Wound healing phases](image)

The second stage of wound healing is the proliferative phase. Epithelialization, angiogenesis, granulation tissue formation, and collagen deposition are the principal steps in this anabolic portion of wound healing. Epithelialization occurs early in wound repair. If the basement membrane remains intact, the epithelial cells migrate upwards in the normal pattern. The epithelial progenitor cells remain intact below the wound, and the normal layers of epidermis are restored in 2-3 days. If the basement membrane has
been destroyed then the wound is reepithelialized from the normal cells in the periphery and from the skin appendages. Angiogenesis, stimulated by TNF-alpha, is marked by endothelial cell migration and capillary formation. The new capillaries deliver nutrients to the wound and help maintain the granulation tissue bed. The migration of capillaries into the wound bed is critical for proper wound healing. The granulation phase and tissue deposition require nutrients supplied by the capillaries, and failure for this to occur results in a chronically unhealed wound. Mechanisms for modifying angiogenesis are under study and have significant potential to improve the healing process.

The final part of the proliferative phase is granulation tissue formation. Fibroblasts differentiate and produce ground substance and then collagen. The ground substance is deposited into the wound bed; collagen is then deposited as the wound undergoes the final phase of repair. Many different cytokines are involved in the proliferative phase of wound repair. The steps and the exact mechanism of control have not been fully elucidated. Some of the cytokines include PDGF, insulin like growth factor (IGF), and EGF. All are necessary for collagen formation.

The final phase of wound healing is the maturational phase. The wound undergoes contraction, ultimately resulting in a smaller amount of apparent scar tissue. The entire process is a dynamic continuum with an overlap of each phase and continued remodeling. Collagen deposition continues for a prolonged period, but the net increase in collagen deposition plateaus after 21 days.
MOLECULAR MECHANISM OF WOUND HEALING

Wound healing involves a complex interaction and equilibrium of cells, cytokines and growth factors working in concert. Two major cellular functions are necessary for a proper wound healing: cell proliferation and motility.

CELL PROLIFERATION

Fundamental part of the answer to the damage in the process of wound healing is the cellular proliferation. The mostly interested cells are fibroblasts and the endothelial cells. Cell proliferation is triggered by the binding of a messenger molecule to a specific receptor of the target cell. In mammalian cells the cascade often is begun from the activation of a receptor with intrinsic tyrosin-kinase activity by interaction with a growth factor, such as EGF (epidermal growth factor), FGF (fibroblast growth factor), PDGF (plateled-derived growth factor) and IGF-1. The interaction with the ligand induces the dimerization of the receptor and the autophosphorylation on tyrosine residues. Phosphorylated tyrosines represent site of protein-protein interaction and act as activation site for intracellular proteins responsible for transduction of the signal from the cellular surface to the nucleus. In particular, there are three main pathways of signalling that have a primary role in the stimulation of the cellular proliferation.

- **RAS/MAPK pathway**

Receptor-linked tyrosine kinases such as the epidermal growth factor receptor (EGFR) are activated by extracellular ligands. Binding of epidermal growth factor (EGF) to the EGFR activates the tyrosine kinase activity of the cytoplasmic domain of the receptor. The EGFR becomes phosphorylated on tyrosines. Docking proteins such as GRB2 contain SH2 domains that bind to the phosphotyrosines of the activated receptor (16). GRB2 binds to the guanine nucleotide exchange factor SOS by way of an SH3 domain of GRB2. When the GRB2-SOS complex docks to phosphorylated EGFR, SOS becomes activated (17). Activated SOS promotes the removal of GDP from Ras. Ras can then bind GTP and become active(1). Activated Ras activates the protein kinase activity of RAF kinase (18), a serine/threonine-selective protein kinase. RAF kinase phosphorylates and activates MEK, another serine/threonine kinase. MEK phosphorylates and activates Extracellular Signal-regulated kinase 1e 2 (Erk 1/2).
Active Erk 1/2 translocates into the nucleus and phosphorylates several transcription factors stimulating cell proliferation (Figure 2).

- **PKC**’s pathway

PKCs proteins belong to a multigenic family including at least 14 isoforms with different catalytic and regulatory proprieties. (19). PKCs can be dived in three subgroups on the basis of their structural features and cofactor dependency. Classical PKCs (α, βI, βII and γ) are Ca²⁺ and diacylglycerol (DAG) dependent; novel PKCs (δ, ε, η, ε θ) are DAG dependent but are Ca²⁺ independent; le atypical PKCs (ζ, λ e τ) are both Ca²⁺ and DAG independent.

PKCs play an important role in several cellular functions such as proliferation, metabolism, vesicular traffic and cytoskeleton organization. In response to growth factors one of the mechanisms of PKCs activation is mediated by phospholypase C e D production of DAG.

- **PI3K/PKB** pathway

PI3K family includes several isoforms divided in three classes based on structural features and regulation mechanisms. (20). The most known class is the Ia that is activated in response to growth factors. Class Ia consisting of heterodimers composed of a 85 kDa regulatory subunit (p85) and of a 110kDa lipidic kinase catalytic subunit. Interaction of p85 with phosphorylated tyrosines on activated growth factors receptors or on adapting proteins induces a conformational change that modulates p110 catalytic activity. (21). Activated PI3K phosphorylates the membrane lipid phosphatidylinositol on 3’ position of inositolic ring induce an increase of phosphatidylinositol 3-phosphate (PI3P), of phosphatidylinositol 3, 4-biphosphate (PI3,4P₂) and of phosphatidylinositol 3,4,5 tri-phosphate (PI3,4,5P₃). Phosphorylate phosphatidylinositol act as second messenger and mediate PI3K effect on cytoskeleton, cell cycle, vesicular traffic, glucose metabolism and cell survival. Phospholipid activated proteins include some Ca²⁺-indipendent PKC isoforms, such as PKCδ and PKCζ (22), p70S6 kinase, small GTPase Rho e Rac and serine-threonine-kinase PKB/Akt.(23) p70S6 kinase phosphorilates 40S ribosomal subunit protein S6, inducing G1-S cell cycle transition; small GTPase Rho e Rac regulate actin cytoskeleton; PKB/Akt phosphorylates and sequestrate into cytosol proapoptotic protein Bad. Thus Bad is unable to associate to
Bcl-2 and to translocate into mitochondria to induce cytochrome C release and to trigger apoptotic signal(24) (Figure 2)

**Figure 2.** Erk 1/2 and Akt/PKB pathway in cell proliferation.

**CELL MIGRATION**

Cell migration at wound site is another fundamental event in wound healing process. The crawling movements of animal cells are among the most difficult to explain at the molecular level. Different parts of the cell change at the same time, and there is not a single, easily identifiable locomotory organelle (analogous to a flagellum, for example). Although actin forms the basis of animal cell migration, it undergoes many different transformations as the cell moves forward, assembling into lamellipodia and microspikes, associating with focal contacts, forming stress fibers, and so on. In broad terms, three distinct processes can be identified in the crawling movements of animal cells: protrusion, in which lamellipodia and microspikes (or filopodia) are extended from the front of the cell; attachment, where the actin cytoskeleton makes a connection with the substratum; and traction, where the body of the cell moves forward.
Protrusion is a function of the leading edge of the cell. Actin-rich lamellipodia and microspikes (or filopodia) extend forward over the substratum, a process that is accompanied by actin polymerization. It seems likely that the protrusion is driven by actin polymerization at the leading edge, although this is still debated. Myosin-I motors attached to the plasma membrane could also drive the cell forward by actively walking along actin filaments. Rapidly motile cells, such as white blood cells, make more diffuse contacts with the substratum. It is thought, however, that similar principles apply to focal contacts of fibroblasts: transmembrane receptors for extracellular matrix proteins link the plasma membrane to the substratum, and actin filaments in the cytoplasm interact with the cytoplasmic domains of these receptors through actin-binding proteins. The details of these important interactions are uncertain, but it is clear that the cell contacts with the substratum must be continually made and broken as the cell moves forward. The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration. During cell migration, both the composition and the morphology of the focal adhesion changes. Initially, small (0.25µm²) focal adhesions called "focal complexes" are formed at the leading edge of the cell in lamellipodia: they consist of integrin, and some of the adapter proteins, such as talin and paxilin. Many of these focal complexes fail to mature and are disassembled as the lamellipodia withdraws. However, some focal complexes mature into larger and stable focal adhesions, and recruit many more proteins such as zyxin. Once in place, a focal adhesion remains stationary with respect to the extracellular matrix, and the cell uses this as an anchor on which it can push or pull itself over the ECM. As the cell progresses along its chosen path, a given focal adhesion moves closer and closer to the trailing edge of the cell. At the trailing edge of the cell the focal adhesion must be dissolved. The mechanism of this is poorly understood and is probably instigated by a variety of different methods depending on the circumstances of the cell. Traction is perhaps the most mysterious part of cell locomotion. In many cases it is thought that the force for cell locomotion is generated near the front of the cell and that the nucleus and bulk cytoplasm are dragged forward passively. The force generation can be viewed in different ways. The leading part of the cell might actively contract like a muscle fiber and thus pull on the back of the cell. In another view polymerization of actin filaments at the front of the cell extends the actin
cortex forward, and the rear of the cell is then carried forward by the contractile force of the resulting cortical tension (Figure 3) (25).

![Figure 3: Cell movement model]

Also cell motility is triggered by binding of signal molecules to specific receptors on cell surface. Ligand-receptor interaction activates several pathway of transduction inside the cell inducing rearrangement in cytoskeleton in order to promote migration. Mitogen activated protein kinase (MAPK) pathway is one of the most important signalling system in cell migration. In particular, JNK (Jun N-terminus kinase), p38 and ERKs play a pivotal role (26).

- **JNK signaling**

  JNK is activated in response to various extracellular stimuli, including tumor necrosis factor (TNF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and lysophosphatidic acid, as well as diverse environmental stresses (27). These activate MAPKKKs: such as MEKK1 and MLK, which phosphorylate and activate two MAPKKs, MKK4 and MKK7. In turn, these phosphorylate the threonine and tyrosine residues within the Thr-Pro-Tyr motif in the JNK activation loop. Accumulating evidence implicates the JNK pathway in regulation of cell migration. First, activation of JNK correlates with an increase in cell migration in
several cell types, for example, JNK activation is closely associated with cell migration induced by EGF, ephrin B1 and CrkII. Second, the signaling molecules that activate JNK are essential for cell migration. MEK kinase 1, an upstream kinase in the JNK pathway, is essential for cell migration and the developmental process of eyelid closure. Third, inhibition of JNK by either the chemical inhibitor SP600125 or the dominant-negative mutant JNK1AF, significantly impairs the rate of migration of several different cell types. Fourth, using a gene knockout approach has been demonstrated that JNK activity plays a crucial role in the migration of fibroblasts in wound healing assays. Active JNK is found in cytoplasmic locations providing evidence for cytoplasmic functions of JNK, in addition to its established nuclear functions. Along with the various well-known transcription factors and apoptosis-related proteins that are substrates for JNK, several cytoskeleton-associated proteins and signaling molecules as well as adaptor proteins have recently been identified as JNK substrates. These include the intermediate filament protein keratin 8, microtubule-associated proteins (MAPs), such as MAP1B, MAP2, DCX and SCG10, the actin-binding protein spir, the protein kinase p90RSK, and the adaptors insulin receptor substrate 1 (Irs-1), p66ShcA and paxillin. Of these, paxillin, spir, DCX, MAP1B and MAP2 are probably directly involved in cell migration. These findings collectively implicate JNK in the control of cell migration in a broad range of cell types and in several developmental processes (28).

- **p38 signaling**

Four isoforms represent the p38 subfamily of MAPKs: p38α, p38β, p38γ and p38δ. The activity of p38 is stimulated by many growth factors, cytokines, and chemotactic substances, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), PDGF, TNF, interleukins, lipopolysaccharide (LPS) and formyl-methionyl-leucyl-phenylalanine (fMLP). The upstream MAPK cascade in this case includes MAPKKKs such as MLK3, DLK and TAK1, which phosphorylate and activate MKK3 and MKK6, which in turn phosphorylate and activate p38. It is well known that p38 is involved in inflammation, apoptosis, cardiomyocyte hypertrophy and cell differentiation. Recently, several studies have suggested it is also involved in the migration of diverse cell types. SB203580 and SB202190, inhibitors of p38, inhibit the migration of smooth muscle cells induced by PDGF, TGFβ and IL-1β, porcine aortic

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endothelial cells challenged with PDGF and VEGF, neutrophils stimulated with fMLP, mast cells treated with stem cell factors and antigen, corneal epithelial cells stimulated with hepatocyte growth factor, mouse embryonic fibroblasts challenged with PDGF and IL-1, NMuMG and MDA-MB-231 mammary epithelial cells treated with EGF and TGFβ1 and NLT neuronal cells stimulated with Gas6 (encoded by growth arrest-specific gene 6). Moreover, p38AF, a dominant negative mutant of p38, also inhibits the migration of smooth muscle cells induced by PDGF, TGFβ and IL-1β, NMuMG and MDA-MB-231 mammary epithelial cells in response to EGF and TGFβ1 and NLT neuronal cells stimulated with Gas6. Taken together, these findings demonstrate that p38 is involved in growth-factor- and cytokine-induced cell migration (28).

- Erk/MAPK signaling

The Erk MAPKs are the most extensively studied subfamily of MAPKs. Erk has been implicated in the migration of numerous cell types. The Erk pathway inhibitors PD98059 and U0126 inhibit the migration of diverse cell types in response to cell matrix proteins, such as fibronectin, vitronectin and collagen, growth factors such as VEGF, FGF, EGF, insulin and other stimuli, such as fetal calf serum and urokinase plasminogen activator (uPA). Moreover, a dominant negative Erk mutant or inhibition of Erk by an antisense strategy also inhibits cell migration. Erk is thus an important factor in the regulation of cell migration. Erk phosphorylates serine or threonine residues followed by proline. The most stringent consensus sequence is Pro-Leu-Ser/Thr-Pro. Identified substrates include several protein kinases, such as p90rsk, MSK1, MNK1/2, myosin light chain kinase (MCLK) and FAK, the protease calpain, paxillin, as well as transcription factors and nuclear proteins. Of these MLCK, calpain, and FAK are most likely to be involved in Erk-mediated cell migration. Erk regulates FAK-paxillin complex sophisticatedly: initially it promotes complex-assembly by phosphorylation of paxillin and then promote disassembly by subsequent phosphorylation of FAK. It is possible that Erk-modulated disassembly of the FAK-paxillin complex is involved in focal adhesion disassembly, but its precise role and the mechanism remain to be completely clarified. Erk also participates in cell migration by suppressing the ability of integrins to bind to their extracellular matrix ligands. It is well known that the Ras-Raf-MEK-Erk pathway regulates integrin activation (the affinity of an integrin for its
substrate), although the molecular mechanism remains to be elucidated. Because dynamic integrin activation is required for cell migration (), Erk might also play an important role regulating cell migration, by regulating integrin activation (Figure 4) (28).

Figure 4. Erk 1/2 pathway in cell migration.
WOUND HEALING IN DIABETES

Diabetic patients frequently have non-healing chronic wounds. This is due, in part, to microcirculation damage typical of diabetes. Intact and functional microcirculation is necessary for an adequate nutrition of tissues, for discarding metabolism’s products and for an effective inflammatory response. Any defect in microcirculation can cause alteration in wound healing process.

Hyperglycemia causes stiffening of vessel’s wall and a reduction of permeability to the blood obstructing inflammatory cells flux to the wound site. Furthermore, insulin absence or insulin resistance causes an ineffective carbohydrates, lipid and protein metabolism, necessary to sustain cellular activity during wound healing (29). Glucose is major source of energy for fibroblasts and polymorphonucleate cells during healing. Inability to use glucose as fuel increases oxidation of fatty acid reducing cell membranes synthesis. Proteins are essential for collagen synthesis. Collagen confers resistance to ECM deposed by fibroblasts at wound site. Has been demonstrated that reduction of chemotaxis, phagocitosys and bactericide activity, (30) increased ROS and decreased antioxidant activity (31) observed in diabetic patients, are also responsible for wound healing impairment. Decreased biodisponibility of growth factors and increased glucorticoids concentration (32) inhibit cell proliferation (33) and stimulate apoptosis (34) reducing granulation tissue formation that is a key step in wound healing process.

FIBROBLASTS DISFUNCTION IN DIABETES

Fibroblasts play a key role in wound healing process. Infact, they produce, secrete and remodel ECM and act as signal cells. It is clear that any impairment of fibroblasts’ function induces an impairment in wound healing. In diabetes, in particular, has been demonstrated an impairment in proliferation, migration and ECM synthesis. In detail, diabetic fibroblasts produce and secrete large amount of matrix metalloprotease (MMPs). Physiologically MMPs promote cell migration and healing, nevertheless increased concentration observed in diabetes act as negative regulator of both process. Moreover (vascular endothelian grow factor) VEGF production is reduced in diabetic fibroblasts. VEGF is fundamental for vascular development during tissue repair; decreased VEGF levels are responsible for a reduced angiogenetic response that impairs the following proliferation and ECM deposition phases of wound healing.(35)
THERAPIES OF DIABETIC FOOT

The optimal therapy for foot ulcers and amputations is prevention through identification of high-risk patients, education of the patient, and institution of measures to prevent ulceration. Despite preventive measures, foot ulceration and infection are common and represent a potentially serious problem. Ulcers may be primarily neuropathic (no accompanying infection) or may have surrounding cellulitis or osteomyelitis. A growing number of possible treatments for diabetic foot ulcers exist, but they have yet to demonstrate clear efficacy in prospective, controlled trials. A recent consensus statement from the ADA identified six interventions with demonstrated efficacy in diabetic foot wounds: 1) off-loading, 2) debridement, 3) wound dressings, 4) appropriate use of antibiotics, 5) revascularization, and 6) limited amputation. Off-loading is the complete avoidance of weight bearing on the ulcer, which removes the mechanical trauma that retards wound healing. Bed rest and a variety of orthotic devices or contact casting limit weight bearing on wounds or pressure points. Surgical debridement is important and effective, but clear efficacy of other modalities for wound cleaning (enzymes, soaking, whirlpools) is lacking. Dressings promote wound healing by creating a moist environment and protecting the wound. Antiseptic agents and topical antibiotics should be avoided. Referral for physical therapy, orthotic evaluation, and rehabilitation may be useful once the infection is controlled. Mild or non-limb-threatening infections can be treated with oral antibiotics (cephalosporin, clindamycin, amoxicillin/clavulanate, and fluoroquinolones), surgical debridement of necrotic tissue, local wound care (avoidance of weight bearing over the ulcer), and close surveillance for progression of infection. More severe ulcers may require intravenous antibiotics as well as bed rest and local wound care. Urgent surgical debridement may be required. New information about wound biology has led to a number of new technologies (e.g., living skin equivalents and growth factors such as basic fibroblast growth factor) that may prove useful. Recombinant platelet-derived growth factor has some benefit and complement the therapies of off-loading, debridement, and antibiotics. Hyperbaric oxygen has been used, but rigorous proof of efficacy is lacking.(1). Another option for treating a patient with a diabetic foot ulcer is the use of platelet releasate (PR). PR is an autologous product obtained by in vitro activation of autologous platelet used in...
conjunction with standard care, which involves covering the wound with saline impregnated gauze and instructing the patient to avoid weight bearing activities on the affected limb (36). A recent study demonstrated that platelet releasate was more effective than standard care (37).

<table>
<thead>
<tr>
<th>Group</th>
<th>No PR: proportion healed by 32 weeks</th>
<th>PR: proportion healed by 32 weeks</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>46.6 (45.2–48.0)</td>
<td>51.9 (47.1–56.7)</td>
</tr>
<tr>
<td>2</td>
<td>46.6 (45.1–48.0)</td>
<td>55.0 (51.6–58.5)</td>
</tr>
<tr>
<td>3</td>
<td>40.4 (38.0–41.0)</td>
<td>49.8 (47.0–52.7)</td>
</tr>
<tr>
<td>4</td>
<td>35.2 (33.7–36.7)</td>
<td>49.1 (46.6–51.6)</td>
</tr>
<tr>
<td>5</td>
<td>31.6 (30.0–33.3)</td>
<td>48.5 (46.5–50.6)</td>
</tr>
<tr>
<td>Overall</td>
<td>41.0 (40.3–41.7)</td>
<td>50.0 (48.7–51.2)</td>
</tr>
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**Table 1:** Effectiveness of PR treatment compared to standard therapy. Patient are collected in groups on basis of growing seriousness of the lesion.

The effect was greatest in those with the most severe wounds, i.e., large wounds that affect deeper anatomical structures. In spite of 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.
AIM OF THE STUDY

Diabetes’ incidence is growing up making it one of the most common health problem in western countries. Chronic complications, in particular, cause a significant decrease in life span and life quality of diabetic patients and have a huge cost to public health worldwide. It was calculated that, in 2006, the US spent an estimated $22.9 billion on direct medical costs related to diabetes complications. Diabetic foot is one of the complications that has major impact on life quality and life span of affected patients. Several therapeutic tools has been used to care it but no one of them is completely effective. Attention has been given to the clinical utilization of individual growth factors, such as PDGF (38), and to autologous platelet factors as useful therapeutic tools to accelerate tissue repair and regeneration. The overall experience with recombinant PDGF in wound healing has not been completely successful (39, 40-42). This is not surprising, however, given the consideration that wound healing is the outcome of an intricated 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. 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 (43). Current therapeutic application of PG includes a number of pathological conditions requiring bony and soft tissue reconstruction (44,45). In fact, beside the important haemostatic 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 (46). In spite of 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. Aim of this work has been to clarify in further detail the molecular mechanisms responsible for effectiveness of TAPs in care of diabetic foot and the mechanisms responsible of its eventual failure.
METHODS

**Materials** - Media, sera, and antibiotics for cell culture were purchased from Invitrogen Ltd. (Paisley, United Kingdom). Rabbit polyclonal ERK 1/2 antibodies, rabbit polyclonal Akt antibodies, rabbit polyclonal antibodies PKCα and antibodies toward the phosphorylated forms of PKCα were from Santa Cruz Biotechnology (Santa Cruz, Calif.), and antibodies toward the phosphorylated forms of the Akt/PKB and ERK 1/2 were from Cell Signal Technology (Beverly, Mass). Mouse monoclonal IGF-1R and phospho-tyrosine antibodies and rabbit polyclonal PDGF-Paxillin antibodies were from Zymed Laboratories (Invitrogen Corporation, Calif.). Rabbit polyclonal fibronectin antibodies were from Chemicon (Millipore Corporation). Western blotting, ECL reagents and radiochemicals were from Amersham (Arlington Heights, Ill.). Electrophoresis reagents were from BioRad. Tyrphostins and mytomycin C were from Sigma-Aldrich (St. Louis, Mo.)

**Cell culture and cell growth** - Shoulder skin fibroblasts were obtained by punch biopsy and cultures established as previously described (47). The cells were grown at 37°C in DMEM supplemented with 10% fetal calf serum in a 5% CO₂-95% 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.

**Platelets’rich plasma and Platelet Gel** - Platelets’rich plasma (PRP) was 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 (48). The preparation procedures for platelets were performed according to standardized methods (49, 50). For PG preparations, thrombin (1:10 vol/vol) and calcium gluconate (1:10 vol/vol) were added to PRP for 5 min at room temperature. 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 cm² aliquots of PG were applied to cells plated in 100 mm diameter dishes.

*Thymidine incorporation* - 10⁵ cells/plate were seeded in 1 ml of DMEM supplemented with 10% fetal bovine serum in six-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 replaced with the same media supplemented with [³H]thymidine (500 nCi/ml). After 1-h incubation, media were removed and cells washed three times with ice-cold phosphate-buffered saline. The cell monolayers were solubilized in 1 ml of SDS (0.1%) solution for 30 min at 37 °C. An equal volume 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 Na₂PO₄, 2 mM Na₃VO₄, 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 5,000 x 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, Mass.). Upon 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) according to the manufacturer’s instructions.

*ERK activity* – ERK activity was assayed as previously described (51). Briefly, cell lysates (200µg 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 Na₄P₂O₇, 2 mM Na₃VO₄, 10% glycerol, 1% Triton X-100), twice more with HNTGVa buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM Na₃VO₄, 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-32P]ATP. Upon 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 activity - Akt activity was assayed in vitro as previously reported (52). Briefly, the cells were solubilized in lysis buffer and lysates were clarified by centrifugation at 5,000 x g for 20 min. 200 μg of the lysates were immunoprecipitated with Akt/PKB antibodies. The precipitates were incubated in a kinase reaction mixture containing 20 mM HEPES [pH 7.2], 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, 5 mM ATP, 0.2 mM EGTA, 1 mM protein kinase inhibitor, 10 μCi of [γ-32P]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% H₃PO₄, and disk-bound radioactivity was quantified by liquid scintillation counting.

Scratch assay - To analyze cell migration, confluent monolayers of cells on well of 6-wells tissue culture dishes were wounded by manually scratching with a pipette tip, washed with PBS, incubated at 37°C. Wound closure was monitored and photographed at 0 and 24 h at the same location using the grid as a marker. Similar assays were performed in the presence of mitomycin C (10 μg/ml; Sigma) to rule out the potential contribution of differences in cell proliferation. Images of areas were collected with a Canon Powershot digital camera coupled to the microscope and percentage of healing was calculated with NIH IMAGE J. These experiments were repeated at least three times with similar results using two different isolations of fibroblasts.
Confocal microscopy - Subconfluent cells on glass coverslips were fixed for 20 minutes with 4% paraformaldehyde (Sigma) in PBS containing 0.9 mM calcium and 0.5 mM magnesium (PBS CM) at room temperature, washed twice in 50 mM NH4Cl in PBS CM and twice in PBS CM. Cells were permeabilized for 5 minutes in 0.5% Triton-X 100 (Bio-Rad) in PBS CM and washed twice, for 10 minutes, in 0.2% gelatin (Sigma) in PBS CM. Cells were then incubated for 1 hour with the primary antibodies diluted in 0.5% BSA (Sigma) in PBS. After three washes with 0.2% gelatin in PBS CM cells were incubated for 20 minutes with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA), diluted 1:50 in 0.5% BSA in PBS. To visualize actin filaments, permeabilized cells were incubated with a 1:70 dilution of rhodamine-conjugated phalloidin (Sigma) for 20 minutes. After final washes with PBS, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in PBS and examined with a Zeiss LSM 510 version 2.8 SP1 Confocal System.
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 (53,43). Seven consecutive diabetic patients with grade II/III ulcers at the lower extremities according to Wagner (54), were treated with local applications of TAPs. Pertinent clinical data of participants has been previously described (48). After two weeks of treatment in 90% of patients the ulceration size was reduced by about 40%. In order to elucidate the molecular mechanisms by which TAPs induce cell growth, the key mediators of survival and proliferation signals activation Akt/PKB and ERK1/2 has been investigated in vivo. Specimens derived from peri-lesional biopsies, taken before (day 0) and after (weeks 1 and 2) repeated topical applications of TAPs, were solubilized and probed with antibodies against phosho-Ser^473^-Akt/PKB and phosho-Thr^202/Tyr^204^-ERK1/2 (Fig. 5).

![Figure 5](image.png)

**Figure 5. Effect of thrombin-activated platelets on ERK 1/2 and Akt/PKB in peri-lesional skin biopsies.** PG was applied twice a week to skin lesions of 12 consecutive diabetic patients, diagnosed with grade II/III ulcers. Peri-lesional skin biopsies were taken at indicated time. The specimens were solubilized and immuno-blotted with specific anti-phospho antibodies (Akt/PKB and ERK1/2). The bar graphs represent means ± S.D. of the densitometric values of the bands obtained by four independent experiments (n = 12). The autoradiographs in the upper insets show a representative experiment.
Both Akt/PKB (Fig. 5A) and ERK1/2 phosphorylation (Fig. 5B) were increased upon 1 week 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, at week 2 Akt/PKB activation 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, topical application of TAPs increases local activation of Akt/PKB and ERK1/2.

**In vitro effect of thrombin-activated platelets on human fibroblasts cell growth.** To analyze the molecular details of TAPs-induced cell growth, 1 x 10⁹ human platelets were activated with thrombin and applied in gel form (1 cm²) to the surface of dishes containing cultured human fibroblasts (1 x 10⁵) 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 and the cells became confluent after 4 days. 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 in the presence of 10% fetal bovine serum (Fig. 6 A).
Figure 6. Effect of thrombin-activated platelets on cell growth in cultured human fibroblasts. A. $10^5$ cells were plated and supplied with complete medium (10% BS) or serum-free media or serum free (-). Where indicated a 1 cm$^2$ aliquot of TAPs, 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 with a Neubauer chamber. Data represent means ± s.d. of six independent experiments in duplicate. B. 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 DMEM 0.25% albumin with or without PG. 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 measured as described in Materials and Methods. The bar graph represents means ± s.d. of three independent experiments in triplicate.

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. 6 B).

**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. 7). Akt/PKB phosphorylation peaked at 15 min, then remaining stable up to 48h (Fig. 7 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. 7 B). Comparable results were obtained by measuring Akt/PKB and ERK1/2 activities (Fig. 7 C).
Figure 7. Effect of thrombin-activated platelets on ERK1/2 and Akt/PKB activation in human fibroblasts. Cells were treated with thrombin-activated platelets for the indicated time and solubilized. Western blot with anti-phosphospecific 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 ± s.d. of three independent experiments in triplicate.

To address whether the activation of Akt/PKB and/or ERK1/2 was required for the TAPs 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. 8), indicating that these molecular pathways are involved in TAPs-induced cell growth.
Figure 8. Role of ERK1/2 and Akt/PKB in TAPs-induced thymidine incorporation. Thymidine incorporation experiments were performed, as previously described in the absence or in the presence of 50 µM PD 98059 and or 100 µM LY294002. The bar graph represents means ± s.d. of three independent experiments in triplicate.

Activation of tyrosine kinase receptors by thrombin-activated platelets. Both Akt/PKB and ERK1/2 are often downstream target of tyrosine kinase signaling (46). Therefore, we analyzed the pattern of tyrosine phosphorylated proteins in human fibroblasts in the absence or in the presence of TAPs (Fig. 9A). Interestingly, TAPs treatment of the cultured cells rapidly (15 min) increased tyrosine phosphorylation of a 205 kDa species, which remained stable up to 48h. In addition, phosphorylation of another major 95 kDa band was detected. Tyrosine phosphorylation of the latter molecular species progressively increased in a time-dependent manner.
Figure 9. Effect of thrombin-activated platelets on receptor tyrosine kinases. A. Thrombin-activated platelets were applied to fibroblasts for the indicated times. Cells were solubilized and western blot with anti-phosphotyrosine antibodies was performed. The experiment has been repeated six times with comparable results. The autoradiograph shows one representative experiment. B. Thymidine incorporation experiments were performed as previously described in the absence or in the presence of specific AG1296 (PDGF-R inhibitor), AG 1024 (IGF1-R inhibitor) or SU1498 (VEGF-R inhibitor). The bar graph represents means ± s.d. of three independent experiments in triplicate.

Platelets are a major source of growth factors, which are bona fide activators of tyrosine kinase receptors (TKR) (53). We then tested the effect of specific TKR inhibitors (tyrphostins) on TAPs-induced cell growth. 10 µM AG1296 (a PDGF-R inhibitor) and 10 µM AG1024 (an IGF-1-R inhibitor) reduced thymidine incorporation by 50% and 60%, respectively (Fig. 9 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 pre-treatment of the cells with tyrphostins (Fig. 10). Akt/PKB activity was reduced by >80% by AG1296 PDGF receptor kinase inhibitor, both upon 15 min and upon 24h TAPs addition. No significant change was observed following AG1024 and SU1498 pretreatment (Fig. 10 A). AG1296 only slightly reduced (about 20%) the acute (15 min) activation of
ERK/12. By contrast, the AG1024 inhibitor decreased by about 50% the activity of ERK1/2 induced by 15 min incubation with TAPs (Fig. 10 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 upon SU1498 pretreatment of the fibroblasts (Fig. 10 B).

Figure 10. 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, AG 1024 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 ± s.d. of three independent experiments in triplicate.
Release of growth factors by platelets and fibroblasts. Since 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. 11). In TAPs derived media, PDGF-AB concentration raised in 15 min, and remained stable up to 48 h (Fig. 11 A). Almost no PDGF was detectable in media from cultured fibroblasts. PDGF levels in media from fibroblasts co-incubated with TAPs were slightly lower than those determined in media from TAPs alone, and the difference was significant (p<0.05) at 24 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. 11 B). No time-dependent changes were observed in these media, however. Different from PDGF, co-incubation 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 which have been pre-exposed for 24 h to either TAPs alone, or to cultured fibroblasts alone or to TAPs-stimulated fibroblasts prior to analyze protein tyrosine phosphorylation (Fig. 12 A). No significant change was detected upon incubation of the cells with conditioned media from fibroblasts alone compared to cells incubated with serum-free media. However,
tyrosine phosphorylation levels of the 205 kDa and of the 95 kDa species were increased by conditioned media from TAPs and from TAPs-stimulated fibroblasts. Interestingly, phosphorylation of the 95 kDa band was further 3-fold enhanced by conditioned media from TAPs-stimulated fibroblasts, as compared to TAPs alone. At variance, tyrosine phosphorylation of the 205 kDa protein was slightly decreased, when the cells were incubated with conditioned media derived from TAPs-stimulated fibroblasts, compared to TAPs alone. The 205 kDa and the 95 kDa bands co-migrated, respectively, with the full-length PDGF-R (Fig. 12 B) and the IGF-1-R beta subunit (Fig. 12 C)

Figure 12. Effect of conditioned media on RTK phosphorylation. Human fibroblasts were incubated for 15 min with media which have been pre-exposed for 24 h to either thrombin-activated platelets (TAPs), or serum-starved cultured fibroblasts (Fibro) or to TAPs-stimulated fibroblast (TAPs+Fibro). Phosphotyrosine blotting (A) were 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.

In addition, TAPs conditioned media increased by 4-fold both Akt/PKB (Fig. 13 A) and ERK1/2 activity (Fig. 13 B). No effect was detectable with conditioned media derived from fibroblasts alone. TAPs-stimulated fibroblasts conditioned media, 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, further enhancing the effect of TAPs alone.
Figure 13. Effect of conditioned media on Akt/PKB and ERK1/2 activation. Human fibroblasts, pre-treated or not with AG 1296 or AG1024, were incubated for 15 min with media which have been pre-exposed for 24 h to either TAPs, or serum-starved fibroblasts or to 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 ± s.d. of three independent experiments in triplicate.

To further assess whether TAPs-stimulated fibroblast-released factors were responsible for the activation of Akt/PKB and ERK1/2, human fibroblasts were pre-treated with AG1296 or AG1024 before stimulation with conditioned media. AG1296 inhibited by >80% Akt/PKB (Fig. 13 A) and by only 20% ERK1/2 (Fig. 13 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 induced respectively, by TAPs and TAPs-stimulated fibroblasts conditioned media.
Characterization of TAPs resistant patients As previously mentioned in about 10% of patients TAPs treatment did not ameliorate wound healing. These patients have been already genetically and clinically characterized in a previous study(6). The common trait among these patients was ped/pea-15 overexpression (Fig. 14).

Figure 14. Evaluation of ped/pea-15 expression in peri-lesional biopsies of TAPs resistant patients. Peri-lesional biopsies were collected from TAPs sensitive and TAPs resistant patients. Specimens were solubilized as previous described (47). Western blot with anti-PED/PEA-15 antibody was performed. Data points are the means of three independent determinations in each individual subject.

PED/PEA-15 effect on wound healing in vitro To verify that ped/pea-15 overexpression could be responsible for TAPs resistance, an in vitro model of wound healing was set up. Fibroblasts were isolated from ped/pea-15 overexpressing mice (Tg) and from non-transgenic littermates (Wt) as previous described (47) and subjected to scratch assays. Confluent monolayer was scratched and photographed at 0 and 24 hours after wounding. Percentage of healing was calculated as described in materials and methods. Hyperglycaemia plays a pivotal role in development of diabetic complications, including wound healing dysfunction (30-35). For this reason, scratch assay was performed in cells cultured both in 5,5 mM glucose (Low glucose-Lg) and in 22,5 mM glucose (High glucose-Hg). Wt and Tg fibroblasts showed only a slight difference in healing capacity in Lg, (Wt 54%±24 vs Tg 50%±15; P=0,27). By contrast, in Hg healing ability of Tg fibroblasts was significantly decreased compared to control (Wt:55%±23 vs Tg:40%±22; P<0,005) (Fig. 15).
Figure 15. **PED/PEA-15 effect on wound healing in vitro.** Fibroblasts were isolated from ped/pea-15 overexpressing mice (Tg) and from non-transgenic littermates (Wt) as previously described (reference TAPs) and subjected to scratch assays. Confluent monolayer was scratched and photographed at 0 and 24 hours after wounding. Percentage of healing was calculated as described in materials and methods. Scratch assay was performed in cells cultured both in 5.5 mM glucose (Low glucose-Lg), in 22.5 mM glucose (High glucose-Hg) and in 22.5 mM glucose in presence of 10 µg/ml mitomycin C. The bar graphs represent means ± S.D. of healing values obtained by four independent experiments (n = 12).

In *vivo* and *in vitro* wound closure is due to both proliferation and motility of the cells. To determine whether the effect of ped/pea-15 was due to alteration of migration or mitosis, the healing capacity of fibroblasts was assessed after treatment with mitomycin C, an irreversible inhibitor of mitosis. Pretreatment with mitomycin C decreased healing percentage of both genotypes. Interestingly Tg fibroblasts’ healing capacity is still reduced compared to the control (Wt: 37%±14 vs Tg: 24%± 8,22;P<0,05) (Fig. 15). These data and the absence of significant differences in thymidine incorporation (data not shown) suggest that ped/pea-15 effect on wound healing is due to alterations of cell motility.
**PED/PEA-15 effect on PKCα and ERK 1/2 activation.** Ped/pea-15 regulates several kinases involved in cell motility such as PKCα and ERK 1/2 (5, 68). In particular, PKCα and Erk 1/2 are also positively regulated by glucose (70,71). Since ped/pea-15 induced wound healing defect is enhanced by high glucose, activation of these two kinases was evaluated by western blot with phospho-specific antibody in Lg and Hg condition (Fig. 16).

![Western blot images for PKCα and ERK1/2 activation](image)

*Figure 16. Effect of PED/PEA-15 on ERK1/2 and PKCα activation.* Wt and Tg fibroblasts cultured in Lg (A and B) and Hg (C and D) were solubilized. Western blot with anti-phosphospecific ERK1/2 (A and C upper panel) and PKCα (B and D, upper panel) antibodies was performed. As control, a western blot with anti total ERK1/2 and PKCα was also performed (A, B, C and D lower panels). Autoradiographs representative of four independent experiments in duplicate are shown.

As expected, both PKCα and ERK 1/2 activation showed a glucose-dependent increase. Nevertheless, ped/pea-15 overexpression, in both culture conditions, further increased activation of these two kinases compared to the control. These data suggest a possible involvement of both kinases in ped/pea-15 induced wound healing alteration.
Effect of PKCα and ERK 1/2 inhibition on PED/PEA-15 induced wound healing alteration

To clarify the role of ped/pea-15 induced hyperactivation of PKCα and ERK 1/2 in wound healing, scratch assays with specific inhibitors of these kinases were performed. Confluent monolayers of Wt and Tg fibroblasts were scratched in Hg in presence of bisindolylmaleimide (an inhibitor of the classical PKC pathway) and PD98059 (an inhibitor of the MEK-ERK1/2 pathway) and percentage of healing was evaluated at 24h (Fig. 17).

![Graph showing the effects of PKCα and ERK 1/2 inhibition on wound healing.](image)

Figure 17. Role of ERK1/2 and Akt/PKB in PED/PEA-15-induced wound healing alteration. Scratch assay experiments were performed, as previously described in the absence or in the presence of 100 nM bisindolylmaleimide (BDM) or 30 μM PD 98059. The bar graph represents means ± s.d. of three independent experiments in triplicate.

Inhibition of classical PKCs reduced healing percentage of both genotypes but it was not able to revert the effect of ped/pea-15 overexpression (Wt 42%±8,8 vs Tg 23%±2,4; P<0,05). By contrast, PD98059 pretreatment reverted almost completely ped/pea-15 induced wound healing alteration (Wt:55%±15; Tg:52%±14).
**PED/PEA-15 effect on cell cytoskeleton** Cell migration is a complex and finely regulated phenomenon. Several cellular structures, including actin filaments, focal adhesion plaques and several components of ECM (such as fibronectin), are continually made and broken as the cell moves forward. To further characterize motility defect induced by ped/pea-15, organization of these components was investigated by immunoflorescence analysis.

To study stress fibres organization, Wt and Tg fibroblasts were stained with rhodamine–phalloidin (Fig. 18 A upper and middle panel). Focal adhesion plaques formation and extracellular matrix component fibronectin were evaluated by immunostaining with specific anti-paxillin (Fig. 18 B upper and middle panel) and anti-fibronectin (Fig. 18 C upper and middle panel) antibodies. In Hg, Tg fibroblasts showed a decrease of stress fibres formation, focal adhesion plaques number and length and altered fibronectin organization in the extracellular space compared to control.

![Figure 18. PED/PEA-15 effect on cell cytoskeleton.](image)

To study stress fibres organization, Wt and Tg fibroblasts were stained with rhodamine–phalloidin (A upper and middle panel). Focal adhesion plaques formation and extracellular matrix component fibronectin were evaluated by immunostaining with specific anti-paxillin (B upper and middle panel) and anti-fibronectin antibodies (C upper and middle panel). Effect of ERK 1/2 inhibition was also evaluated (A, B, C, lower panel). Images representative of four independent experiments are shown.
To understand if ERK 1/2 hyperactivation was responsible even for ped/pea-15 induced cytoskeleton alterations, stress fibres formation, focal adhesion plaques number and fibronectin organization was evaluated in presence of 30 µM PD98059 (Fig. 18 A, B, and C lower panel ). Erk 1/2 inhibition reverts ped/pea-15 induced cytoskeleton alterations. These data, together with scratch assays results, suggest that the synergistic effect of glucose and ped/pea-15 in alteration of cell motility is mediated by Erk 1/2.
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 (53). According to recent reports (55-57), treatment with autologous platelets represents an important therapeutic tool for diabetic patients with ulcers at the lower extremities. I sought therefore to investigate whether activated platelets could induce a cellular growth response when applied to ulcerative skin lesions of diabetic individuals. I found that the growth-related molecules Akt/PKB and ERK1/2 were activated in the peri-lesional skin following TAPs applications. I 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 is consistent with recent evidence reporting the use of platelet extracts for culturing stem cells of mesenchimal origin (58) and support the possible utilization of human platelet factors as a substitute for animal serum for cell-based therapeutic applications. The effect of PG, however, was cell-specific, as we failed to observe a similar growth induction in human umbilical vein endothelial cells (data not shown).

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 (59), 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-co-cultured 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 hours. At variance, ERK1/2 activity progressively increased, reaching maximal activation only upon 48 h of TAPs exposure. This sustained activation of ERK1/2 was also detected in the bioptical specimens of the ulcerative lesions upon 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 (60), 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 levels time-dependently increased in the supernatant of TAPs-stimulated human fibroblasts. These same conditioned media were also capable to stimulate IGF-1 receptor tyrosine phosphorylation and ERK1/2 activation when added to recipient fibroblasts, while conditioned media from isolated fibroblasts or TAPs preparations were not. These data are consistent with the hypothesis that IGF-1 autocrine production by fibroblast is needed to propagate TAPs proliferative signals. This 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 (61-63).

It is conceivable that platelet factors stimulate fibroblast production and release of IGF-1, 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, suggesting 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 (64). Consistently, Loot et al. (65) 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.

PG treatment was able to ameliorate wound healing in a large percentage of patients. Nevertheless it was not effective in 100% of cases. To clarify the molecular mechanisms responsible of PG resistance represents therefore an important issue to improve its effectiveness. In my study approximately 10% of patients undergoing TAPs treatment had no significant results compared to standard treatment. Interestingly, ped/pea-15 overexpression was found virtually in peri-lesional biopsies of all resistant patients. Indeed, previous reports indicate that the ped/pea-15 gene is hyperexpressed in fibroblasts derived from diabetic patients and in their first degree relatives (6). Moreover, Ped/Pea-15 overexpression leads to abnormal glucose tolerance, insulin resistance and impaired insulin secretion in transgenic mice, which progress to overt diabetes if the animals are fed an high fat diet (7). In addition it has been recently shown that ped/pea-15 expression also regulates motility of both normal and neoplastic cells(66, 67). I therefore sought to investigate if ped/pea-15 overexpression could be responsible for PG treatment failure. To this aim, an in vitro model of wound healing was set up. Fibroblasts isolated from both transgenic mice overexpressing ped/pea-15 and from non transgenic littermates were subjected to scratch assay. Since in
development of diabetic complication hyperglycemia plays a pivotal role, scratch assay were performed in culture media containing both 5,5 mM and 22,5 mM glucose, representative of euglicemic and hyperglicemic condition, respectively. Interestingly, ped/pea-15 induces significant alteration of wound healing only in high glucose. This is not due to difference in proliferative ability of the cell type but, most likely, to alteration in motility as demonstrated by thymidine incorporation and scratch assay in presence of mitosis inhibitors. Cell motility is a complex and tightly regulated phenomenon. Several kinases are involved in its regulation. I focused my attention on PKCα and Erk 1/2. Both kinases are positively regulated by ped/pea-15 and by glucose. Scratch assay in presence of specific inhibitors of these kinases demonstrated that ped/pea-15 induced wound healing alteration was mediated by Erk 1/2. Ped/pea-15 binds, activates and keeps into the cytosol Erk 1/2. Cytosolic activity of Erk 1/2 is responsible for suppression of integrin activation (68). Indeed, ERK 1/2 inhibition by PD 98059 reverts not only extracellular fibronectin disorganization but also stress fibres formation, number and length of focal adhesion plaques. Active integrins trigger activation of several downstream target such as RhoA and components of adhesion plaques such as FAK. RhoA GTPase is responsible for organization of stress fibres (69), while cysolic kinase FAK is a key component of assembly of focal adhesion plaques. Furthermore, it has been recently demonstrated that Erk regulates FAK-paxillin complex sophisticatedly: initially it promotes complex-assembly by phosphorylation of paxillin and then promote disassembly by subsequent phosphorylation of FAK (28). Then, an increase of ERK 1/2 cytosolic activity could perturb normal focal adhesion plaque assembly and turnover. Thus, these data suggest that ped/pea-15 induced cell motility defect could be mediated by increased ERK 1/2 cytosolic activity and suppression of integrin activation.


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32. Hunger-Glaser I., Salazar E.P., Sinnett-Smith J., Rozengurt E. Bombesin, lysophosphstidic acid, and episermal growht factor rapidly stimulate focal


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