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PDGF AND SIGNAL TRANSDUCTION IN HEPATIC STELLATE CELLS

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1. ABSTRACT

Platelet-derived growth factor (PDGF) is one of the most potent mitogen for cultured HSC isolated from rat, mouse, or human liver. Phosphotyrosines on the activated PDGF receptor operate as high affinity binding sites for several molecules involved in the downstream propagation of the signal, including the sequential activation of Raf-1, MEK and extracellular-signal regulated kinase (ERK). Nuclear translocation of ERK is associated to the phosphorylation of several transcription factors, including Elk-1 and SAP, and represents an absolute requirement for triggering a proliferative response. Phosphatidylinositol 3-kinase (PI 3-K), is another molecule recruited by the activated PDGF receptor. In human HSC cultures, PI 3-K activation is necessary for both mitogenesis and chemotaxis induced by PDGF. In addition, PI 3-K is involved in the activation of the Ras-ERK pathway in human HSC, although it is not strictly necessary, since established PI 3-K inhibitors inhibit ERK activation only by 40-50%. Therefore, in HSC, PI 3-K regulates PDGF-related mitogenesis and cell migration by pathways that are at least in part independent of ERK activation. Accumulated evidence indicates that the induction of replicative competence by PDGF is dependent on the maintenance of sustained increase in $[Ca^{2+}]_i$ due to calcium entry rather than from the release from intracellular stores. In addition, stimulation with PDGF increases the activity of the Na^+/H^+ exchanger in rat or human HSC with consequent sustained changes in intracellular pH.

2. DISCUSSION

Growth factors, cytokines, chemokines, oxidative stress products and other soluble factors play a role in the activation of hepatic stellate cells (HSC) and in the

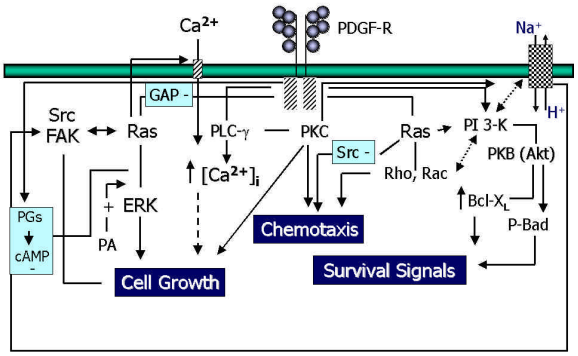

Figure 1. PDGF signaling. PDGF binds to a receptor with intrinsic tyrosine kinase activity. Receptor dimerization leads to autophosphorylation with formation of hygh affinity binding sites for signaling proteins with SH-2 or PTB domains. The downstream pathways are differentially implicated in the regulation of the biologic activities of PDGF.

erpetuation of their activated phenotype during active hepatic fibrogenesis (1,2). In the presence of chronic liver tissue damage/inflammation, these factors directed at specific cell targets, are simultaneously active in the tissue and are, at least in part, responsible for the fibrogenic outcome of the wound healing process.

Among other polypeptide growth factors potentially involved in the chronic wound healing response, platelet-derived growth factor (PDGF), a dimer of two polypeptide chains referred to as A- and B-chain, is the most potent mitogen for cultured HSC isolated from rat, mouse, or human liver (3-5). Of the three possible dimeric forms of PDGF (-AA, -AB, and -BB), PDGF-BB is most potent in stimulating HSC growth and intracellular signaling, in agreement with a predominant expression of PDGF-receptor β (or type B) subunits compared to PDGF-receptor α (or type A) subunits in activated HSC (5). Importantly, co-distribution of PDGF with cells expressing PDGF receptor subunits has been demonstrated following both acute and chronic liver tissue damage (6,7), thereby confirming an active role of this growth factor in liver repair and fibrosis. In addition, PDGF is pro-fibrogenic in conditions where inflammation is less evident such as experimental cholestatic liver injury (8,9). In this setting, PDGF synthesis and release is sustained by proliferating bile duct cells. In addition, in liver tissue obtained from patients with chronic liver diseases, expression of PDGF and its receptor subunits appears strictly correlated with the extent of necroinflammation and fibrosis (7).

Based on its importance to stellate cell growth, considerable effort has been invested in understanding the intracellular signaling events elicited by the interaction of PDGF with its receptor. Figure 1 illustrates the major signaling pathways and biologic effects elicited by the interaction of PDGF with its membrane receptors, i.e. cell proliferation and migration. This response is the net result of the activation of both positive and negative intracellular signals, and, accordingly, each pathway leading to a specific effect is often provided with an autoregulation loop. PDGF receptors, which have intrinsic tyrosine kinase activity, dimerize and become auto-phosphorylated on tyrosine residues upon binding to their ligand (10). The phosphotyrosines on the activated receptor operate as high affinity binding sites for several molecules involved in the downstream propagation of the signal, which bind through src-homology-2 (SH-2) domains or phosphotyrosine-binding (PTB) domains (11,12). Association of the PDGF receptor with the adapter protein Grb2 leads to recruitment of the exchange factor mSos with the consequent recruitment and activation of Ras. This event is followed by the sequential activation of Raf-1, MEK and extracellular-signal regulated kinase (ERK) (13). Two isoforms of ERK have been described, and are referred to as ERK-1 (or $p44^{MAPK}$) and ERK-2 (or $p42^{MAPK}$) (14). Nuclear translocation of ERK is associated to the phosphorylation of several transcription factors, including Elk-1 and SAP, and represents an absolute requirement for triggering a proliferative response (15). In cultured human HSC, there is activation of the ERK pathway followed by increased expression of *c-fos* in response to PDGF (16-18). The activation of this pathway is necessary for PDGF-induced cell proliferation and, accordingly, the pharmacological blockade of signaling molecules upstream of ERK (e.g. MEK) leads to a dose-dependent inhibition of cell growth. This observation is supported by the reduction in the downstream activation of the proto-oncogene *c-fos* and of the AP-1 complex binding activity that follows the inhibition of PDGF-induced ERK activation (18). Inhibition of ERK is associated with a reduction of STAT-1 activation induced by PDGF, although the potential role of the cross talk occurring between ERK and STAT-1 in PDGF-induced cell growth is presently unclear. Work by Reeves et al. indicate that PDGF-induced generation of the lipid second messenger phosphatidic acid (PA) contributes to ERK activation in rat HSC, an action attributed to a positive interaction of PA with signaling molecules upstream of ERK (19).

ERK activation in rat HSC occurs following *in vivo* liver injury induced by the acute administration of CCl_4 (18). In this model, increased ERK activity temporally precedes HSC proliferation and peaks at 48 hrs after the administration of the toxin. Remarkably, this time point is associated with maximal availability of PDGF in acutely injured liver tissue (6), and precedes HSC proliferation, which begins at 48 hrs and peaks at 72 hrs after liver damage.

Studies which employ other drugs to interfere with pathways upstream of ERK provide additional evidence for a key role of ERK in mediating PDGF-induced cell growth in HSC. Pre-incubation of HSC with drugs increasing intracellular cAMP levels such as pentoxifylline, a phosphodiesterase inhibitor, leads to a remarkable reduction in the PDGF-induced ERK phosphorylation and activity, *c-fos* expression, and mitogenesis (20). Other examples of upstream ERK antagonists are compounds that increase prostaglandin (PG) E_2 synthesis, which act via an increase in intracellular cAMP (21-23). Interestingly, stimulation of HSC with

PDGF leads to increased synthesis and release of PGE_2. This causes in turn an autocrine increase in intracellular cAMP leading to a self-limitation of PDGF mitogenic potential (21,22). Increased levels of intracellular cAMP may inhibit PDGF-induced cell growth with two main mechanisms: A. inhibition of Raf kinase, an upstream activator of ERK, occurring through phosphorylation of Raf-1 by cAMP-activated protein-kinase A (PKA) (24,25), and B. inhibition of STAT1 activation, as demonstrated by Kawada and co-workers (26).

Phosphatidylinositol 3-kinase (PI 3-K), another molecule that is recruited by the activated PDGF receptor, is comprised of a 85 kDa regulatory subunit, equipped with two SH-2 domains, and a catalytic 110 kDa subunit (27). PDGF stimulation leads to the association of PI 3-K with the activated receptor, and to tyrosine phosphorylation of p85 but not of p110. The downstream effectors of PI 3-K activation are only partially known and include protein kinase Cζ, ribosomal S6 kinase, and protein kinase B (c-Akt). Nevertheless, this pathway is sufficient to transduce PDGF-dependent mitogenic signals (28) and to be necessary for cell chemotaxis (29). Thus, in human HSC cultures, PI 3-K activation is necessary for both mitogenesis and chemotaxis induced by PDGF (30). The in vivo relevance of this finding is suggested by the recruitment of the p85 subunits by the PDGF-receptor and activation of PI 3-K following acute CCl_4-induced liver damage in the rat. Wortmannin, a fungal metabolite that binds and non-competitively inhibits PI 3-K, induces a dose-dependent inhibition of PDGF-BB-induced PI 3-K activation in HSC with a maximal effect at 100 nM. This concentration, which does not affect either PDGF receptor autophosphorylation or the physical association between the PI 3-K p85 subunit and the receptor, virtually abolishes PDGF-induced mitogenesis and chemotaxis in HSC, indicating a functional involvement of this pathway. Similar observations have been made with other PI 3-K inhibitors such as LY294002 (31). In addition, PI 3-K is involved in the activation of the Ras-ERK pathway in human HSC, although it is not strictly necessary, since both wortmannin and LY294002 inhibit ERK activation only by 40-50% (16,31). Therefore, in HSC, PI 3-K regulates PDGF-related mitogenesis and cell migration by pathways that are at least in part independent of ERK activation.

Although all three PDGF isoforms are able to activate PI 3-K, PDGF-BB is more potent than PDGF-AA. Accordingly, dose-response experiments indicate that the ability of PDGF-AA or -BB to activate PI 3-K is fairly correlated with their mitogenic potential towards HSC. However, PDGF-AA is unable to stimulate HSC chemotaxis in spite of its ability to promote mitogenesis and PI 3-K activation. This finding may be consequent to the activation of divergent pathways following the autophosphorylation of PDGF-α or -β receptor subunits (32). On the other hand, these data also suggest that activation of PI 3-K is necessary, but not sufficient, for inducing chemotaxis in HSC.

In addition to the involvement in cell growth and migration, growth factor-induced PI 3-K activation may contribute to the downstream signaling that regulates cell survival. Current evidence suggests that the "survival" or anti-apoptotic action of PI 3-K is mediated by the activation of c-Akt (also referred to as protein kinase B - PKB), a signaling protein whose activity is regulated by several upstream events, and particularly the generation of phoshoinositides by PI 3-K (33). It has been shown that in human activated HSC, PDGF-BB can activate the c-Akt pathway and its downstream targets regulating cell survival (34). Importantly, activation of the c-Akt pathway is a PI 3-K-dependent event that is reversed by PI 3-K inhibitors.

In general, early signaling events (i.e. observed within 5-20 min after growth factor stimulation) may be responsible for one or more biologic effects occurring after several hours. Indeed, pharmacological interference with these early events is often followed by abrogation or reduction of the biologic effect. However, it is increasingly evident that a reagent that neutralizes signaling enzymes is able to inhibit the biologic effect even when applied several hours after the addition of the growth factor. This and other observations suggest that the biologic effect of a growth factor may be dependent on the activation of one or more intracellular signals occurring with a cyclic and/or reiterated pattern between the interaction of the growth factor and its receptor (early signaling) and the completion of the biologic effect (intermediate and late signaling). This modality has been extensively elucidated for the relationship between Ras/ERK activation and the progression of the cell cycle induced by PDGF (35) and likely applies to other pathways such as PI 3-K (36). It is further supported by recent data obtained in human HSC, showing that PDGF triggers a biphasic activation of ERK, with a late peak at 15-24 hrs after addition of the stimulus (37).

In addition to specific intracellular signaling pathways that involve protein phosphorylation, PDGF-signaling relies also on changes in $[Ca^{2+}]_i$ and pH. In particular, in HSC and other cells sustained changes in $[Ca^{2+}]_i$ and intracellular pH are necessary for the correct articulation of pathways involving protein phosphorylation. The mitogenic potential of different PDGF dimeric forms is proportional to their effects on $[Ca^{2+}]_i$ in activated rat and human HSC (5,38). The increase in $[Ca^{2+}]_i$ induced by PDGF in HSC is characterized by two main components : 1) a consistent and transient increase (peak increase), due to calcium release from intracellular stores following the activation of phospholipase C (PLC)γ and the consequent PIP_2 hydrolysis, and 2) a lower but longer lasting increase (plateau phase) due to an influx from the external medium. Accumulated evidence indicates that the induction of replicative competence by PDGF is dependent on the maintenance of sustained increase in $[Ca^{2+}]_i$ due to calcium entry rather than from the release from intracellular stores (39,40). Accordingly, stimulation of human HSC with PDGF in the virtual absence of extracellular calcium results in an almost complete abrogation of the mitogenic effect of this growth factor, and supports the view that the plateau phase of the increase in $[Ca^{2+}]_i$ is essential for eliciting full PDGF-induced replicative competence in this cell type (41). Extracellular calcium entry induced by PDGF was

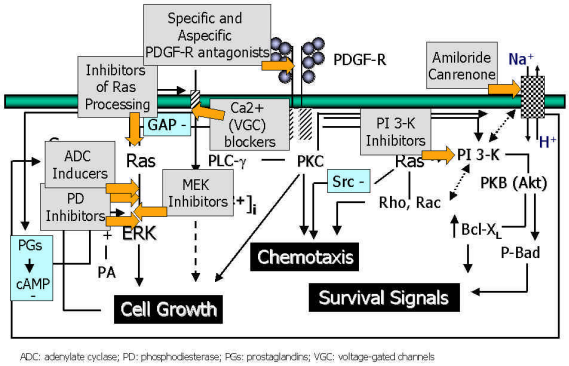

Figure 2. Pharmacological agents affecting PDGF intracellular signaling.

originally ascribed to the opening of low threshold voltage-gated calcium channels consistent with "T" type designation (40). Subsequently, this channel has been better characterized and defined as a PDGF-receptor-operated non-selective cation channel controlled by the tyrosine kinase activity of the PDGF-R and, particularly, by the activation of Ras through Grb2-Sos (42). The existence of this PDGF-receptor-operated channel in activated human HSC is suggested by the functional uncoupling between PDGF-R and this calcium channel caused by the inhibition of Ras processing following incubation of HSC with GGTI-298, an inhibitor of protein geranylgeranylation (43).

Stimulation with PDGF increases the activity of the Na^+/H^+ exchanger in rat or human HSC with consequent sustained changes in intracellular pH (44-46). This increased activity appears to occur through calcium-calmodulin and protein kinase C dependent pathways (45). Inhibition of the activity of the Na^+/H^+ exchanger by pretreatment with amiloride inhibits PDGF-induced mitogenesis, thus indicating that changes in intracellular pH induced by this growth factor are essential for its full biologic activity (47). Recent data suggest that PDGF-induced Na+/H+ exchanger activity is linked to the activation of PI 3-K, and is blocked by pre-incubation with PI 3-K inhibitors. Furthermore, inhibition of the Na+/H+ exchanger leads to the interruption of downstream signaling events essential for growth factor-mediated cytoskeletal reorganization such as PDGF-induced focal adhesion kinase (FAK) phosphorylation (48).

A final issue concerning PDGF signaling in HSC is the relationship occurring between the activation of PDGF receptors and the action of products of oxidative stress, including reactive aldehydes (HAKs). HAKs, an particularly 4-hydroxy-2,3-nonenal (HNE), exert direct pro-fibrogenic effects via an upregulation of the pro-collagen type I gene. This effect is mediated through a peculiar signal pathway based on activation and nuclear translocation of c-Jun NH_2-terminal kinases (JNKs), upregulation of c-jun and increased AP-1 binding (49). HNE, as well as other HAKs at the same low concentrations induce procollagen type I synthesis, abolish PDGF-BB mitogenic signaling in human HSC (50). This occurs through specific inhibition of the intrinsic tyrosine kinase activity associated with the PDGF-β receptor subunit, while autophosphorylation of other receptors, such as PDGF-α receptor or EGF receptor is not affected. These observations indicate that a complex interplay may occur among a PDGF signaling and products of oxidative stress in conditions characterized by chronic inflammation typical of liver disease.

Figure 2 summarizes the groups of pharmacological agents shown to affect one or more intracellular signaling pathways activated by PDGF in HSC. Although often highly effective in *in vitro* experiments and to a certain extent in animal models of liver fibrosis, most of these agents will not be likely suitable for therapeutic applications in humans. This because the main signaling pathways involved in PDGF-induced biological effects are common to other growth factors acting possibly with positive effects on other cell types within the same tissue. Therefore, a "global" inhibition of these pathways would likely lead to detrimental effects, unless an appropriate "cell-targeting" is feasible. Along these lines, recent studies have shown that modified human serum albumin (HSA) with cyclic peptide moieties peptides recognizing cytokine binding sites or binding sites for different ECM components may be applied as a carrier to deliver antifibrotic agents to HSC, thus strongly enhancing the effectiveness and tissue selectivity of drugs included in the complex and released inside the cell after internalization (51,52).

In recent years, studies have begun to address the multiple potential interactions of cells with the microenvironment. The major advances reflect elucidation of the fine tuning occurring upon cell adhesion and the consequent cytoskeletal organization. Key elements in these responses are: a) the specificity of integrin receptors and their downstream signaling; b) the cross talk between integrin and cytokine signaling; c) the relationship between the above mechanisms and the organization and tension of the cytoskeleton.

Binding of cell to ECM is mediated, at least in part, by cell surface receptors belonging to the integrin family. Integrins are heterodimeric transmembrane proteins that consist of an α subunit and β subunit. Activated HSC express several integrin β_1-associated α subunits. A particularly high expression has been demonstrated for $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (53,54). Protein phosphorylation is one of the earliest events detected in response to integrin stimulation, and in particular tyrosine phosphorylation is a common response to integrin engagement in many cell types (55,56). Several protein tyrosine kinases have been implicated in integrin-related signaling events. Focal adhesion kinase (FAK), plays a central role in integrin-mediated signal transduction (57). In addition to its activation by integrins, FAK is also activated by several growth factors. FAK phosphorylation is stimulated by mitogenic neuropeptides such as bombesin, vasopressin, and by PDGF and endothelin (ET)-1. Indeed, many of the signaling proteins regulated by integrins are also involved in signal transduction pathways activated by growth factor receptors, indicating that synergistic interactions between

growth factor and integrin signaling pathways are involved in the regulation of cell proliferation, adhesion and migration. For example, PI 3-K co-immunoprecipitates with tyrosine-phosphorylated FAK in response to cell adhesion. Tyrosine phosphorylated PLCγ may be a transducer molecule in integrin-mediated signaling pathways. Adhesion of human HSC to ECM proteins does not result in PLCγ tyrosine phosphorylation (58). Nevertheless, adhesion of HSC induces interactions between PLCγ and cellular proteins undergoing tyrosine phosphorylation, one of which has been identified as FAK, suggesting that adhesion of HSC is followed by recruitment of PLCγ to phosphorylated FAK. Since PLCγ also physically associates with the PDGF-receptor, there may be cross-talk between this receptor and proteins of the focal adhesion complex. Indeed, stimulation of HSC with PDGF-BB leads to clustering of PDGF-β receptor subunits in areas possibly corresponding to focal adhesion complexes. Along these lines upon autophosphorylation, PDGF-receptors are co-distributed with FAK, thus suggesting a potential functional cross-talk between these signaling molecules (43). In addition, experimental evidence indicates that Ras plays a key role in the cross-talk between PDGF-receptor and FAK in human HSC (43). Therefore, it appears that multiple receptor systems can synergize with integrins to regulate biological phenomena such as cell proliferation, cell motility, and the signaling proteins activated by these synergistic agents are common to different receptor pathways. However, more recent advances indicate that cell adhesion and the consequent signaling events are necessary but not sufficient to provide a complete control of cell functions, and in particular their response to growth factors and cytokines. Indeed, a modern concept, defined in its complexity with the term "cellular tensegrity architecture" (59), implies that in the presence of growth factor stimulation and adequate adhesion to the substratum, the cell is unable to progress into the cell cycle if restricted in its spreading. This view is supported by several observations indicating that lack of cytoskeletal tension, obtainable only with full cell spreading, is equivalent to cytoskeletal disruption and lead to the inability to enter the cell cycle. This general key mechanism appears relevant for the regulation of the molecular signaling cascades operating in the context of the structural and mechanical complexity of living tissue and their pathophysiological alterations, including hepatic fibrogenesis.

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Abbreviations: ECM: extracellular matrix; ET-1: endothelin 1; ERK: extracellular-regulated kinase; FAK: focal adhesion kinase; HAKs: reactive aldehydes; HNE: 4-hydroxy-2,3-nonenal; HSC: hepatic stellate cells; JNK: c-Jun NH_2-terminal kinase; PDGF: platelet-derived growth factor; PKA: protein kinase A; PI 3-K: phosphatidylinosytol 3-kinase; PLC: phospholipase C

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