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Platelet derived growth factor recruits lactosylceramide to induce cell proliferation in UDP Gal:GlcCer: β1 $\rightarrow$ 4Galactosyltransferase (GalT-V) mutant Chinese hamster ovary cells

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Recent molecular cloning studies have suggested the presence of at least two β4Gal transferase genes (β4GalT-V and β4GalT-VI) that may encode lactosylceramide synthase but whether they are functional *in vivo* and whether they mediate growth factor induced phenotypic change such as cell proliferation is not known.

Our previous studies lead to the suggestion that various risk factors in atherosclerosis such as oxidized LDL, shear stress, nicotine, tumor necrosis factor-α converge upon LacCer synthase to induce critical phenotypic changes such as cell proliferation and cell adhesion [1]. However, whether platelet-derived growth factor also recruits LacCer synthase in mediating cell proliferation is not known. Here we have employed a Chinese hamster ovary mutant cell line Pro$^-$5Lec20 to determine whether this enzyme physiologically functions to mediate cell proliferation.

We show that PDGF stimulates the activity of UDP galactose:glucosylceramide, β1,4galactosyltransferase. The activity of LacCer synthase increased about 2.5 fold within 2.5–5 min of incubation with PDGF in both wild type and Pro$^-$5Lec20 cells. Concomitantly, there was an increase in the generation of superoxide radicals, p^{44}MAPK phosphorylation and cell proliferation in CHO cells. D-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a potent inhibitor of GlcCer synthase/LacCer synthase impaired PDGF mediated induction of LacCer synthase activity, superoxide generation, p^{44} MAPK activation and cell proliferation in Pro$^-$5Lec20 cells. PDGF-induced superoxide generation was also mitigated by the use of diphenylene iodonium; an inhibitor of NADPH oxidase activity that is required for superoxide generation. This inhibition was bypassed by the addition of lactosylceramide. Thus, β4GalT-V gene produces a bona fide LacCer synthase that can function *in vivo* to generate LacCer. Moreover, this enzyme alone can mediate PDGF induced activation of a signal transduction cascade involving superoxide generation, p^{44}MAPK activation, phosphorylation of Akt and cell proliferation. *Published in 2005.*

Keywords: **lactosylceramide, UDP-Galactose:glucosylceramide β1,4galactosyl transferase, Chinese hamster ovary cells, cell proliferation, platelet- derived growth factor**

Abbreviations: **GSL, glycosphingolipid; PDGF, platelet derived growth factor; LacCer, lactosylceramide; MAPK, mitogen-activated protein kinase; DPI, diphenylene iodonium; D-PDMP, D-phenyl-2-decanoylamino-3-morpholino-1-propanol.**

Introduction

Previously, glycosphingolipids (GSL) were largely known to be components of cell membranes. However, recently these compounds have been accorded functional roles in multiple signal transduction pathways that lead to critical phenotypic changes in cells such as cell proliferation, adhesion and apoptosis [1–5]. Lactosylceramide is a member of the GSL family and plays a pivotal role as a precursor in the biosynthesis of lactose series of GSL e.g. the gangliosides, globotriosylceramide and sulfatides. The level of LacCer is elevated in proliferative disorders in man such as atherosclerosis, polycystic kidney disease and in renal cancer [1,6,7].

Lactosylceramide is synthesized by an enzyme UDP-Galactose:glucosylceramide β1,4galactosyl transferase. The activity of LacCer synthase is elevated in several proliferative diseases above and thus may explain the biochemical basis of elevated level of LacCer in these pathological tissues [8]. Previous studies indicate that several risk factors in atherosclerosis

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such as oxidized low density lipoproteins, fluid shear stress, nicotine and pro-inflammatory cytokine such as tumor necrosis factor-α (TNF-α) can stimulate the activity of LacCer in cultured human aortic smooth cells and human umbilical vein endothelial cells. LacCer generated as a consequence of LacCer synthase activation led to an increase in cell proliferation and cell adhesion [6,8,9]. Although it was shown that LacCer is synthesized by a LacCer synthase (GalT-2) recently using human β4galactosyltransferase-I (which is recognized to function in lactose biosynthesis) as the query sequence, investigators have identified seven gene family members [10]. Based on an *in vitro* enzymatic assay we found that the human GalT-2/β4GalT-VI is an ortholog of the rat brain LacCer synthase. In addition, another gene β4GalT-V has ~68% nucleotide sequence homology with β4GalT-VI gene, presumably encoding for a novel LacCer synthase.

However, based on *in vitro* enzymatic assay using a diverse panel of acceptor sugar substrates another group of investigators concluded that β4GalT-V functions in O-linked glycan biosynthesis. Thus, there is controversy in the literature. The purpose of this manuscript is two folds. First, to resolve this conflict in the literature; to demonstrate that β4GalT-V indeed is a bona fide LacCer synthase. Second, to employ a Chinese hamster ovary cell line Pro$^-$5Lec20 [11] to demonstrate that this enzyme can transduce platelet derived growth factor (PDGF) signaling leading to cell proliferation.

LacCer synthase was originally referred to as GalT-2 [12]. It is now recognized that LacCer synthase is a member of the β4-galactosyltransferase gene family, which now contains seven members. In the emerging new nomenclature, LacCer synthase is also referred to as β4GalT-VI or β4GalT-6. We will denote LacCer synthase as GalT-2/β4GalT-VI (the corresponding human gene has been mapped to chromosome 18q). A second member of the β4-galactosyltransferase gene family (β4GalT-V, which is located on human chromosome 20) potentially encodes a second LacCer synthase; based on *in vitro* assay, recombinant β4Gal T-V can in fact synthesize LacCer. We will refer to this second LacCer synthase as GalT-2/β4GalT-V. Lastly, we will use "LacCer synthase" when we do not know which gene is responsible for the observed/measured enzymatic activity.

Materials and methods

Materials

β4GalT-V mutant Chinese hamster ovary cells Pro$^-$5Lec20, missing the β4GalT-II and VI genes, were obtained as a gift from Dr. Pamela Stanley, Albert Einstein University, New York [11]. All chemicals, reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. D-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) was purchased from Matreya Inc. (Pleasat Gap, PA). UDP-[^{14}C]Galactose (specific activity 50 mCi/mmol), [14C]Palmitic acid (50–60 mCi/mmol) and [^{3}H]Thymidine (specific activity 20–30 Ci/mmol) were purchased from American Radiolabeled Company, St.Louis, MO. Anti-MAPK antibody (specific for p^{44}MAPK and p^{42}MAPK) were obtained from Upstate Biotechonology Inc. (Lake Placid, NY). Phospho-Akt (Thr 308) antibody and phospho-p44/42 MAP kinase polyclonal antibody were purchased from Cell Signaling Technology (Beverly, MA**).** Polyclonal antibody against GalT-2 were prepared and characterized as described before [13]. BCA protein assay kit was obtained from Pierce, Rockford, IL.

Incubation of CHO cells with platelet derived growth factor

Wild type CHO cells and Pro$^-$5Lec20 cells previously characterized by Dr.Pamela Stanley [11] and by RT-PCR in our laboratory, were grown to confluence. Next, fresh medium was added with or without PDGF, D-PDMP, LacCer, *N*-acetylcysteine. Following incubation (as described in individual legends) cells were harvested and subjected to various assays below.

Preparation of buffy coat

Confluent cultures Pro$^-$5Lec20 cells were washed with PBS, harvested and homogenized in 0.1 M K_2HPO_4-$KHPO_4$ buffer, pH 6.65, containing 5 mM $MgCl_2$, 0.5 sucrose and 1 μg/ml aprotinin and leupeptin. The homogenate was centrifuged at 1000 g for 10 min. To 5 ml-polyalomer tube 3 ml of 1.3 M sucrose in the same buffer was pipetted and homogenate was carefully layered on the top of sucrose solution. The samples were centrifuged for 2 h at 10,600 $\times$ *g* at 4°C. The layer at the interface of homogenate and sucrose solution (buffy coat) was removed and used for further experiments.

Western immunoblot assay

After incubation cells were washed with ice-cold phosphate—buffered saline containing 1 mM Na_3VO_4 and then lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 1% Triton X-100, 0.5% Nonidet P-40 and aprotinin, pepstatin, leupeptin (1 μg/ml each).The detergent-insoluble material was separated by centrifugation (10,000 $\times$ *g* 15 min, 4°C), and the soluble supernatant fraction was used as the enzyme source. The enzyme preparation (50 μg/well protein) was subjected to electrophoresis by a 4–15% SDS-PAGE under denaturing conditions. The protein was then transferred onto a nitrocellulose membrane, and probed with anti-MAPK antibody or anti-ppAkt antibody as described earlier [9].

Determination of LacCer synthesis in Pro$^-$5Lec20 cells

Cells were metabolically labeled with [^{14}C] palmitate (2 μCi/ml) for 24 h at 37°C. Next, the cells were washed with fresh medium and incubated for 2 h with or without D-PDMP (10 μM). Next, PDGF (5 ng/ml) was added and incubation was continued at 37°C. At the indicated time intervals, cells were washed three times with PBS and lipids were

extracted with hexane-isopropanol (3:2 by volume) for 15 min at room temperature. The procedure was repeated and lipid extracts were pooled and dried in nitrogen. Glycosphingolipids were fractionated from the total lipid extracts by the use of silicic acid column chromatography and separated by high-performance thin layer chromatography.The plates were calibrated with standard glycosphingolipids. Following development in chloroform-methanol-water (100:42:6 by volume), the gel areas corresponding to LacCer were scrapped and radioactivity measured using a Beckman scintillation counter. The solvent extracted cell culture dish was solubilized over night with 1 M NaOH and then suitable aliquots withdrawn for the measurement of protein using the BCA protein assay kit.

Lactosylceramide synthase activity measurement

Briefly, the LacCer synthase assay mixture contained 100 μl of enzyme preparation, 20 μM of cacodylate buffer (pH 6.8), 1.0 mM Mn^{2+}/Mg^{2+}, 0.2 mg/ml Triton X-100, 30 nmol of GlcCer, and 0.1 mmol of UDP-galactose. Assays without exogenous GlcCer served as blanks and were subtracted from all corresponding data points. The assay was terminated by the addition of 25 μmol of EDTA plus 2.5 μmol of KCl. Chloroform/methanol (2:1, v/v) and 5 μg of GSL were added and the products were isolated and separated by Whatman SG-81 paper chromatography by developing in chloroform/methanol/water (60:17:2, v/v/v). Chromatogram areas corresponding in migration with standard LacCer were cut, and radioactivity was measured in a Beckman LS-3800 scintillation spectrometer using the background subtract setting and automatic quench setting [14].

Cell proliferation assay

Pro$^-$5Lec20 cells were grown to confluence in 96 well sterile plastic trays. Next, fresh medium 100 μl and various amounts of agonists/inhibitors were added and incubation continued for 18 h. [^{3}H]Thymidine (1 μCi/ml) was added next and following incubation for 6 h the reaction was terminated by repeated washing of monolayers with PBS. The incorporation of [^{3}H]Thymidine into DNA was measured by scintillation spectrometry as described previously [15].

Measurement of superoxide generation

Lucigenin, an acridylium compound that emits light upon reduction and interaction with $O_2^{\cdot-}$ was used to measure $O_2^{\cdot-}$ production. Briefly, cultured Pro$^-$5Lec20 cells were harvested, and cell pellets were suspended in a balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 35 mM phosphoric acid, and 20 mM HEPES, pH 7.4). The viability of the suspended cells as determined by the trypan blue exclusion principle was >90%. To measure $O_2^{\cdot-}$ production, preincubated cells were added to a 96-well plate containing dark-adapted lucigenin (500 μM) in balanced salt solution. Next, inductors of $O_2^{\cdot-}$ generation were added and photon emission was measured for 15 min in a scintillation counter (Packard TOP counter, Wellesley, MA). The amount of $O_2^{\cdot-}$ produced at each time point was calculated by comparison with a standard curve generated using xanthine/xanthine oxidase.

Statistical analysis

Results are expressed as mean $\pm$ SD. Statistical analysis was performed with Student's t-test and values of $^*p \leq 0.05$ were considered significant.

Result

Platelet- derived growth factor stimulates LacCer synthase activity in wild type CHO cells and Pro $^-$5Lec20 cells

As shown in Figure 1 in wild type CHO cells PDGF exerted a time-dependent increase in the activity of LacCer synthase. Maximum increase in LacCer synthase activity 2.5 -fold compared to control occurred 2.5–5 min following incubation with PDGF. Interestingly, in Pro$^-$5Lec20 cells PDGF also exerted a similar time kinetics of increase in LacCer synthase activity observed in wild type CHO cells. These data suggest that β4GalT-V functions as a LacCer synthase.

Platelet- derived growth factor stimulates and D-PDMP decreases the endogenous level of LacCer in Pro$^-$5Lec20 cells

Metabolic labeling of cells with [^{14}C] Palmitate followed by treatment with PDGF revealed that PDGF exhibits a dose-dependent increase in LacCer synthesis. Moreover, pretreatment of cells with D-PDMP inhibits increase in LacCer mass (Figure 2).

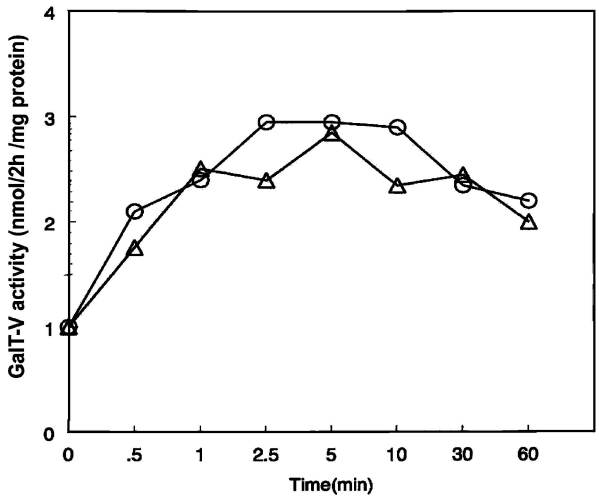

Figure 1. Effect of platelet derived growth factor on LacCer synthase activity in wild type CHO cells and β4GalT-V mutant CHO cell line Pro$^-$5Lec20. Confluent culture of wild type CHO cells and mutant Pro$^-$5Lec20 CHO cells were incubated with platelet derived growth factor (5 ng/ml). At indicated time interval cells were harvested, homogenized and the activity of LacCer synthase measured as described [12]. O–wild type CHO cells; Δ—mutant Pro$^-$5Lec20 cells.

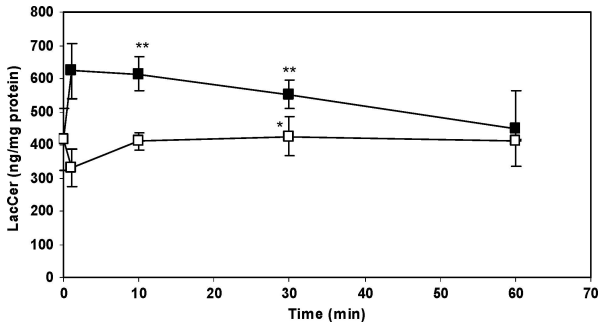

Figure 2. Effect of platelet derived growth factor on LacCer mass in Pro^{-}5Lec20 cells. Confluent culture of Pro^{-}5Lec20 cells was metabolically radiolabeled by culturing the cells in the presence of [^{14}C]Palmitate (2 μCi/ml) for 24 h. D-PDMP (10 μM) was added to corresponding wells for 2 h, next PDGF (5 ng/ml) was added to all wells and cells incubated for indicated time point. After washing and extraction the samples were proceeded to columns and HPTL chromatography as described in Materials and Methods. Data are presented as mean $\pm$ SD; $^{*}P < 0.05$ vs control. $^{**}P < 0.01$ vs control.

Activation of superoxide ($O_2^{\cdot-}$) production by PDGF

The treatment of Pro^{-}5Lec20 cells with PDGF (5 ng/ml) markedly increased $O_2^{\cdot-}$ production ($\sim$5-fold) in 5 min. Pretreatment of cells with D-PDMP (10 mM) blocked the PDGF-induced increase in $O_2^{\cdot-}$ production (Figure 3A). Preincubation of Pro^{-}5Lec20 cells with DPI (5 mM), an inhibitor of NAD(P)H-oxidase, also significantly decreased $O_2^{\cdot-}$ production but to a lesser extent than D-PDMP (Figure 3A).

Lactosylceramide alone (5 mM) stimulated the production of $O_2^{\cdot-}$ (Figure 3B) as compared to nonstimulated cells. Preincubation of Pro^{-}5Lec20 cells with D-PDMP (10 mM) attenuated the LacCer induced increase in $O_2^{\cdot-}$ production (data not shown). These findings reveal that PDGF-induced LacCer production is an upstream signaling reaction. But once endogenous LacCer is formed then it can generate $O_2^{\cdot-}$ production.

Platelet-derived growth factor stimulates p^{44}MAPK in Pro^{-}5Lec20 cells

Western immunoblot assay revealed that in Pro^{-}5Lec20 cells PDGF stimulates the level of phosphorylated form of p^{44}MAPK. This was abrogated by D-PDMP and bypassed by LacCer. Diphenylene iodonium, an inhibitor of NAD(P)H oxidase activity (DPI) abrogated PDGF induced p^{44}MAPK

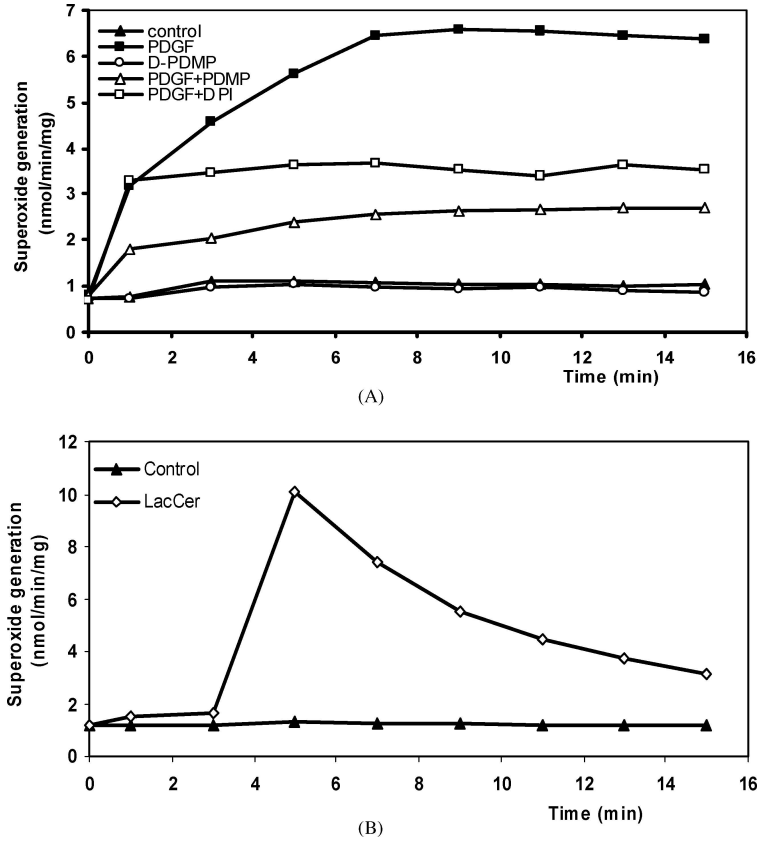

Figure 3. PDGF stimulates Pro^{-}5Lec20 CHO cells to generate superoxide. (A) Confluent culture of Pro^{-}5Lec20 cells were pretreated with D-PDMP (10 μM) for 2 h or DPI (5 μM) for 30 min. PDGF (5 ng/ml) was added and lucigenin chemiluminescence was monitored for 15 min. The PDGF-induced superoxide generation was blocked by D-PDMP and inhibited by NAD(P)H inhibitor DPI. (B) Superoxide generation was measured in the presence and absence of lactosylceramide (5 μM).

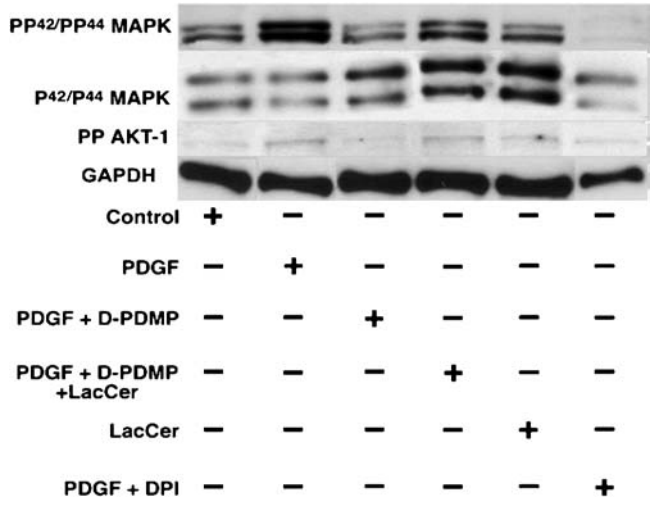

Figure 4. Effect of platelet derived growth factor on mitogen - activated protein kinase and pp-Akt in Pro$^-$5Lec20 cells. Confluent culture of wild and mutant CHO cells were incubated with or without PDGF (5 ng/ml), D-PDMP (10 μM) and with or without 15 μM *N*-acetylcysteine, 5 μM diphenylene iodonium and lactosylceramide (5 μM). Following incubation for 10 min, cells were harvested and subjected to Western immunoblot assay employing antibody specific for p^{44} MAPK and p^{42} MAPK.

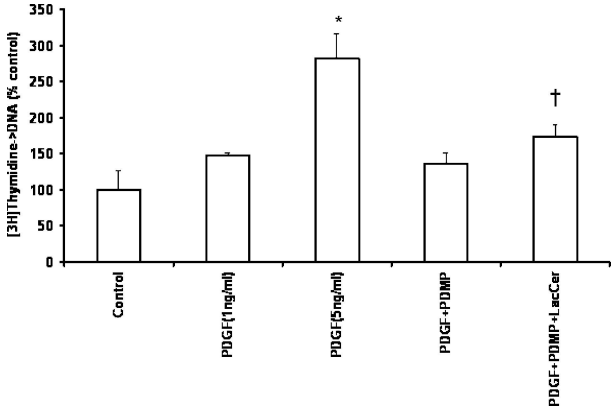

Figure 5. Effect of platelet- derived growth factor on the proliferation of Pro$^-$5Lec20 cells. Confluent culture of wild type and Pro$^-$5Lec20 cells grown in 96 well sterile plastic trays were incubated with 1–5 ng PDGF and with or without D-PDMP (10 μM) or lactosylceramide for 18 h. Next, [^{3}H]Thymidine (1 μCi/ml)was added and incubation was continued for an additional 6 h. The incorporation of radioactivity was measured by scintillation spectrophotometer as described in Material and Methods. Data are presented as mean ± SD; $^*P < 0.05$ vs control; $^\dagger P < 0.05$ vs PDGF + D-PDMP.

phosphorylation (Figure 4). Moreover, preincubation of cells with DPI markedly decreased PDGF induced p^{44}MAPK level. Western immunoblot assay revealed that PDGF stimulated the phosphorylated form of AKT-I (Figure 4). LacCer alone stimulated pp-Akt protein expression as well as bypassed the inhibitory effect of D-PDMP. In contrast, PDGF did not alter the expression of SAPK, PTEN and pp38 MAPK (data not shown).

Platelet derived growth factor stimulates proliferation in Pro$^-$5Lec20 cells

Our cell proliferation assays revealed that PDGF exerted a concentration-dependent increase in the proliferation of Pro$^-$5Lec20 cells (Figure 5). Pre-incubation of cells with D-PDMP completely abrogated PDGF induced cell proliferation and this was bypassed by the inclusion of LacCer.

Discussion

The major findings in the present study were the following. First, both wild type and Pro$^-$5Lec20 cells express significant LacCer synthase activity that is stimulated by platelet-derived growth factor. Second, Pro$^-$5Lec20 cells expressing β4GalT-V but not β4GalT-VI are able to recruit this enzyme to mediate PDGF induced generation of LacCer, superoxide generation, MAPK activation and cell proliferation. Finally, D-PDMP abrogated PDGF induced signaling that led to cell proliferation and this was bypassed by preincubation of cells with LacCer.

Using human β4GalT-I, which is recognized to function in lactose biosynthesis, as the query sequence, Shaper and others [16] have identified seven gene family members. The human GalT-2/β4-GalT-VI is the ortholog of the rat brain LacCer synthase originally cloned by Nomura [17]. The unanticipated result is that the GalT-2/β4GalT-V gene also encodes a protein, which based on *in vitro* assay, has LacCer synthase activity and has ~68% identity at the amino acid level with β4GalT-VI; the highest sequence identity between any other β4Galtransferase raised the speculation that β4GalT-V may also encode a LacCer synthase. Interestingly, the mRNA for GalT-2/β4GalT-VI was expressed predominantly in the adult human brain as well as in the kidney and pancreas, whereas in fetal tissues, it was expressed in the kidney, brain, lung, and liver. In contrast, the GalT-2/β4GalT-V was expressed among all the tissues investigated in both adults and in fetal tissue. Thus, GalT-2/β4GalT-V is constitutively expressed. In contrast, GalT-2/β4GalT-VI has a tissue restrictive expression. This observation has been independently reported by others [17] and is in conflict with another report [18].

We have contributed a body of literature suggesting that LacCer may be implicated in mediating oxidized LDL, nicotine, epidermal growth factor, shear stress and tumor necrosis factor induced phenotypic changes leading to cell proliferation and cell adhesion [1,8]. Because of the availability of Pro$^-$5Lec20 cells that exclusively express β4GalT-V gene and LacCer synthase we examined whether this may be involved in PDGF induced cell proliferation.

We found that PDGF stimulated cell proliferation in mutant CHO Pro$^-$5Lec20 cells in a concentration-dependent manner and this was also about 3 fold as compared to control. Although the wild type CHO cells have both the β4GalT-V and

β4GalT-VI LacCer synthases and the Pro$^-$5Lec20 cells express only β4GalT-V LacCer synthase the 3-fold stimulation of cell proliferation induced by PDGF is similar and within the range of cell proliferation induced by PDGF in human aortic smooth muscle cells [8].

We found that PDGF stimulated LacCer synthase activity in Pro$^-$5Lec20 cells in a time-dependent fashion and was similar to our previous observation in which oxidized-LDL stimulated LacCer synthase activity within 2.5 min and reached saturation in about 10 min. Moreover, PDGF induced increase in the activity of LacCer synthase was compromised by preincubation of cells with D-PDMP.

Previous studies have shown that D-PDMP is a non-specific inhibitor of GlcCer synthase, LacCer synthase as well as higher homologs of LacCer [19,20].We found that PDGF-induced increase in cell proliferation was inhibited by D-PDMP. This finding implicates that the β4GalT-V LacCer synthase in Pro$^-$5Lec20 can mediate PDGF induced cell proliferation. Our previous studies have shown that TNF-α [21] and nicotine [22] induced increase in the endogenous biosynthesis of LacCer level activates NAD(P)H oxidase to generate superoxide $O_2^{\cdot-}$. In turn, $O_2^{\cdot-}$ stimulates p^{44}MAPK activation, c-fos expression and cell proliferation in human aortic smooth muscle cells [9]. Here we show that in Pro$^-$5Lec20 cells PDGF recruits β4GalT-V LacCer synthase to produce LacCer to generate $O_2^{\cdot-}$, activate p^{44}MAPK, ppAkt and induce cell proliferation. Our present studies also revealed that the alternate, signaling intermediates such as SAPK, PTEN, pp38 MAPK are not implicated in PDGF induced cell proliferation via the LacCer synthase pathway (data not shown), although their participation in signal transduction pathways that regulate gene expression in response to growth modulators are well known in some normal and cancer cell lines [23–26].

Our studies point to the conclusion that β4GalT-V is a bona fide LacCer synthase that can function *in vivo* in cultured cells to mediate platelet derived growth factor induced cell proliferation. The essential component of this signaling pathway is superoxide that is generated due to LacCer mediated activation of NAD(P)H oxidase and the MAP kinase cascade as well as the Akt.

Acknowledgments

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