Receptor-mediated attenuation of insulin-like growth factor-1 activity by galactose-1-phosphate in neonate skin fibroblast cultures: Galactosemia pathogenesis

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Abstract

Background. The pathogenesis of classical galactosemia, a rare metabolic disorder associated with developmental complications in neonates and children due to inherited deficiency of galactose-1-phosphate (Gal-1-P) uridylyltransferase (GALT), is known to be mediated by elevated Gal-1-P levels and involves a cascade of cytokines, reactive oxygen species (ROS) and growth factors.

Objectives. To examine ex vivo the effect of Gal-1-P on the mitogenic activity of different growth factors, particularly insulin-like growth factor-1 (IGF-1), known to regulate growth and development from the fetal stage to adulthood.

Material and methods. Fibroblasts derived from the foreskin of 3–8-day-old healthy neonates were cultured for 1–14 days with 0–20 mM galactose or 0–10 mM Gal-1-P and then stimulated with 5% fetal bovine serum (FBS) or 50 ng/mL of platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) or IGF-1 for 24 h. DNA synthesis was measured and protein expression of PDGFR, FGFR and IGF-1R was assessed with western blotting.

Results. Supra-physiological concentrations of galactose significantly decreased FBS- and IGF-1-induced BrdU incorporation. The presence of Gal-1-P (5–10 mM) in culture medium for 7–14 days significantly ($p < 0.01$) decreased IGF-1-, PDGF- and FBS-stimulated DNA synthesis. While treatment with Gal-1-P selectively and significantly ($p < 0.01$) reduced the protein expression of IGF-1 receptor, galactose treatment did not have any marked effect on examined growth factor receptors.

Conclusions. This study demonstrates that Gal-1-P impairs IGF-1 activity through IGF-1-receptor impairment, thereby providing a new insight into the molecular mechanisms of galactosemia pathogenesis.

Key words: neonates, growth factors, DNA synthesis, IGF-1 receptor, galactosemia

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Introduction

Classical galactosemia is a metabolic disorder which results from a genetic aberration in galactose-1-phosphate uridylyltransferase (GALT), an enzyme that catalyzes the formation of uridine diphosphate galactose from galactose-1-phosphate (Gal-1-P).[1] The clinical features of classical galactosemia range from cataract, hypoglycemia, hepatomegaly, and hypotonia to sepsis. Clinical symptoms develop in infants during the neonatal period due to consumption of galactose[2–4] and an early withdrawal of galactose from the diet is known to remarkably reduce the incidence of serious illness among neonates. Dietary restrictions can prove to be life-saving in the first few weeks of life but do not protect from galactosemia-related long-term complications like cognitive impairment or neurological or speech abnormalities. Toxic levels of Gal-1-P in the cells due to continued endogenous galactose synthesis in diet-restricted cases or from abnormal galactosylation of proteins[5–7] lead to mental retardation, motor abnormalities, dyspraxia, and hypergonadotropic hypogonadism. Though galactosemia-induced cellular dysfunction has been reported to involve the participation of various intracellular and extracellular biomolecules such as reactive oxygen species (ROS), cytokines and growth factors,[8] the exact molecular mechanism of pathogenesis remains unclear. We recently reported that the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system is impaired in patients with classical galactosemia and may contribute, at least in part, to increased risk of infection and sepsis due to impaired bactericidal superoxide anion production by the leukocytes.[9] In addition to the reported role of ROS and cytokines in cellular events of galactosemia, endocrine participation has also been suggested in the pathogenesis of galactosemia, as children with classical galactosemia are reported to have low levels of circulating insulin-like growth factor-1 (IGF-1) levels.[10]

We reported earlier that galactosemia is associated with a nitric oxide (NO)-mediated downregulation of IGF-1, a mitogen with a key role in the growth and function of various human organs and tissues.[11] Growth hormone, IGF-1, epidermal growth factor (EGF) and fibroblast growth factor (FGF) are known to play a vital role in growth and development at different stages of childhood.[12,13] The IGF-I is a peptide that regulates bone growth, cellular differentiation, and metabolic activities, and plays a role in the functioning of the cardiovascular system.[14] A major amount of the circulating IGFs is of hepatic origin, however, physiologically, production of IGF-I also occurs in other tissues, such as growth plate and bone, with various autocrine and paracrine functions.[15] Though previous studies have demonstrated that IGF-1 production is mitigated under galactosemic conditions along with decreased levels of IGF-1 levels in plasma, our main goal in this study was to investigate if Gal-1-P has any effect on growth factor activity, particularly that of IGF-1.

This study was carried out according to the protocols of the Institutional Ethical Review Committee of Kuwait University, Kuwait (approval No. MK 01/16).

Material and methods

Material

Galactose, Gal-1-phosphate and other chemicals used for the biochemical assays were purchased from Sigma-Aldrich (St. Louis, USA). The chemicals and materials required for the cell cultures, including fetal bovine serum (FBS), were procured from Invitrogen–Thermo Fisher Scientific (Carlsbad, USA). The electrophoresis reagents were purchased from BioRad Laboratories (Hercules, USA), whereas the antibodies for western blot analysis were procured from Santa Cruz Biotechnology (Santa Cruz, USA). The fibroblasts used in this study were cultured from the skin explants of foreskin samples collected from 3–8-day-old healthy neonates.

Methods

Cell cultures and their treatment with experimental materials

Fibroblasts were cultured in a growth medium, Dulbecco's modified Eagle medium (DMEM) F-12 supplemented with 10% FBS and penicillin/streptomycin (5U, 5 μg/mL, respectively), in a humidified atmosphere of 5% CO_2 and 95% air. The cell cultures were grown to confluence before their use for various experiments. Cell cultures were washed twice with RPMI culture medium before the start of each experiment. The cells were treated with varying concentrations (0–20 mM) of galactose and Gal-1-P (0–10 mM) for 1–14 days and the cultures were replenished with galactose/Gal-1-P every 24 h. Lipofectamine was added as a permeating agent to the cell cultures in experiments with the polar molecule Gal-1-P. Growth factors (50 ng/mL), namely platelet-derived growth factor (PDGF), FGF and IGF-1, were added to the culture medium for 24 h in the presence or absence of galactose/Gal-1-P to examine DNA synthesis. Following treatment of the cell cultures with the experimental agents, culture supernatants were collected and cells were harvested and homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing protease inhibitors. In the control experiments, 5–20 mM of mannitol was added to the cell cultures in order to examine its effect on mitogen-induced DNA synthesis and growth factor receptors.

BrdU incorporation

DNA synthesis in the cell cultures was assayed by measuring bromodeoxyuridine (BrdU) incorporation into the cellular DNA. The cell cultures were grown in the presence or absence of galactose or Gal-1-phosphate for

1–14 days and mitogens (FBS and growth factor) were added for 24 h along with BrdU, provided in kits purchased from Calbiochem (Merck, Kenilworth, USA).

Western blot analysis

Following treatment of the fibroblast cultures with the various experimental agents, cell homogenates were prepared and lysed using 50 mM Tris-based buffer (pH 7.6) which contained 5 mM EGTA, 150 mM NaCl, 1% Triton 100, 2 mM Na_3VO_4, 50 mM sodium fluoride solution (NAF), 1 mM phenylmethanesulfonyl fluoride (PMSF), 20 μM phenylarsine, 10 mM sodium molybdate, 10 μg/mL leupeptin, and 8 μg/mL aprotinin. Cell lysates prepared from different treatment groups were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in equal amounts of protein and transferred onto a nitrocellulose membrane. The membranes were then incubated with monoclonal antibodies to FGF receptor (FGFR), platelet-derived growth factor (PDGF) receptor and IGF-1 receptor, and washed 3 times before incubation with secondary antibodies conjugated to horseradish peroxidase. The SuperSignal chemiluminescence method was used to detect protein bands and equal loading of the proteins was checked through detection of β-actin using primary rabbit anti-human β-actin antibody. The bands were analyzed and quantified using densitometry.

The amount of protein in each cell homogenate was measured using Bio-Rad protein assay kit (catalogue #500-0006).

Statistical analysis

The results were analyzed using GraphPad Prism software v. 6 (GraphPad, San Diego, USA). The data is presented as mean ± standard deviation (SD) of number of experiments and the mean values were compared using Student's t-test for significance of variance between the different study groups.

Results

Effect of galactose on growth factor-induced BrdU incorporation into DNA

The addition of 1–20 mM of galactose for 1–14 days induced varied DNA synthesis in response to stimulation by FBS (Fig. 1), with no significant effect of 1-day exposure to galactose. Treatment of the cell cultures with supraphysiological concentrations (10–20 mM) of galactose for 7–14 days significantly ($p < 0.01$) impaired FBS-induced DNA synthesis when compared to cells stimulated with FBS without galactose treatment. The addition of 10 mM galactose to the cell cultures for 1–14 days did not have any significant effect on FGF- or PDGF-induced DNA synthesis (Fig. 2); however IGF-1-induced DNA synthesis was markedly impaired by exposure of the cells to galactose for 7 days or longer, as BrdU incorporation was significantly ($p < 0.01$) reduced compared to the cells treated with IGF-1 in a galactose-free medium. Interestingly, the control experiments using cells cultured in the presence of 20 mM mannitol revealed that growth factor-induced DNA synthesis was not markedly altered (data not shown).

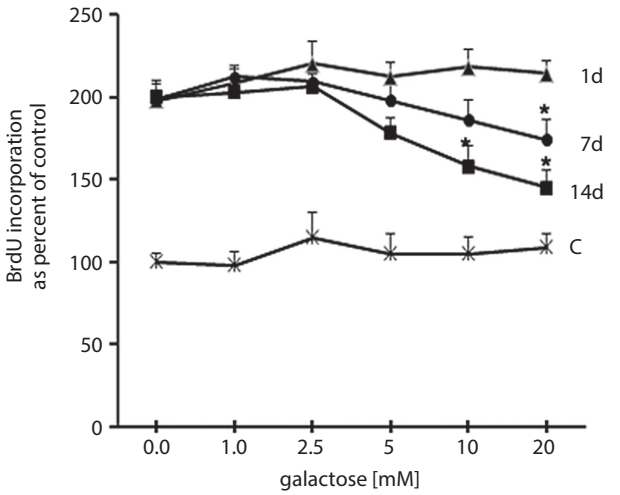

Fig. 1. BrdU incorporation (shown as percent of control) in skin fibroblasts stimulated with FBS for 24 h following treatment with varying concentrations (0–20 mM) of galactose for 1 day (rectangle points), 7 days (round points) or 14 days (square points). The control (star points) is represented by cells treated with varying concentrations of galactose in a mitogen-free medium (MFM). BrdU incorporation in the control cells was 0.58 ±0.04 absorbance units (Au) at 450 nm per 5×10^3 cells. The data shown in graph is mean ±SD of at least 6 measurements of BrdU incorporation carried out in duplicate. Statistical significance * $p < 0.01$ when compared to cells stimulated with FBS in the absence of galactose for the respective period of treatment (1, 7 or 14 days)

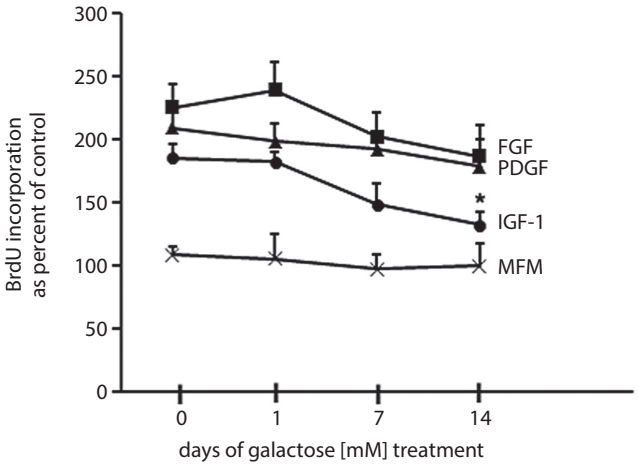

Fig. 2. BrdU incorporation (shown as percent of control) in skin fibroblasts cultured in the presence of 10 mM galactose for 1–14 days and then stimulated for 24 h with 50 ng/mL of PDGF (triangle points), FGF (square points) or IGF-1 (round points). The star points represent cells cultured in the presence of galactose for 1–14 days but not stimulated with any growth factor for BrdU incorporation. The data shown in the graph is the mean ±SD of at least 6 measurements of BrdU incorporation carried out in duplicate. Statistical significance * $p < 0.01$ when compared to cells stimulated with the respective growth factor in the absence of galactose

Effect of galactose-1-P on IGF-1- or FGF- or PDGF-induced BrdU incorporation into DNA

The addition of physiological or supraphysiological concentrations of Gal-1-P (0.5–10 mM) to the culture medium for 24 h did not have any marked effect on basal (control) or FBS-induced DNA synthesis in the fibroblast cells (Fig. 3). Chronic treatment (7–14 days) with supraphysiological concentrations of Gal-1-P (2.5–10.0 mM) significantly reduced ($p < 0.01$) FBS-induced BrdU incorporation in the fibroblast cells while lower concentrations of Gal-1-P did not have any notable effect (Fig. 3). The addition of Gal-1-P to the culture medium at supraphysiological concentrations for 14 days was observed to markedly impair growth factor-induced DNA synthesis in fibroblasts, as BrdU incorporation was significantly ($p < 0.01$) reduced in the cells stimulated with IGF-1 or PDGF (Fig. 4). Selective impairment of growth factor-induced DNA synthesis was also observed in cells cultured with 5 mM Gal-1-P for 7 days, where IGF-1-induced BrdU incorporation was significantly reduced without any marked effect on the mitogenic activity of PDGF and FGF (Fig. 4). Supraphysiological concentrations of Gal-1-P did not have any remarkable effect on basal (mitogen-free culture medium) DNA synthesis except for a notable, but statistically non-significant, reduction in BrdU incorporation following 14 days of treatment.

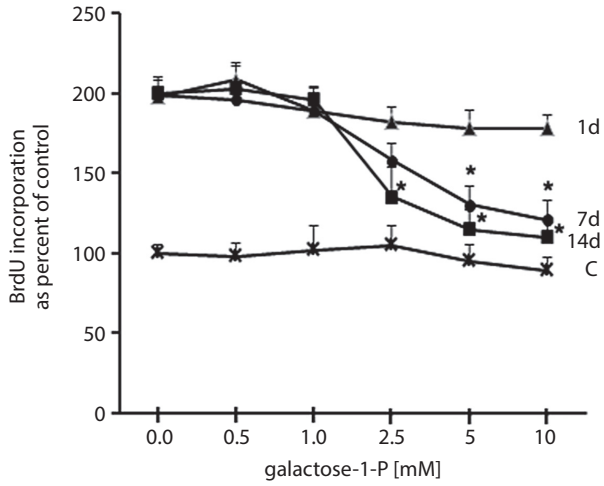

Fig. 3. BrdU incorporation (shown as percent of control) in skin fibroblasts stimulated with FBS for 24 h following treatment with varying concentrations (0–10 mM) of Gal-1-P for 1 day (rectangle points), 7 days (round points) or 14 days (square points). The control (star points) is represented by cells treated with varying concentrations of Gal-1-P in a mitogen-free medium (MFM). The data shown in the graph is the mean ±SD of at least 6 measurements of BrdU incorporation carried out in duplicate. Statistical significance * $p < 0.01$ when compared to cells stimulated with FBS in the absence of Gal-1-P for the respective period of treatment (1, 7 or 14 days)

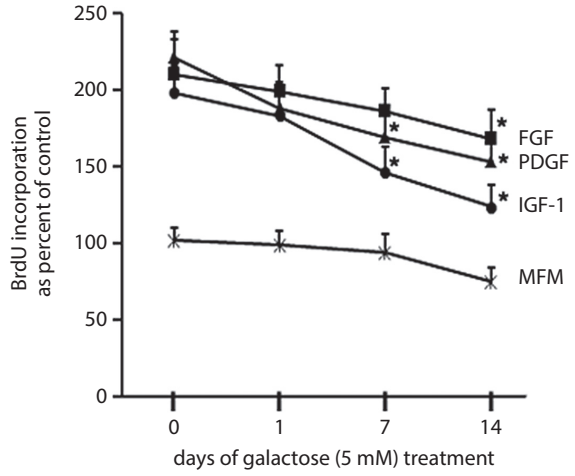

Fig. 4. BrdU incorporation (shown as percent of control) in skin fibroblasts cultured in the presence of 5 mM Gal-1-P for 1–14 days and then stimulated for 24 h with 50 ng/mL of PDGF (triangle points), FGF (square points) or IGF-1 (round points). The star points represent cells cultured in the presence of Gal-1-P for 1–14 days but not stimulated with any growth factor for BrdU incorporation. The data shown in the graph is the mean ±SD of at least 6 measurements of BrdU incorporation carried out in duplicate. Statistical significance * $p < 0.01$ when compared to cells stimulated with the respective growth factor in the absence of Gal-1-P

Effect of galactose and Gal-1-P on IGF-1 receptor and FGF receptor

Protein expression of IGF-1 receptor was significantly ($p < 0.01$) reduced in fibroblasts cultured in the presence of 5 mM Gal-1-P for 14 days (Fig. 5). Galactose treatment did not have any remarkable effect on the protein expression of FGFR or IGF-1R, while the impairment effect of Gal-1-P on IGF-1R was selective as FGFR protein levels remained unaltered. Neither Gal nor Gal-1-P had any marked effect on PDGF receptor expression (data not shown).

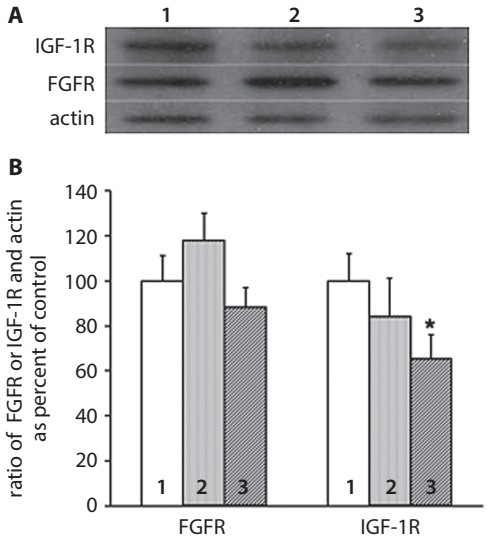

Fig. 5. Western blot analysis for protein expression of FGFR and IGF-1R in the fibroblast cells cultured in the presence of 10 mM of galactose (lane and bar 2) or 5 mM of Gal-1-P (lane and bar 3) for 14 days. The control (lane and bar 1) represents cells without any treatment. The values shown are the mean ±SD of 6 measurements. Statistical significance * $p < 0.01$ when compared to the control (bar 1). The ratio of the band densities of FGFR/actin or IGF-1R/actin are shown as a percent of the control

Discussion

Endocrine disturbances have been reported to occur in galactosemia, where IGF-1 and its binding proteins are known to contribute toward pathogenesis, and our previous studies[10,11] on the molecular mechanisms have also demonstrated that galactose/Gal-1-P downregulate the gene expression of IGF-1, a key determinant in the growth and development of children. The bioactivities of growth factors such as IGF-1 and growth hormone are important, in addition to their available content in the body tissues and fluids, for their role in the endocrine system during various developmental stages in children. This study establishes that the mitogenic activity of certain growth factors is profoundly impaired, possibly through receptor impairment, under galactosemic conditions. A marked decrease in FBS-induced mitogenic activity after chronic exposure of fibroblasts to supraphysiological concentrations of galactose under ex vivo conditions indicates some modifications in the cascade of molecular events. Our findings that exposure of fibroblast cultures to sub-/supraphysiological concentrations of galactose for 1–14 days did not affect basal (absence of a mitogen) DNA synthesis rules out any direct inhibitory effect of galactose on cell growth/survival. The fact that exposure to supraphysiological concentrations of galactose for up to 7 days did not have any marked effect on FBS-simulated DNA synthesis further points to some molecular modifications in the chronic presence of galactose or its metabolic derivatives where profound impairment of mitogen-induced DNA synthesis was observed. It is well-documented that Gal-1-P, a major metabolite of galactose that accumulates in the tissues and body fluids of galactosemia patients,[16] is responsible for the detrimental effects. Profound impairment of growth factor-induced DNA synthesis by prolonged exposure to Gal-1-P strongly signals that the galactosemia-induced effects on growth factor activity might be mediated by this metabolite.

Though the exact mechanism of pathogenesis remains unknown, molecular galactosylation is a well-reported biochemical feature of galactosemia, which leads to the altered structure and function of cells and its membranes.[17] The observed effect of chronic hypergalactosemic exposure on mitogen-/IGF-1-stimulated DNA synthesis may possibly be due to some kind of molecular galactosylation. The mitogenic activity of a growth factor is dependent upon its respective receptor, which triggers a cascade of signal transduction events to regulate cell growth and survival.[18] The selective impairment of growth factor activity by Gal-1-P as observed in this study suggests a differential regulation of the function of various growth factor receptors under galactosemic conditions. A marked impairment of IGF-1-induced DNA synthesis associated with significantly decreased IGF-1 receptor protein in fibroblasts exposed to chronic treatment with Gal-1-P suggests that either Gal-1-P downregulates the gene expression of IGF-1 receptor or post-translational modifications might be responsible for the reduction in IGF-1 receptor protein under galactosemic conditions. The fact that FGF receptor levels are increased, though not significantly, and PDGF receptor levels remained unaltered in the fibroblasts after chronic treatment with Gal-1-P indicates that either Gal-1-P selectively regulates the gene expression of various proteins or post-translational modifications such as molecular galactosylation differentially affect the structure and function of various membrane proteins including growth factor receptors. Other factors that might have deleterious effects on cell membrane components include cellular oxidative stress due to an imbalance of cellular ROS/reactive nitrogen species and cytokines.[19]

Increased levels of nitrites in the serum of galactosemia patients, as shown in our previous study, in addition to other reports regarding the overproduction of NO and the formation of nitrotyrosine residues in galactosemic tissues, may cause, at least in part, some of the post-translational modifications in membrane receptor proteins. In view of our earlier study, showing the profound effect of Gal-1-P on NO production in comparison to galactose signifies that Gal-1-P may likely be the major molecular trigger to induce deleterious changes in membrane receptor protein, in addition to galactosylation and other factors, to bring about the observed impairment of growth factor activity. Galactose alone did not have any marked effect on IGF-1-induced DNA synthesis, however its metabolite, Gal-1-P significantly reduced IGF-1 receptor protein and profoundly impaired IGF-1-induced DNA synthesis, clearly establishing that Gal-1-P is the key galactose metabolite to regulate growth factor function under hypergalactosemic conditions.

Conclusions

The selective impairment observed of IGF-1 mitogenic function by Gal-1-P through receptor modulation unravels a likely new molecular mechanism of galactosemia pathogenesis.

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