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Folate Protection of Congenital Heart Defects Linked with Canonical Wnt Signaling and Epigenetics

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Abstract

Purpose of review—Environmental factors, such as drugs, chemicals, or abnormal concentrations of natural metabolites induce birth defects. Environmental effects on cardiogenesis have been little studied in contrast to neurogenesis. This review presents evidence on three environmental factors, alcohol, the drug lithium (Li), and the metabolite homocysteine (HCy), impacting the Wnt/β-catenin pathway during cardiac development and folate (FA) protection.

Recent findings—Animal and epidemiological studies have shown that FA protects the embryo from birth defects. New animal studies demonstrate that FA prevents cardiovascular defects induced by Li, HCy, or alcohol, but protection occurs at a higher concentration than currently used in vitamin supplements. The data indicate that FA in combination with myo-inositol may further reduce the risk of birth defects. Discussion is presented of the cell specification stages that are impacted resulting in cardiac defects, how Wnt/β-catenin signaling is involved, and how FA and myo-inositol additively may protect embryonic pathways. The possible epigenetic role of folate in Wnt/β-catenin signaling is described.

Summary—This review will enable better counseling of women by defining, during early pregnancy, a susceptible window of embryonic exposure leading to a high risk of cardiac defects, and provides a therapeutic means and the necessary timing for prevention of environmentally induced birth defects.

Keywords

folate; inositol; Wnt/β-catenin; alcohol; birth defects

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Editor's Note:
It is a pleasure to join Kersti Linask, PhD to summarize her recent data in the rapidly changing field of cardiac development and the causes of congenital heart defects. Dr Linask holds the Mason endowed chair in Heart Development at the University of South Florida. This editorial article is the basic science basis for the hope that, someday, congenital heart disease could be reduced with prenatal supplementation of the maternal diet.

Introduction

Birth defects are the leading cause of infant mortality. Among all birth defects, cardiac anomalies are the most prevalent. The causes of birth defects can be divided between those due to genetic factors, as caused by mutations in specific genes or chromosomal abnormalities [1], or those induced by environmental causes, as by drugs, environmental chemicals, or by abnormal concentrations of natural metabolites [2-5]. This article will provide an overview of new cell and molecular data from studies in vertebrate models on underlying mechanisms associated with induction of congenital heart defects by environmental factors during a critical early window of development and new evidence on cardiac protection by folate and myo-inositol supplementation.

A critical early time period of development for induction of cardiac birth defects

The vertebrate heart arises from two bilateral cardiac fields that are specified during gastrulation [6]. The primary or first heart field gives rise to the left ventricle and part of the right. The second heart field gives rise to the anterior part of the right ventricle and to the outflow tract. The left ventricle differentiates first. The second heart field, although specified early during gastrulation [7-9], contributes much later to the heart, after the primary heart tube is already present and beating. As the outflow is adding onto the heart, the heart tube takes a rightward direction, becomes C-shaped, to initiate the process of looping that brings the developing atria in the posterior part of the heart forming region anteriorly, to be positioned cephalad to the ventricles. Looping ends with the formation of the four-chambered heart [10]. These early stages of cardiac cell specification, differentiation, and looping are critical in the development of a structurally and functionally normal heart [11].

Cardiac cells become specified several days before a tubular heart forms. In the human embryo, cardiac cell specification occurs during the second week after fertilization. A straight, tubular beating heart is present on embryonic day 21 post-fertilization. In mouse embryogenesis, specification takes place between days 6.5 to 7.5 days of gestation in a gestational period of 21 days. In the chick embryonic model, specification takes place in the first day after fertilization and a straight, tubular beating heart is present by 34 hrs of embryonic development. The gestational period that encompasses specification stages of development is critical in that it occurs early in gestation at a time that pregnancy may yet be undetected. As a result, a woman may not be taking precautions to protect the embryo from exposure to environmental factors, as smoking, alcohol, or drugs, all of which can impact dominant early cell signaling pathways to perturb and delay cardiomyocyte specification. As a result, this delay, even by one exposure, perturbs the timing of normal developmental cell interactions, thus leading to valve and myocardial dysfunction, as well as to placental abnormalities and intrauterine growth retardation.

The normal metabolite homocysteine at elevated serum levels [12-16], the mood-stabilizing drug lithium [17,18], and alcohol (ethanol) [19-25], all have been shown in epidemiological studies to be associated with cardiac and neural birth defects. Data from our lab have shown that all three factors impact an early critical period of heart development during cardiac cell specification [17,26-28]. Folate (FA) deficiency can lead to birth defects not only in association with the heart, but also of other organ systems. FA supplementation protects the early embryo from the adverse effects of elevated homocysteine, lithium, or alcohol [15,26,28-31]. Although FA feeds into the one-carbon metabolic cycle resulting in synthesis of the methyl donor S-adenosylmethionine, the underlying mechanism of FA embryonic protection has been unknown. Our data suggest that alcohol, lithium and hyperhomocysteinemia inhibit cardiac cell differentiation by potentiation of Wnt/β-catenin

signaling to inhibit induction of early cardiac genes important in initiating cardiogenesis. This suggests that FA protection would involve suppression of canonical Wnt signaling and possibly through epigenetic regulation via histone and DNA methylation.

Potentiation of Wnt/β-catenin signaling leads to induction of cardiac birth defects

Canonical Wnt/β-catenin signaling is an important growth factor pathway in cell fate decisions of a number of tissues within the embryo, including of the heart [27,32,33], neural tissue [34], and neural crest cells [35-37]. This signaling pathway is involved in cell specification, cell proliferation, and morphogenesis. Wnt/β-catenin signaling also is important in stem cell biology for cell fate determination and differentiation, including of cardiac cells [38,39]. Misregulation of this pathway in the embryo can lead to multiple defects during embryogenesis and different steps of this signaling pathway appear to be targets for environmental factors in the induction of cardiac birth defects [17,26,28]. A recent review provides more detail on the complexities of the canonical and noncanonical Wnt pathways [40].

In the canonical pathway after Wnt binds to a Frizzled receptor, a cascade of events is initiated at the plasma membrane that is transduced by *Dishevelled* (*Dvl*) on the intracellular side of the plasma membrane and leads downstream to an inactivation of a complex of proteins that includes axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β). Due to the inactivation, β-catenin downstream in this pathway is not phosphorylated, becomes stabilized and accumulates in the cytoplasm. Subsequently, it can translocate to the nucleus where β-catenin serves as a transcriptional coactivator of transcription factors of the TCF/LEF family to activate numerous downstream target genes. Importantly, the mechanism of β-catenin regulated gene transcription in light of data in tumorigenesis, supports a central role for histone 3 lysine 4 (H3K4) methylation at Wnt responsive genes [41]. These findings also implicate a role for APC via methylation in the turnover and cycling of Wnt coregulator complexes at target genes. Thus, one carbon metabolism and methylation reactions are important in β-catenin regulation of gene expression, suggesting an important role for epigenetic mechanisms in canonical Wnt signaling. If the canonical Wnt pathway is not active, cytoplasmic β-catenin is phosphorylated by the axin/APC/GSK3β complex, is polyubiquitinated, and undergoes proteasome-mediated degradation. In addition to β-catenin stabilization, Wnt signaling induces the formation of phosphoinositides (PIs), i.e, phosphorylated derivatives of phosphatidylinositol (PtIns) which are second messengers in Wnt signal transduction. Phosphatidylinositol derivatives regulate fundamental biological processes, including cell growth and survival, membrane trafficking, cytoskeletal reorganization, migration, and ion channel activation [42,43]. PIs are accessible to PI kinases and phosphatases capable of adding and removing phosphate groups, respectively, and to phospholipases that cleave lipids. The localization of PIs in distinct cellular compartments provides a mechanism for fine-tuning membrane trafficking and controlling the correct sequence of signaling events. Thus, these signaling pathways are associated with numerous cell processes and function in a cell context and developmental stage dependent manner. When perturbed even acutely, Wnt and PtIns can impact and delay important cellular processes to induce birth defects.

β-catenin expression is important in the cardiac compartment formation during early vertebrate embryogenesis along with N-cadherin [44,45]. Subsequently, β-catenin was reported to be required for *Islet 1*(*Isl1*) expression in cardiac progenitor cells, directly regulating the Isl1 promoter [46]. *Isl1* is expressed early during gastrulation, slightly medial-lateral to the primary cardiogenic crescent, Hex-expressing, region [47]. *Isl1* is considered a marker for the cardiac progenitors of the second heart field. Reported evidence indicates that

canonical Wnt signaling has differential effects during different time periods, i.e., inhibitory during cardiac specification and stimulatory later in development of the outflow region of the heart [27,32,33,44,48]. The signals are transduced in a temporal, cell-context dependent manner to result in rapid changes in gene transcription. In the early heart fields, Wnt signaling initially is active and then is down-regulated by the Wnt antagonist Dickkopf-1 (*Dkk-1*), in order for induction of *Hex* gene expression and cardiac commitment [48]. Potentiation of Wnt/β-catenin signaling during cardiac commitment becomes *inhibitory* [49]. We have demonstrated that Wnt3a exposure directly, or the drug lithium [17] that mimics Wnt signaling [50], or the metabolite homocysteine (HCy), all three molecules similarly suppressed the expression of the two canonical Wnt-regulated genes, *Hex* and *Isl1* that are involved in the specification of the primary and second heart fields at the gastrulation stages we targeted [26]. Other investigators using transgenic approaches arrived at the same conclusion that canonical Wnt signaling is important during discrete windows of mammalian cardiogenesis [32,33]. We also demonstrated that exposure to one binge-drinking level of alcohol during the same early window of gastrulation in the mouse on embryonic day 6.75 has the same effect as exposing embryos to one exposure of Li or HCy on ED 6.75. Alcohol exposure during gastrulation resulted in the induction of valve and myocardial defects as determined by noninvasive Doppler ultrasound on ED 15.5 [28]. The cardiac defects included tricuspid and semilunar valve anomalies resulting in valve regurgitation. Also the embryonic hearts also displayed compromised myocardial function. In terms of morphometric measurements, the experimentally exposed embryos and their placentas were smaller in comparison to similar aged control embryos and placentas in the untreated group. Taken together, the results in both chick and mouse models on acute embryonic exposure to Li, HCy, and alcohol indicate canonical Wnt signaling is potentiated and cardiogenic gene expression is delayed. Thus the normal signaling events are delayed to disrupt the timing of developmental signaling events necessary for normal valve, myocardial, and placental development [51-53].

Folate and *myo*-inositol protection of the embryo to prevent cardiac anomalies

The preventive effect of FA in the development of neural anomalies is generally accepted and has been corroborated by numerous studies. A Hungarian randomized clinical trial suggested that multivitamin or FA supplementation might reduce the risk for some types of heart defects as well, but at higher concentrations than used for neural tube defects_[54,55]. In a more recent time trend analysis in Quebec (between 1990-2005), this study reported that in the seven years after FA fortification of grain products in 1998, there was a significant 6% decrease per year in prevalence of severe congenital heart defects [56].

To investigate the possibility that folate may suppress the potentiation of canonical Wnt/β-catenin signaling by the above-described environmental factors, we analyzed in both the chick and mouse models whether folic acid supplementation would reverse the inhibitory effects of Li, HCy, and alcohol exposure on *Hex* and *Islet-1* gene expression in the early heart fields and protect heart development. FA supplementation in the chick returned *Hex* and *Islet-1* gene expression to normal, and often to higher than control, levels [26,28]. The addition of *myo*-inositol in combination with FA resulted in even better protection of the chick embryo to the adverse effects of the environmental factors [26,28]. *Myo-Inositol* is a water-soluble vitamin that is important in the inositol-lipid cycle that provides metabolic substrates for processes including signal transduction, as within the Wnt pathway, steroid synthesis, and intracellular calcium regulation. There is evidence that inositol depletion results in the cell from an inhibition of GSK3β, a key intermediary in the Wnt pathway [57]. Lithium also targets GSK3β to result in its inhibition, thus mimicking canonical Wnt

pathway effects [58]. Taken together, it would be expected that potentiation of Wnt/β-catenin would also lead to inositol depletion.

In the mouse model, FA supplementation was provided daily in the diet at a concentration of 10 mg/kg beginning with morning after conception [26]. This is a higher level than used in perinatal vitamins in human pregnancy, i.e.,400 μg/day. The 10 mg/kg concentration in the mouse study gave near complete protection from the adverse effects of a binge-level alcohol exposure or of acute exposure to lithium and elevated homocysteine during gastrulation stages of gestation [26,28]. Without FA supplementation exposure on ED 6.75 lead to 63% (HCy exposure) and 66% (Li) of embryos displaying valve defects [26]. With alcohol, the effects were more severe with 87% of the embryos having cardiac defects [28]. Supplementation at a moderate dose, i.e., 6.2 mg/kg, provided only partial protection [28]. The mouse FA supplementation studies suggest that higher doses of FA than currently used may provide better protection against birth defects during human pregnancy as well, when provided at conception. Complete protection against human neural tube defects is defined as red blood cell FA concentration above 900 nmol/l [59]. However, 40% of women of child-bearing age and 36% of pregnant women exhibited RBC folate levels below 900 nmol/L[59]. For protection against cardiac defects the RBC folate levels may be needed at even higher concentrations than 900 nmol/L. In a comparative analysis of steady state folate concentrations that were achieved with intake of 5 mg or 1.1 mg FA daily for 30 weeks, significant differences in RBC folate were detected between the two groups at weeks 4,6,12 and 30: The 5 mg FA group had higher blood FA levels showing a faster rate of FA accumulation, compared with the 1.1mg folic acid [60]. Inositol has been shown to prevent folate-resistant neural tube defects in the mouse [61] and has shown efficacy in reducing the incidence of diabetic embryopathy in diabetic rats [62]. *myo*-Inositol leads to changes in expression of hundreds of genes in numerous essential pathways [63] and does so at the cytoplasmic level, while FA epigenetic regulation of β-catenin signaling and possibly of other pathways by methylation reactions would target multiple genes and pathways at the nuclear level [41]. In summary the data suggest that a higher concentration of FA than currently used for neural tube defects in combination with *myo*-inositol would be more effective for protection of cardiac, and possibly for neural tube, defects than folate alone.

Epigenetic regulation

Epigenetics relates to heritable changes in gene function that occur independently of alterations in primary DNA sequence. Several recent reviews provide an overview of different aspects of epigenetics [64,65]. The best characterized epigenetic modifications are DNA methylation and histone modifications. DNA methylation in promoter regions tends to result in gene silencing [66]. Methyl-CpG binding domain proteins can reinforce silencing by recruiting co-repressor complexes that include histone deacetylases (HDACs) or histone methyltransferases. Because of the folic acid metabolic cycle leading to S-adenosylmethionine, an important biological methyl donor, this suggests that one-carbon metabolism, as well as the methylation states of H3K9, H3K4, and H3K27, histone markers of silent or repressed chromatin [67] maybe important in genetic regulation of Wnt signaling. This appears to be the case as based on evidence from a number of studies indicating that β-catenin functions as a framework to link the LEF/TCF proteins to specific chromatin remodeling complexes that mediate trimethylation of histones. The combined studies highlight a key role for H3K4Me3 in Wnt-regulated transcription [41,68]. A new report demonstrated that alcohol exposure altered DNA methylation profiles in mouse embryos at early neurulation [69]. Another study reported on a correlation between chronic alcohol use and demethylation of normally hypermethylated imprinted regions in sperm DNA [70]. We have new data using human umbilical cord blood (UCB1) stem cells showing that ethanol and Li induce DNA hypermethylation of CpG islands in the Wnt

antagonist Dkk-1 promoter (unpublished). This would have the effect of repressing Wnt antagonism, to potentiate Wnt signaling which we observed in our embryonic exposure studies. These results, taken together with the environmental exposure analyses indicate that cardiac defects rescued by FA most likely involve one-carbon metabolism and epigenetic mechanisms. Whether embryonic and fetal exposure to environmental toxicants will have long term transgenerational effects remains to be determined. Epigenetic mechanisms have been linked with adult diseases, as cancer [71,72], diabetes [73], obesity [74], and neurodevelopmental disorders [75].

Conclusion

As described above, even before pregnancy is often recognized, there is an early period of cardiac, as well as neural, development that is especially susceptible to epigenetic influences. Knowing also these early sensitive periods of gestation between weeks 2 and 3 post-fertilization of human pregnancy provides a window of opportunity to improve maternal-child health by targeting folate/myo-inositol supplementation to this time period and at high enough concentrations to provide the greatest benefit to protect the embryo against environmental factors. For the future, unraveling the epigenetic processes promises to have great potential for elucidating molecular mechanisms of environmentally induced congenital birth defects and in defining the basis of embryonic protection by folate/myo-inositol supplementation.

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