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ORIGINAL SCIENTIFIC ARTICLES

Malonylcarnitine in Newborns with Non-syndromic Cleft Lip with or without Cleft Palate

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Abstract

Aim Malonyl-CoA is regarded as a key signaling molecule in mammalian cells. It is converted to acetyl-CoA, and to a lesser extent, to malonyl acid and malonylcarnitine (C3DC). Availability of carnitine has been reported to be essential for the developing fetus. The objectives of the present study were to analyze associations of malonylcarnitine, acetylcarnitine (C2), and free carnitine (C0) in subjects with orofacial clefts.

Methodology We performed a retrospective analysis of carnitine concentration obtained from a newborn screening program carried out in our institution. Concentrations of whole blood malonylcarnitine, acetylcarnitine, and free carnitine were measured using tandem mass spectrometry. The study group consisted of 51 children with non-syndromic cleft lip with or without cleft palate. In total, 106 healthy children without congenital anomalies served as controls. Cut-off points were established using likelihood ratio values.

Results The mean concentration of malonylcarnitine in the cleft group was lower than that of the control group, 0.048 μmol·L^{-1} *vs.* 0.058 μmol·L^{-1}, respectively (P=0.009). In patients with orofacial cleft, low malonylcarnitine levels (≤0.047 μmol·L^{-1}) were 1.7 times more predominant than in healthy individuals (P=0.03). The mean concentration of acetylcarnitine was also lower in affected newborns in comparison to controls, 33.8 μmol·L^{-1} *vs.* 37.8 μmol·L^{-1}, respectively (P=0.026). After analysis of acetylcarnitine and free carnitine concentrations, the likelihood ratio test did not indicate valuable cut-off points.

Conclusion The study provides initial data indicating a potential association between decreased malonylcarnitine and abnormal palatogenesis.

Keywords cleft palate, malonyl-CoA, carnitines, fatty acids, biotin

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Introduction

Non-syndromic cleft lip with or without cleft palate (CL/P) is one of the most common congenital abnormalities in humans. Population-based estimates of the prevalence of CL/P are 4 to 13 per 10 000 births (Vanderas, 1987). Moreover, about 12% of all embryos or fetuses aborted nowadays have an orofacial cleft (Weingartner *et al*., 2007). The etiology of these defects is complex and associated with both genetic and environmental factors. At the molecular level, recent studies in chicks and rodents have identified specific roles for several major signaling pathways, including Shh, Fgf, and Bmp pathways, in facial morphogenesis (Lipinski *et al*., 2010). In humans, research suggests that various growth factors (*e.g.* TGFA, FGF10, FGFR1), receptors, and transcription factors are important in CL/P development (Mostowska *et al*., 2010). The CL/P-affected children have to undergo several

invasive medical procedures, and therefore it is a imperative for a better understanding of abnormal palatogenesis and identification of modifiable risk factors.

Malonyl-CoA is regarded as a key signaling molecule in mammalian cells (Saggerson, 2008). Both acetyl-CoA carboxylase 1 (ACC1) and acetyl-CoA carboxylase 2 (ACC2) catalyze the incorporation of bicarbonate into acetyl-CoA to form malonyl-CoA. ACC1 is belived to function primarily, but not exclusively, as a provider of malonyl-CoA for fatty acid elongation, which is catalyzed by fatty acid synthase. ACC2 is thought to function more as regulator of β-oxidation (Saggerson, 2008). Malonyl-CoA is a potent inhibitor of carnitine palmitoyl transferase 1 (CPT1), which conjugates fatty acids to carnitine, also called vitamin B_T (Fraenkel, 1953), allowing their subsequent mitochondrial import. Malonyl-CoA plays an important role in balancing fuel usage. Carnitine is essential for the membrane transport and subsequent intramitochondrial β-oxidation of long-chain fatty acids, and it also forms esters with several medium- and short-chain fatty acids.

The activities of ACC1 and ACC2 are regulated in response to a variety of metabolic and environmental signals (Tong, 2005), and their product malonyl-CoA may participate in a diverse range of physiological or pathological responses, including associated with diabetes and ischemia, which are well-known risk factors for orofacial clefts (Watkinson and Millicovski, 1983; Spilson *et al*., 2001; Evers *et al*., 2004; Jones *et al*., 2008). Sensitivity of CPT1 to inhibition by malonyl-CoA may alter during embryogenesis (Murray *et al*., 1999). Homozygous deficiencies of CPT1 and ACC1 are lethal in mouse mutants (Nyman *et al*., 2005; Abu-Elheiga *et al*., 2005). Carnitine probably might diminish the regulatory effect of malonyl-CoA (Bird and Saggerson, 1985). Recently, availability of carnitine has been reported to be essential for the developing human fetus and dynamic features of plasma carnitine profile during pregnancy suggest an extraordinary active participation of this amino acid in fetal metabolism (Oey *et al*., 2006; Talian *et al*., 2007; Abdelrazik *et al*., 2009).

Malonyl-CoA is mostly converted back to acetyl-CoA by malonyl-CoA decarboxylase. It may be also hydrolysed to free malonyl acid while some may be estrified to malonylcarnitine (C3DC). Inborn deficiency of malonyl-CoA decarboxylase strongly increases malonylcarnitine (Salomons *et al*., 2007). It was assumed that malonylcarnitine level might reflect malonyl-CoA homeostasis.

Acylcarnitine analysis of dried blood spots using tandem mass spectrometry (MS/MS) to detect disorders of fatty acid oxidation and some disorders of organic acid metabolism is one of the most important advancements in neonatal screening (Rinaldo *et al*., 2004). During catabolism on first days after an infant's birth, all of the transporters and enzymes required for fatty acid β-oxidation are highly induced. MS/MS offers the possibility of multimetabolite analyses in a single analytical run. The newborn screening program provided by our institute quantifies carnitines, which may reflect malonyl-CoA homeostasis (Salomons *et al*., 2007). Thus, the aim of the present study was to analyse malonylcarnitine, acetylcarnitine (C2), and total free carnitine (C0) in newborns with CL/P and in healthy controls.

Materials and Methods

Study population

All patients with isolated cleft lip with or without cleft palate (CL/P) attending our institution and unrelated healthy children without congenital anomalies of similar age attending three local primary care pediatricians were considered for inclusion for the study. Inclusion criteria were as follows: (1) singleton pregnancy, (2) gestational age at delivery ≥36 weeks and/or birth weight >2 000 g, which have long been recognized as important determinants of newborn health, and (3) delivery in the years 2004–2007 in hospitals located in the area covered by the MS/MS Newborn Screening Program provided by our institution. Case eligibility was ascertained from detailed medical records. Finally, we performed retrospective analysis of carnitine concentrations in 51 newborns with non-syndromic cleft lip, with or without cleft palate (cleft group), and 106 healthy newborns without congenital anomalies (control group). All participants were white Caucasians. The study protocols were approved by the local

Ethics Committee.

MS/MS analysis

The dried blood spots ("Guthrie cards") were generally collected three days after birth. Discs were punched from dried blood spots on a filter paper. Specimens were extracted in pure methanol containing known concentrations of stable isotopically enriched carnitines. MS/MS analyses of the carnitines as butyrated esters were performed on a tandem mass spectrometer (SCIEX Api 2000, Concord, Canada) configurated with liquid chromatography for sample handling. The flag (>0.25 μmol·L^{-1}) for concentrations of malonylcarnitine indicating the possibility of inborn malonyl-CoA decarboxylase deficiency was set such that 0.02% of all screened newborns by our laboratory would be flagged.

Statistical Methods

Statistical methods for malonyl-, acetyl-, and free-carnitine concentration analysis as a continuous variable included the *t*-test and Spearman correlation analysis. Cut-off points were established using likelihood ratio values (the ratio of the maximum probability of a result using two different hypotheses). The *chi-square* test was used to investigate the relationship between categorical parameters. Statistical significance was interpreted as *P* values <0.05. All statistical analyses were performed using the SPSS version 12.0.1 for Windows.

Results

Slightly more case newborns were males (67%) compared with the control group (62%), *P*>0.05. Mean gestational age at delivery of cases and controls were the same (39 weeks). Birth weights of CL/P newborns were, in mean, 140 g lower than that in not affected children (*P*=0.05). None of newborns in cleft group, as well as controls, flagged for whole blood malonylcarnitine, which might indicate inborn deficiency of malonyl-CoA decarboxylase. The mean (SD) concentrations of malonylcarnitine and acetylcarnitine were lower in newborns with CL/P compared to newborns without congenital anomalies; 0.048 (0.021) μmol·L^{-1} *vs*. 0.058 (0.024) μmol·L^{-1} (*P*=0.009) and 33.8 (9.3) μmol·L^{-1} *vs*. 37.8 (10.7) μmol·L^{-1} (*P*=0.026), respectively. The mean free carnitine level in cases was insignificantly higher than in controls, 25.5 (7.3) μmol·L^{-1} *vs*. 24.1 (8.1) μmol·L^{-1}, respectively (*P*>0.05). The likelihood ratio test indicated one malonylcarnitine level cut-off point: 0.047 μmol·L^{-1}. We therefore analysed two groups of individuals with malonylcarnitine levels: (1) ≤0.047 μmol·L^{-1} (*n*=64) and (2) >0.047 μmol·L^{-1} (*n*=93). In patients with CL/P, low malonylcarnitine levels were 1.7 times more predominant than in healthy individuals, 29/51 (57%) *vs*. 35/106 (33%), respectively (*P*=0.03). During analysis of acetylcarnitine and free carnitine concentrations, the likelihood ratio test did not indicate valuable cut-off points. The levels of malonylcarnitine were significantly correlated with acetylcarnitine concentrations, *r*=0.484 (*P*<0.001) but not with free carnitine, *r*=0.086 (*P*=0.287). Acetylcarnitine and free carnitine levels were highly correlated, *r*=0.599 (*P*<0.001). There were no significant correlations between concentrations of carnitines and clinical variables such as birth weight or gestational age at delivery.

Discussion

To our knowledge, the present report is the first study investigating malonylcarnitine in patients with isolated structural malformations. We found an association between low level of whole blood malonylcarnitine and the risk of CL/P in newborns. Acetylcarnitine levels were also lower in affected children, despite similar concentrations of free carnitine in the cleft group and controls. The molecular mechanisms of abnormal palatogenesis are poorly understood. The relatively high and fluctuating frequency of orofacial clefts is attributable to the sensitivity of facial development to enviromental exposures? (Weingartner *et al*., 2007). It is well-accepted that orofacial cleft may be caused by the simultaneous interaction of the several genetic factors and environmental conditions. However, the exact conditions on which these interaction take place remain to be elucidated. It remains unclear whether decreased concentrations

of malonylcarnitine and acetylcarnitine may be causally associated with risk of CLP or are only correlated epiphenomena.

It is noteworthy that there is strong evidence for the utilization of lipids as an energy substrate by early embryos (Sturmey *et al*., 2009; El-Shahat *et al*, 2010). Malonyl-CoA plays a key role in control of energy balance in mammalian cells, but its function in developing embryo deserves further studies (Saggerson, 2008). Recently Downs *et al*. (2009) demonstrated that malonyl-CoA as well as carnitine are important regulators of oocyte meiotic maturation in mice. ACC1 and ACC2, which form malonyl-CoA, are biotin (vitamin H)-dependent enzymes. It is intriguing that biotin is concentrated in the area of palatogenesis in chicken embryos and the vitamin deficiency affects the proliferation of human embryonic palatal cells (Taniguchi and Watanabe, 2007; Takechi *et al*., 2008). Fatty acids belong to the growing list of metabolic signals with physiologically relevant actions (Obici *et al*., 2003). Results of the studies on chicks suggested that the alternations in fatty acid metabolism might cause malformations occurring in biotin deficiency (Bain *et al*., 1988; Watkins *et al*., 1989). Even marginal biotin deficiency, which affects the expression of more than 2000 genes in human cells, is teratogenic in some mammalian species (Zempleni, 2005). Vitamin H deficiency is one of the most potent clefting factors even when the dams do not show any signs of biotin deficiency (Mock, 2005; Watanabe *et al*., 2009). In normal human pregnancy, vitamin H deficiency develops frequently; however, it develops in a marginal, clinically asymptomatic form (Mock, 2005). It is noteworthy that marginal maternal biotin deficiency may produce severe reductions in fetal carboxylases in experimental animals (Sealey *et al*., 2005).

Our findings suggest that the metabolic pathway of malonyl-CoA is disturbed in CL/P-affected patients; however, the potential role of biotin-dependent carboxylases has yet to be elucidated. It is impossible to conclude whether malonyl-CoA or its metabolites directly affect palatogenesis. Further studies are warranted to validate these presumptions. It seems reasonable to test polymorphic variants of genes involved in malonyl-CoA metabolism as CL/P risk factors. There are no previously published clinical or experimental studies on the role of malonyl-CoA pathway in teratogenesis, and thus we are unable to compare our results with data from other studies.

Associations between maternal diabetes mellitus, hyperhomocysteinemia, and chemicals exposures, which are known to affect both antioxidant systems as well as free radical generation, and an increased risk of having a child with orofacial cleft, have been reported (Spilson *et al*., 2001; Weingartner *et al*., 2007). Carnitine has antioxidant activity that combines both free radical scavenging and metal-chelating properties (Abdelrazik *et al*., 2009). In animal husbandry, the use of supplements containing carnitine for reproduction and pregnancy is widely recognized (Ramanau *et al*., 2008).

This study is limited in many ways. First, the number of patients with clefts who were screened by newborn MS/MS is small; thus, extrapolation to larger populations must be cautious. Second, nutritional status of participants was not recorded while the newborn dried spots were taken. Therefore, influence of feeding problems on metabolic stress in some cases was possible. Third, the study design has retrospective nature and we lacked the ability to access additional information on maternal diet, supplements use, and weight gain during pregnancy, which might interfere with carnitine and fatty acid metabolism. Fourth, the MS/MS assessments were carried out after delivery, and only in newborns, which may have lowered the possibility of finding teratologically pertinent feto-maternal changes. From gametogenesis, throughout pregnancy, the conceptus is compelled to adapt to changes in its environment, determined by maternal nutritional status and metabolism. It is currently impossible to decipher whether lower malonylcarnitine levels in some newborns with CL/P were associated with low maternal malonylcarnitine, decreased biotin intake, or an increased utilization of the vitamin. Moreover, we should keep in mind that MS/MS methods were developed to diagnose markedly elevated malonylcarnitine levels in inherited malonyl-CoA decarboxylase deficiency.

This study had also some notable strength. The investigated population was ethnically homogeneous, mostly omnivorous, and from an area where preconceptional supplements use is low. Data of carnitines assessments were from a population

based MS/MS Newborn Screening Program, but we were able to establish phenotypic cleft type in all cases.

In conclusion, this is the first report of malonylcarnitine level in newborns with orofacial cleft. Despite its limitations, the study provides initial data indicating a potential association between low malonylcarnitine and acetylcarnitine concentrations and CL/P risk. It is important to stress the need for further studies of metabolic profiles of CL/P-affected children, which might throw new light on nutritional intervention strategies to reduce risk of orofacial clefts.

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