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Prenatal Diagnosis of Molybdenum Cofactor Deficiency by Assay of Sulphite Oxidase Activity in Chorionic Villus Samples

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Summary: Molybdenum cofactor deficiency is characterized by the absence of sulphite oxidase, xanthine dehydrogenase and aldehyde oxidase, the three known enzymes in man that require the cofactor for their activity. Prenatal diagnosis of the deficiency may be performed by assay of sulphite oxidase activity in cultured amniocytes. However, the activity in amniocytes is low and large numbers of cells are required for reliable assessment. We show that sulphite oxidase is present at high levels in chorionic villi obtained at 10–14 weeks gestation and can be assayed directly in the biopsy sample without cell culture. This assay has been applied to two pregnancies at risk for molybdenum cofactor deficiency with successful diagnoses of an unaffected and an affected fetus.

Molybdenum cofactor deficiency (McKusick 25215) is a rare inborn error of metabolism (Johnson *et al.*, 1980; Wadman *et al.*, 1983; Johnson and Wadman, 1989) characterized by a combined deficiency of three enzymes that require the molybdenum cofactor for activity: sulphite oxidase (EC 1.8.2.1), xanthine dehydrogenase (EC 1.2.1.37), and aldehyde oxidase (EC 1.2.3.1). The absence of sulphite oxidase, in par-particular, leads to severe neurological sequelae and most often to death at an early age.

The molybdenum cofactor of these enzymes is a complex of the metal with an unusual pterin termed molybdopterin (Figure 1) (Johnson and Rajagopalan, 1982; Johnson *et al.*, 1984; Kramer *et al.*, 1987). The isolated cofactor is unstable and for this reason cannot be used as a therapeutic agent to correct the deficiency disease. The complex structure of the cofactor would indicate that a multistep pathway is required for its biosynthesis; studies are currently in progress to define this biosynthetic pathway and to identify the specific molecular defect in affected individuals (Johnson *et al.*, 1989a). In the course of these studies, it is possible that stable biosynthetic

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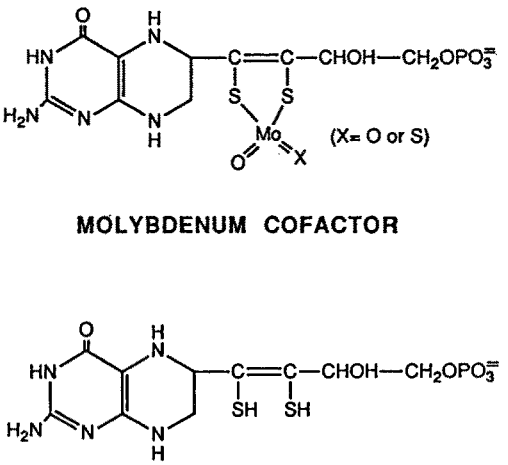

Figure 1 The structure of the molybdenum cofactor present in sulphite oxidase, xanthine dehydrogenase and aldehyde oxidase and the structure of molybdopterin. The pterin ring systems are shown in the fully reduced (tetrahydro) state

intermediates may be identified which could serve to correct the deficiency in some patients. At present, however, there is no effective treatment or cure.

Prenatal diagnosis of molybdenum cofactor deficiency has previously been accomplished by assay of sulphite oxidase in cultured amniocytes (Ogier *et al.*, 1983). However, the activity of sulphite oxidase in fibroblasts and amniocytes is very low, and weeks of cell culture are generally required to obtain a sufficient number of cells for assessment of enzyme activity. In this study we have evaluated the effectiveness of assay of sulphite oxidase in chorionic villi for prenatal diagnosis of molybdenum cofactor deficiency. While these studies were in progress, a report appeared summarizing the first attempted diagnosis of this disease by CVS assay (Gray *et al.*, 1990). In this case, the absence of sulphite oxidase activity in the chorionic villus sample was taken as suggestive evidence for an affected fetus, although a delay in transport of the sample that could have resulted in inactivation of sulphite oxidase and the absence of any reported values for sulphite oxidase in control chorionic villi made the diagnosis tentative. Subsequent assays showed elevated *S*-sulphocysteine in the amniotic fluid and the absence of sulphite oxidase in cultured amniocytes and in fibroblasts and liver from the aborted fetus. In the studies reported here, we define normal values for sulphite oxidase in chorionic villi from two control pregnancies and one pregnancy at risk in which the fetus was unaffected. In addition, we confirm the earlier observation that sulphite oxidase activity is absent in chorionic villi from an affected pregnancy. These findings establish that it is possible to diagnose molybdenum cofactor deficiency, or a specific deficiency of sulphite oxidase, by assay of this enzyme directly in uncultured chorionic villus tissue and that, with proper handling of the chorionic villus sample, no confirmatory assays of amniotic fluid or cultured amniocytes are required.

CASE DESCRIPTIONS

In these studies two pregnancies at risk for molybdenum cofactor deficiency in two separate families were monitored. In the first family studied, both parents were of Dutch origin and were unrelated. The index case (K.N.) was their third child after the birth of two healthy daughters. He presented from birth with frequent severe convulsions that were unresponsive to anticonvulsants. Molybdenum cofactor deficiency was suggested by analyses of sulphur and purine metabolites and was confirmed by the finding of no detectable sulphite oxidase activity in cultured fibroblasts. The patient died at 9 days of age. The mother (M.N.) was monitored in her fourth pregnancy by assay of sulphite oxidase activity in chorionic villus tissue as described below.

In the second family studied, both parents were Vietnamese and were unrelated. The index case (W.T.) was their first child and was diagnosed with molybdenum cofactor deficiency at 8 months of age when she presented with developmental delay, seizures and bilateral dislocation of the lenses. The pattern of sulphur and purine metabolite excretion was consistent with a combined deficiency of sulphite oxidase and xanthine dehydrogenase; assay of cultured fibroblasts confirmed the absence of sulphite oxidase activity (Bamforth *et al.*, in press). Analysis of urine from this patient revealed the absence of urothione, the normal metabolic excretory product of the molybdenum cofactor (Johnson and Rajagopalan, 1982), and the presence of a molybdopterin precursor that could be oxidized to a characteristic fluorescent species, Compound Z (Bamforth *et al.*, in press; Johnson *et al.*, 1989a,b). The presence of this precursor, and fibroblast complementation studies, indicated that W.T. was of the group B complementation class with a defect in the terminal enzyme required for synthesis of molybdopterin (Johnson *et al.*, 1989a). A second, unmonitored pregnancy led to the birth of a normal child. In the third pregnancy, the mother (N.T.) was monitored by assay of sulphite oxidase in cultured amniocytes (Bamforth *et al.*, in press). The results indicated that the fetus was affected, and an abortion was carried out. The diagnosis was confirmed by the finding of no detectable sulphite oxidase activity in fibroblasts and in liver tissue from the aborted fetus. The fourth pregnancy was monitored by assay of sulphite oxidase activity in chorionic villus tissue as described below.

MATERIALS AND METHODS

Chorionic villi were separated from a control placenta obtained at 14 weeks gestation by therapeutic abortion carried out as the result of fetal death *in utero*. The tissue was rinsed with tissue culture medium, blotted dry and divided into 50–200 mg samples. The chorionic villus samples obtained from pregnancies at risk for molybdenum cofactor deficiency were immediately dissected free of maternal decidua and snap-frozen in liquid nitrogen, then stored at −70°C. The material was shipped on solid carbon dioxide for subsequent assay. Individual samples were weighed and homogenized at 4°C in 1 ml 0.01 mol/L Tris-HCl, pH 8.5, containing 0.01 mmol/L EDTA using a ground-glass homogenizer. After the addition of 50 μl 1% (w/v) sodium

deoxycholate and 10 μl 50 mmol/L sodium cyanide, the samples were centrifuged at 12 000*g* for 3 min at 4°C. One millilitre of the supernatant was applied to a PD-10 gel filtration column (Pharmacia) equilibrated with 0.1 mol/L Tris-HCl, pH 8.5, containing 0.1 mmol/L EDTA (assay buffer). The column was washed with 1.5 ml of the same buffer and the sample was eluted with 2 ml buffer. Aliquots of 200–400 μl were assayed for sulphite oxidase activity at 25°C by monitoring the reduction of cytochrome *c* at 550 nm. The assay cuvettes contained 50 μl 1 mmol/L cytochrome *c* (Sigma, Type VI, dissolved in assay buffer), extract and assay buffer to a final volume of 1 ml. The blank rate of cytochrome *c* reduction was recorded. After 4–5 min a 10 μl aliquot of 50 mmol/L sodium sulphite was added, and the increase in absorbance at 550 nm was again recorded for the same time period. Sulphite oxidase activity was calculated as the difference between the rate observed in the presence of sulphite and the blank rate. A unit of sulphite oxidase activity is defined as enzyme sufficient to produce an increase at 550 nm of 1.0 absorbance unit per min at 25°C. Activity is expressed as units per gram tissue (wet weight).

RESULTS

Sulphite oxidase activity in control chorionic villus tissue: Sulphite oxidase activity in the control villus tissue was 0.89 unit/g. The activity was not altered in tissue samples that were stored frozen at −70°C for periods of several months.

Sulphite oxidase activity in chorionic villus tissue from M.N.: Sulphite oxidase activity, measured in a 115 mg biopsy sample from M.N. obtained at 10–11 weeks, was found to be 1.09 units/g. A control biopsy sample (51 mg) shipped from The Netherlands at the same time was found to contain 1.04 units of sulphite oxidase per gram. These values were in good agreement with that of the control monitored above, and the fetus at risk was judged to be unaffected with molybdenum cofactor deficiency. This diagnosis was confirmed with the birth of a normal infant.

Sulphite oxidase activity in chorionic villus tissue from N.T.: Assay of sulphite oxidase in biopsy material from N.T. (25 mg) obtained at 12 weeks revealed no detectable activity. Control chorionic villi assayed on the same day were found to contain 0.97 unit/g. A very slight stimulation in the rate of cytochrome *c* reduction was seen upon addition of sulphite to a cuvette containing extract from N.T. but was no greater than the stimulation seen upon adding sulphite to a mixture of cytochrome *c* and assay buffer and thus could be attributed to a very low rate of non-enzymatic reduction of cytochrome *c* sulphite. This was confirmed by testing the effect of addition of a preparation of polyclonal antibodies directed against sulphite oxidase purified from human liver. The antiserum produced complete inhibition of the activity of sulphite oxidase in the control extract but had no effect on the low non-enzymatic rate of cytochrome *c* reduction seen with buffer alone or with the extract from N.T. From these results the fetus at risk was judged to be affected with molybdenum cofactor deficiency, and the pregnancy was terminated at 14 weeks by prostaglandin induction.

For confirmation of the diagnosis, a sample of liver tissue was obtained from the aborted fetus and assayed for sulphite oxidase activity along with control liver tissue from an aborted fetus of approximately the same gestational age. No activity was detected in the fetal liver sample from the N.T. pregnancy, while activity in the control was found to be 5.16 units/g.

DISCUSSION

The results documented above demonstrate that molybdenum cofactor deficiency can be unequivocally and efficiently diagnosed by direct assay of sulphite oxidase activity in chorionic villus biopsy material. The enzyme is present in the tissue, as obtained, at high levels and can be quantitated without difficulty in samples of 25–50 mg. Sulphite oxidase activity is fully retained in samples that have been frozen at −70°C; thus, biopsy samples that have been frozen in liquid nitrogen and shipped on solid carbon dioxide are suitable for prenatal diagnosis. Since there is no need to culture cells for expression of activity, the assays can be done immediately, and the status of the fetus at risk can be determined at an early gestational age. The CVS assay of sulphite oxidase is superior to enzyme assay in cultured amniocytes which has been used to date for diagnosis of molybdenum cofactory deficiency. Amniocytes are obtained significantly later in the pregnancy, and the very low levels of sulphite oxidase in these cells necessitate weeks of cell culture to obtain sufficient material for an accurate assessment of enzyme activity.

The observation that sulphite oxidase is expressed at a level of approximately 1 unit/g in chorionic villus as early as 10–11 weeks gestation and 5 units/g in liver of 14 weeks serves to emphasize the essential nature of this enzyme during prenatal development. The enzyme in normal adults is present at highest levels in liver, where it ranges from 10 to 25 units/g (Wadman *et al.*, 1983). Newborns and young children have been found to contain similar levels of the enzyme in liver. Thus, although the enzyme levels do increase somewhat with age from that found at 14 weeks, it appears that a definite portion of the adult level of activity is present already at this early stage in development. In contrast to the early development of sulphite oxidase activity that has been documented in these studies, no xanthine dehydrogenase activity was detected in the chorionic villus or fetal liver samples examined. Similar patterns of developmental appearance of the two molybdoenzymes have been noted in more detailed studies carried out in rats (Cohen *et al.*, 1974). In this species, sulphite oxidase activity was clearly present at birth and showed a rapid rise to adult levels within a few days of birth. Xanthine dehydrogenase, on the other hand, was low at birth and did not increase significantly until weaning at 21 days.

Biochemical analysis of patients that exhibit a deficiency of sulphite oxidase has revealed that nearly all are characterized by an underlying defect in the molybdenum cofactor required by this enzyme. However, a very small number of patients display a defect in sulphite oxidase yet produce functional molybdenum cofactor, xanthine dehydrogenase and aldehyde oxidase (Johnson and Wadman, 1989). These individuals exhibit essentially the same clinical symptoms as those with the combined deficiency, again stressing the essential nature of sulphite oxidase. Although these individuals

represent at present a small patient population, obligate heterozygous carriers of this form of sulphite oxidase deficiency could also benefit from the prenatal diagnosis technique described in this article.

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REFERENCES

Bamforth, F. J., Johnson, J. L., Davidson, A. G. F., Wong, L. T. K., Lockitch, G. and Applegarth, D. A. Biochemical investigation of a child with molybdenum cofactor deficiency. *Clin. Biochem.*, in press

Cohen, H. J., Johnson, J. L. and Rajagopalan, K. V. Molecular basis of the biological function of molybdenum. Developmental patterns of sulfite oxidase and xanthine oxidase in the rat. *Arch. Biochem. Biophys.* 164 (1974) 440–446

Gray, R. G. F., Green, A., Basu, S. N., Constantine, G., Condie, R. G., Dorche, C., Vianey-Liaud, C. and Desjacques, P. Antenatal diagnosis of molybdenum cofactor deficiency. *Am. J. Obstet. Gynecol.* 163 (1990) 1203–1204

Johnson, J. L. and Rajagopalan, K. V. Structural and metabolic relationship between the molybdenum cofactor and urothione. *Proc. Natl. Acad. Sci. USA* 79 (1982) 6856–6860

Johnson, J. L. and Wadman, S. K., Molybdenum cofactor deficiency. In Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. (eds.) *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw-Hill, New York, 1989, pp. 1463–1475

Johnson, J. L., Waud, W. R., Rajagopalan, K. V., Duran, M., Beemer, F. A. and Wadman, S. K. Inborn errors of molybdenum metabolism. Combined deficiencies of sulfite oxidase and xanthine dehydrogenase in a patient lacking the molybdenum cofactor. *Proc. Natl Acad. Sci. USA* 77 (1980) 3715–3719

Johnson, J. L., Hainline, B. E., Rajagopalan, K. V. and Arison, B. H. The pterin component of the molybdenum cofactor. Structural characterization of two fluorescent derivatives. *J. Biol. Chem.* 259 (1984) 5414–5422

Johnson, J. L., Wuebbens, M. M., Mandell, R. and Shih, V. E. Molybdenum cofactor biosynthesis in humans: identification of two complementation groups of cofactor-deficient patients and preliminary characterization of a diffusible molybdopterin precursor. *J. Clin. Invest.* 83 (1989a) 897–903

Johnson, J. L., Wuebbens, M. M. and Rajagopalan, K. V. The structure of a molybdopterin precursor. Characterization of a stable, oxidized derivative. *J. Biol. Chem.* 264 (1989b) 13440–13447

Kramer, S. P., Johnson, J. L., Ribeiro, A. A., Millington, D. S. and Rajagopalan, K. V. The structure of the molybdenum cofactor: characterization of di-(carboxamidomethyl)molybdopterin from sulfite oxidase and xanthine oxidase. *J. Biol. Chem.* 262 (1987) 16357–16363

Ogier, H., Wadman, S. K., Johnson, J. L., Saudubray, J. M., Duran, M., Boue, J., Munnich, A. and Charpentier, C. Antenatal diagnosis of combined xanthine and sulphite oxidase deficiencies. *Lancet* 2 (1983) 1363–1364

Wadman, S. K., Duran, M., Beemer, F. A., Cats, B. P., Johnson, J. L., Rajagopalan, K. V., Saudubray, J. M., Ogier, H., Charpentier, C., Berger, R., Smit, G. P. A., Wilson, J. and Krywawych, S. Absence of hepatic molybdenum cofactor: an inborn error of metabolism leading to a combined deficiency of sulphite oxidase and xanthine dehydrogenase. *J. Inher. Metab. Dis.* 6 (Suppl. 1) (1983) 78–83