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Hyperglycosylated hCG and pregnancy failures

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ABSTRACT

Considerable evidence indicates that one third of early pregnancy failures, spontaneous abortions and biochemical pregnancies, are due to chromosomal abnormalities, and two thirds are due to inappropriate implantation. These findings led us to investigate the role of hyperglycosylated hCG, an important pregnancy implantation signal, in pregnancy failures. We used urinary hCG determinations to evaluate a total of 127 pregnancies on the day of implantation, as marked by a positive urinary hCG. These included 81 normal term pregnancies, 18 spontaneous abortion pregnancies, and 28 biochemical pregnancies. Of the normal term pregnancies, the mean ± standard deviation concentration of hyperglycosylated hCG was 5.4 ± 4.3 mIU/ml equivalents, and the percentage of hyperglycosylated hCG was 88 ± 17%. All term pregnancies produced hyperglycosylated hCG > 51%. Of the 18 cases that spontaneously aborted, both the mean hyperglycosylated hCG (1.9 ± 2.0 mIU/ml equivalents) and the percentage of hyperglycosylated hCG (41 ± 33%) were significantly lower than in the normal pregnancy group. Only 4/18 spontaneously aborting pregnancies produced more than 51% hyperglycosylated hCG on the day of implantation. Similarly, of the 28 biochemical pregnancies, both the mean hyperglycosylated hCG (0.63 ± 1.3 mIU/ml equivalents) and the percentage of hyperglycosylated hCG (21 ± 29%) were significantly lower than in the normal pregnancy group. Only 4/28 pregnancies produced more than 51% hyperglycosylated hCG. Low hyperglycosylated hCG concentrations are associated with pregnancy failure. Whether this association is a primary cause of pregnancy failure or is simply a marker for an abnormal conceptus requires further investigation.

1. Introduction

It has long been known that approximately 25% of pregnancies end very early after a short rise in hCG as "biochemical pregnancies" (Wilcox et al., 1988, 1999) well before the possibility of the ultrasound detection of pregnancy. Another 17% of clinical or established pregnancies terminate in spontaneous abortions in the first and second trimesters of pregnancy (Wang et al., 2004; Gray and Wu, 2000). It is well accepted that at least one-third of these pregnancy failures are due to conceptus chromosomal abnormalities (Norwitz et al., 2001; Semprini and Simon, 2000). Other alleged factors leading to pregnancy failure include hormonal abnormalities, infection, environmental exposures (e.g., caffeine or smoking), diabetes, collagen vascular disease, T cell and immune defects, although a causal relationship between pregnancy loss and many of these factors is little more than speculation (Licciardi, 2006). Recent research by two independent groups suggests that two thirds of pregnancy failures are due to inappropriate implantation (Norwitz et al., 2001; Semprini and Simon, 2000). Thus, we sought to investigate the role of hyperglycosylated hCG, the pregnancy implantation signal, in pregnancy failure.

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Hyperglycosylated hCG is a large variant of the hormone hCG distinguished by having large carbohydrate side chains. Hyperglycosylated hCG is made exclusively by cytotrophoblast cells (Kovalevskaya et al., 2002; Cole et al., 2006a,b). The large carbohydrate side chains limit subunit folding exposing structures similar to those found in transforming growth factor β (TGFβ) (Lapthorn et al., 1994; Laub and Jennissen, 2003). Thus, hyperglycosylated hCG competes with TGFβ for binding to appropriate receptors (Cole et al., 2006b; Cole and Butler, 2012).

TGFβ receptor ligand binding blocks cell apoptosis (Hamade et al., 2005; Cole and Butler, 2012), promotes cell growth (Cole et al., 2006a,b), and promotes collagenase and metalloproteinase production, or cell invasive enzyme synthesis (Murphy et al., 1987; Bany et al., 2000; Bai et al., 2005; Shibahara et al., 2005). Only hyperglycosylated hCG, and not hCG, drives invasion by cytotrophoblast cells in nude mice models (Cole et al., 2006a,b). And hyperglycosylated hCG, and not hCG, drives normal pregnancy cytotrophoblast primary culture cells to invade Matrigel cell invasion chamber membranes (Cole et al., 2006b). Given these properties, it is not surprising that hyperglycosylated hCG is produced and is present in serum at the time of pregnancy implantation, 3rd to 4th weeks of invasion (O'Connor et al., 1998). Cytotrophoblast cells producing hyperglycosylated hCG are the principle cells on blastocysts, and the cells that come into contact with the decidua at the time of pregnancy implantation (Guibourdenche et al., 2010; Handschuh et al., 2007b).

One can conclude from the aforementioned that hyperglycosylated hCG is a primary component of cytotrophoblast invasion (Cole et al., 2006a,b) and thus plays a key role in implantation (O'Connor et al., 1998; Guibourdenche et al., 2010; Handschuh et al., 2007b; Cole, 2010). We hypothesized that poor implantation resulting in pregnancy failure (Norwitz et al., 2001; Semprini and Simon, 2000) might be due to inadequate production of hyperglycosylated hCG.

2. Methods

Over a period of four years, we collected 215 women attempting to achieve pregnancy and who volunteered for a urine collection research program offered by the USA hCG Reference Service. The urine collection program was funded by Church and Dwight, Inc. solely for the collection of newly pregnant samples for evaluating home pregnancy devices. The study included an agreement that urine results could be independently used for unbiased research. The study was managed by the University of New Mexico Human Research Review Committee (protocol number 04-132). All volunteers used home ovulation screening devices to aid them in the achievement of pregnancy. Fifteen individuals withdrew from the program because of ovulation disorders (no LH peak), and another 22 women withdrew of their own accord and were not seen further. Thus, there were 137 pregnancies among the 178 fruitful volunteers. In 10 pregnancies, a lack of samples on the day of implantation or a lack of complete assay data led to exclusion, leaving 127 pregnancies among 168 subjects. Samples were available for this study from 81 women achieving normal term pregnancy, 18 with spontaneously aborting pregnancy and 28 with biochemical pregnancies (127 pregnancies in total). Biochemical pregnancies were defined as those terminating after a short rise in hCG (Wilcox et al., 1988, 1999), well before the possibility of the ultrasound detection of pregnancy. Of these volunteers the average age and standard deviation of volunteers was 28.8 ± 4.4 years, and the range was 18–37 years. The pregnancy volunteers included 78 nulliparous women, 28 secundigravidas, and 21 multiparous women.

Urines were tested daily during this program for luteinizing hormone (LH), hCG, and hyperglycosylated hCG through up to six menstrual cycles, and, when pregnancy was achieved, until 8 weeks' gestation. Total hCG and LH were measured using the Siemens Immulite 1000 automated test. This test has been calibrated for urine application as described previously (Cole and Khanlian, 2009). Hyperglycosylated hCG was determined using the specific microtiter plate assay with antibody B152 as the capture and antibody 5008 with peroxidase label as the tracer, as described previously (Cole et al., 2006b).

The day of pregnancy implantation was estimated from urine hCG results from all 127 pregnancy cases as the day of first detectable hCG production, using a sensitive total hCG test, as first proposed by Wilcox et al. (1999). Each patient's assay results were stored in a separate Microsoft Excel worksheet, and then analyzed together for this report in an independent spreadsheet. Means, standard deviations, and *t*-test significance values were all determined in Microsoft Excel.

Calculation of percentage hyperglycosylated hCG normalizes values for urine concentration inasmuch as both hyperglycosylated hCG and total hCG are subject to urine dilution. Thus, the percentage of hyperglycosylated hCG was calculated as: hyperglycosylated hCG/total hCG

3. Results

Urine samples were collected and tested from the day of pregnancy implantation from 127 pregnancies: 81 were normal term (64%); 18 spontaneously aborted (16 during the first trimester and two during the second trimester of pregnancy; 14%); and 28 were biochemical pregnancies (22%).

The mean ± standard deviation total hCG concentration (hCG + hyperglycosylated hCG) in the 81 term pregnancies was 5.3 ± 4.8 mIU/ml (Fig. 1). The hyperglycosylated hCG mean concentration was 5.4 ± 4.3 mIU/ml equivalents (Fig. 2). With normalizing individual hyperglycosylated hCG concentration to individual total hCG concentrations (hyperglycosylated hCG ÷ total hCG), the 81 term pregnancies produced 88 ± 17% hyperglycosylated hCG (Fig. 3). In each of the 81 term outcome cases the ratio of hyperglycosylated hCG to total hCG was >51%.

In the 18 cases that spontaneously aborted, the total hCG mean concentration was 4.2 ± 4.2 mIU/ml, which was not significantly different from that of the successful term pregnancies (Fig. 1). However, the hyperglycosylated hCG mean concentration, 1.9 ± 2.0 mIU/ml equivalents, was significantly lower than that of the successful term pregnancies ($P < 0.001$; Fig. 2). With normalization of individual

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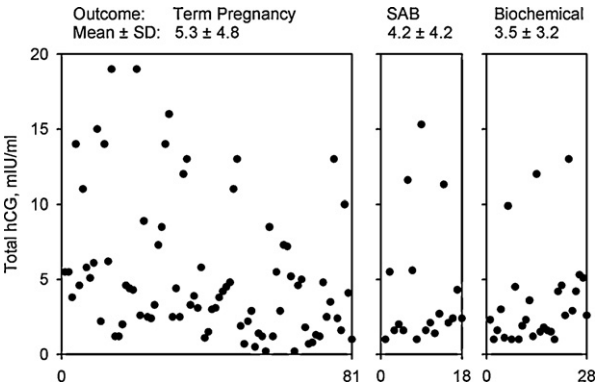

Fig. 1. Total hCG concentration on the day of implantation in 81 normal term outcome pregnancies, 18 spontaneous abortion outcome pregnancies (SAB), and 28 biochemical outcome pregnancies.

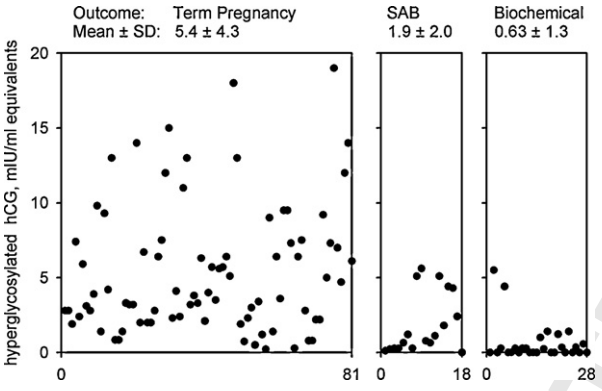

Fig. 2. Hyperglycosylated hCG concentration on the day of implantation in 81 normal term outcome pregnancies, 18 spontaneous abortion outcome pregnancies (SAB), and 28 biochemical outcome pregnancies.

hyperglycosylated hCG concentration to individual total hCG concentrations (hyperglycosylated hCG/total hCG), the mean percentage for the spontaneous abortion group was 41 ± 33%, statistically lower than that of the successful term outcome group (Fig. 3). Just four of the

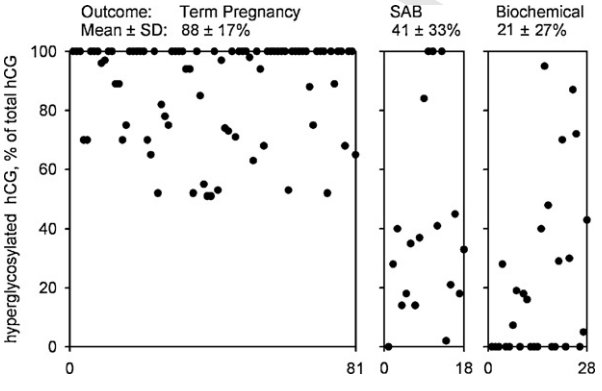

Fig. 3. Proportion of hyperglycosylated hCG (% of total hCG) on the day of implantation in 81 normal term outcome pregnancies, 18 spontaneous abortion outcome pregnancies (SAB), and 28 biochemical outcome pregnancies.

18 spontaneously aborting pregnancies (22%) produced hyperglycosylated hCG at a ratio of more than 51%.

In the 28 cases of biochemical pregnancy, the total hCG mean concentration was 3.5 ± 3.2 mIU/ml, which was not significantly different from that of successful term outcomes. The hyperglycosylated hCG mean concentration was only 0.63 ± 1.3 mIU/ml equivalents, significantly lower than that of successful term outcomes ($P < 0.001$) (Fig. 2). With normalization of individual hyperglycosylated hCG concentration to individual total hCG concentrations (hyperglycosylated hCG/total hCG), the mean percentage for the biochemical pregnancy group was 21 ± 29% of hyperglycosylated hCG, which is statistically lower than that of the successful term outcome group (Fig. 3). Just four of the 28 biochemical pregnancies (14%) produced hyperglycosylated hCG at a ratio of more than 51% (Fig. 3).

A significant difference was also observed between the two groups of failing pregnancies. Comparing spontaneous abortions and biochemical pregnancies, hyperglycosylated hCG mean concentration (Fig. 2) was significantly lower in the biochemical pregnancy group (0.63 ± 1.3 mIU/ml vs. 1.9 ± 2.0 mIU/ml, $P = 0.012$), as was the mean hyperglycosylated hCG (21 ± 27% vs. 41 ± 33%, $P = 0.048$; Fig. 3).

4. Discussion

Recent research shows that approximately one third of pregnancy failures are due to major chromosomal abnormalities, and that two thirds are due to ineffective or inefficient implantation of pregnancy (Norwitz et al., 2001; Semprini and Simon, 2000). Using sensitive urine hCG testing, we have demonstrated that on the day of implantation, pregnancies destined to fail as spontaneous abortions or biochemical pregnancies are marked by low production of hyperglycosylated hCG, in terms of both mean production and hyperglycosylated hCG as a proportion of total hCG. Our results infer that diminished hyperglycosylated hCG production by a blastocyst at the time of implantation is at least a harbinger of pregnancy failure and in some circumstances, plays a primary role in pregnancy failure.

Experts believe that hyperglycosylated hCG is an important signal for cytotrophoblast cells to become invasive in pregnancy implantation (Cole et al., 2006a,b; Cole, 2010; Guibourdenche et al., 2010; Handschuh et al., 2007a,b; Sasaki et al., 2008). Failure to produce an appropriate amount or proportion of glycosylated hCG could well lead to suboptimal or wholly inadequate implantation. That some 17% of pregnancy failures in our series produce glycosylated hCGs similar to that of successful term outcomes could be explained by alternative mechanisms of pregnancy failure, such as conceptus chromosomal abnormalities.

If inadequate hyperglycosylated hCG plays a causative role in pregnancy failures, what are the mechanisms for this? Hyperglycosylated hCG is perhaps the most heavily glycosylated glycoprotein in humans (Cole, 2010). Production requires N-acetylglucosaminidase 4 and 6, two rare glycosyltransferase enzymes critical for the N- and O-linked oligosaccharides on hyperglycosylated hCG. Future

studies should explore possible alterations in these pathways due to infections or environmental agents.

References

Bai, S.X., Wang, Y.L., Qin, L., Xiao, Z.J., Herva, R., Piao, Y.S., 2005. Dynamic expression of matrix metalloproteinases (MMP-2, -9 and -14) and the tissue inhibitors of MMPs (TIMP-1, -2 and -3) at the implantation site during tubal pregnancy. Reproduction 129, 103–113.

Bany, B.M., Harvey, M.B., Schultz, G.A., 2000. Expression of matrix metalloproteinases 2 and 9 in the mouse uterus during implantation and oil-induced decidualization. J. Reprod. Fertil. 120, 125–134.

Cole, L.A., 2010. Hyperglycosylated hCG. A review. Placenta 8, 653–664.

Cole, L.A., Butler, S.A., 2012. Hyperglycosylated hCG, hCGβ and hyperglycosylated hCGβ: interchangeable cancer promoters. Mol. Cell. Endocrinol. 349, 232–238.

Cole, L.A., Khanlian, S.A., 2009. The need for a quantitative urine hCG assay. Clin. Biochem. 42, 676-83.23.

Cole, L.A., Khanlian, S.A., Riley, J.M., Butler, S.A., 2006a. Hyperglycosylated hCG in gestational implantation and in choriocarcinoma and testicular germ cell malignancy tumorigenesis. J. Reprod. Med. 51, 919–925.

Cole, L.A., Dai, D., Butler, S.A., Leslie, K.K., Kohorn, E.I., 2006b. Gestational trophoblastic diseases: 1. Pathophysiology of hyperglycosylated hCG-regulated neoplasia. Gynecol. Oncol. 102, 144–149.

Gray, R.H., Wu, L.Y., 2000. Subfertility and risk of spontaneous abortion. Am. J. Public Health 90, 1452–1454.

Guibourdenche, J., Handschuh, K., Tsatsaris, V., Gerbaud, M.C., Legul, F., Muller, D., Evain-Brion, D., Fournier, T., 2010. Hyperglycosylated hCG is a marker of early human trophoblast invasion. J. Clin. Endocrinol. Metab. 95, E240–E244.

Hamade, A.L., Nakabayashi, K., Sato, A., Kiyoshi, K., Takamatsu, Y., Laoag-Fernandez, J.B., Ohara, N., Maruo, T., 2005. Transfection of antisense chorionic gonadotropin β gene into choriocarcinoma cells suppresses the cell proliferation and induces apoptosis. J. Clin. Endocrinol. Metab. 90, 4873–4879.

Handschuh, K., Guibourdenche, J., Tsatsaris, V., Guesnon, M., Laurendeau, I., Evain-Brion, D., Fournier, T., 2007a. Human chorionic gonadotropin expression in human trophoblasts from early placenta, comparative study between villous and extravillous trophoblastic cells. Placenta 28, 175–184.

Handschuh, K., Guibourdenche, J., Tsatsari, V., Guesnon, M., Laurendeau, I., Evain-Brion, D., Fournier, T., 2007b. Human chorionic gonadotropin produced by the invasive trophoblast but not the villous trophoblast promotes cell invasion and is down-regulated by peroxisome proliferator-activated receptor-a. Endocrinology 148, 5011–5019.

Kovalevskaya, G., Genbacev, O., Fisher, S.J., Caceres, E., O'Connor, J.F., 2002. Trophoblast origin of free isoforms: cytotrophoblasts are the primary source of choriocarcinoma-like hCG. Mol. Cell. Endocrinol. 194, 147–155.

Lapthorn, A.J., Harris, D.C., Littlejohn, A., Lustbader, J.W., Canfield, R.E., Machin, K.J., 1994. Crystal structure of hCG. Nature 369, 455–461.

Laub, M., Jennissen, H.P., 2003. Identification of the anthelix motif in the TGF-ß superfamily by molecular 3D-Rapid Prototyping. Materialwissensc. Werkstofft. 34, 1113–1119.

Licciardi, F., 2006. The dreaded biochemical pregnancy. Infertility Blog 2006-General Information. Available at: http://infertilityblog.blogspot.com/2006/02/dreaded-biochemical-pregnancy.html. Retrieved (13.06.11).

Murphy, G., Reynolds, J.J., Whitham, S.E., Docherty, A.J., Angel, P., Heath, J.K., 1987. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. Eur. Mol. Biol. Org. J. 6, 1899–1904.

Norwitz, E.R., Schust, D.J., Fisher, S.J., 2001. Implantation and the survival of early pregnancy. N. Engl. J. Med. 345, 1400–1408.

O'Connor, J.F., Ellish, N., Kakuma, T., Schlatterer, J., Kovalevskaya, G., 1998. Differential urinary gonadotrophin profiles in early pregnancy and early pregnancy loss. Prenat. Diagn. 18, 1232–1240.

Sasaki, Y., Ladner, D.G., Cole, L.A., 2008. Hyperglycosylated hCG the source of pregnancy failures. Fertil. Steril. 89, 1871–1886.

Semprini, A.E., Simon, G., 2000. Not so efficient reproduction. Lancet 356, 257–258.

Shibahara, K., Ayustawati, T.S., Kikuchi, K., Hirano, Y., Suzuki, M., 2005. Serum matrix metalloproteinase and tissue inhibitor of metalloproteinase concentrations in infertile women achieved pregnancy following IVF-ET. Am. J. Reprod. Immunol., 186–192.

Wang, J.X., Norman, R.J., Wilcox, A.J., 2004. Incidence of spontaneous abortion among pregnancies produced by assisted reproductive technology. Hum. Reprod. 19, 2272–2277.

Wilcox, A.J., Weinberg, C.R., O'Connor, J.R., Baird, D.D., Schlatterer, D.D., Canfield, R.E., Armstrong, G.E., Nisula, D.C., 1988. Incidence of early pregnancy loss. N. Engl. J. Med. 319, 189–194.

Wilcox, A.J., Baird, D.D., Weinberg, C.R., 1999. Time of implantation of the conceptus and loss of pregnancy. N. Engl. J. Med. 340, 1796–1799.

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