

7-30-2008

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Testicular embryonal carcinoma cell proliferation *in vitro*:
Effect of B152, an anti-hyperglycosylated hCG monoclonal antibody

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Abstract

Hyperglycosylated human chorionic gonadotropin (hCG-H), a variant of hCG, has been shown to promote the growth and invasion of trophoblast cells in early pregnancy placental implantation, choriocarcinoma and testicular germ cell malignancies. In a previous study (15), hCG-H was found to have a significant effect on the growth of a choriocarcinoma cell line, JEG-3, and a testicular embryonal carcinoma cell line, NTERA. It was also shown that, B152, an anti-hCG-H specific monoclonal antibody, significantly inhibited the growth of these cells *in vitro* and *in vivo* murine models. In this study, we quantified the amount of hCG-H produced by two embryonal carcinoma cell lines, n2102Ep and NTERA 2.clone D1 (NT2/D1). We then investigated the impact of B-152 on the proliferation of each cell line as well as JEG-3.

Over a 72 hour period, n2102Ep was found to produce 0.04 mIU of hCG per 10,000 cells and 0.004 of hCG-H per 10,000 cells. The proportion of hCG-H produced by n2102Ep was 10.0%. NT2/D1 produced 0.02 mIU of hCG per 10,000 cells and 0.002 mIU of hCG-H per 10,000 cells. The proportion of hCG-H produced by NT2/D1 was 11.5%. We then cultured these cell lines and JEG-3 in seven different concentrations of B152 ranging from 0.0 $\mu\text{g/mL}$ to 40 $\mu\text{g/mL}$ for 72 hours. Cell proliferation was determined by measuring the absorbance of each cell line and determining a percentage of proliferation relative to a control without additional B152. Cell proliferation decreased with increasing concentrations of B152 for all three cell lines with significance determined by Bartholomew's Test of increasing means: n2102Ep (P value <0.001), NT2/D1 (P value 0.005), and JEG-3 (P value 0.001).

Results suggest that B152 had a negative effect on the cell proliferation of n2102Ep, NT2/D1, and JEG-3. We conclude that hyperglycosylated hCG appears to inhibit the growth of

two embryonal carcinoma cell lines, n2102Ep and NT2/D1, and confirm earlier studies in which B-152 inhibited JEG-3 growth.

Human chorionic gonadotropin (hCG) is a dimeric glycoprotein composed of an α -subunit bound non-covalently to a β -subunit. The heterogeneity of hCG and more than 30% of its molecular weight is due to oligosaccharide side chains. The hCG α -subunit, which has the same polypeptide sequence as the α -subunit found in follicle-stimulating, luteinizing, and thyroid-stimulating hormones, is made up of a 92 amino acid sequence with 2 N-linked oligosaccharides. The hCG β -subunit is composed of 145 amino acids with 2 N-linked and 4 O-linked oligosaccharides (1,2). Although six genes for the hCG β -subunit exist on chromosome 19, only two, CG β 3 and CG β 6, express the hCG β -subunit under the regulation of cyclic AMP (3, 4). hCG and its variants are produced by the normal placenta in pregnancy, gestational trophoblastic diseases, and testicular germ cell malignancies (2,5,17,23).

Testicular germ cell neoplasms account for greater than 90 percent of all testicular malignancies. Though relatively rare and accounting for 1 percent of all malignancies in men, testicular germ cell tumors are the most common neoplasms in young adult males (24). The American Cancer Society had estimated that there would be 7,920 new cases of testicular cancer diagnosed in 2007 (6). In males, germ cell tumors typically afflict young men in the third decade of life, and have a five fold greater incidence in Caucasian males versus African-American males (7,24,27). Risk factors for germ cell tumors of the testis include cryptorchidism, race, environment, estrogenic exposure in early development, and geography (24).

Non-seminomatous (testicular choriocarcinoma, yolk sac carcinoma, embryonal carcinoma, and teratoma) and seminomatous germ cell testicular malignancies produce hCG. This hormone serves as a serum tumor marker and is used in the diagnosis and clinical management of these tumors (25). Hyperglycosylated hCG (hCG-H), an hCG variant, has been found to be produced by a testicular embryonal carcinoma cell line, NTERA (15). This hormone

variant functions independently of regular hCG with different biological functions, structure, metabolism, and it is produced by cytotrophoblast cells instead of syncytiotrophoblast cells, which produce regular hCG (8,10,23).

Hyperglycosylated hCG is the principal form of hCG produced by cytotrophoblast cells at the time of and two weeks following implantation in pregnancy, in gestational trophoblastic diseases (invasive hydatidiform mole, quiescent gestational trophoblastic disease, and choriocarcinoma), and in testicular germ cell malignancies (5,8,9,15,17,18,20,21,23). It functions as an autocrine and blocks TGF-beta activity, which allows for increased cytotrophoblast proliferation. It also promotes hemochorial implantation in the second trimester thereby possibly preventing preeclampsia. The uses of hCG-H as a biomarker include serum tumor marker with 100% sensitivity and 100% specificity for certain actively invasive neoplasia, a marker for Down Syndrome pregnancy, and a predictor of pregnancy failure (10,14,18,23,28).

The hCG-H produced by choriocarcinoma or testicular germ cell malignancies is greater in size (>40,000 vs. 36,700 Da) than hCG produced during pregnancy. This larger size is attributed to the presence of larger and more complex oligosaccharides on choriocarcinoma hCG (1, 13, 30). The hCG found in pregnancy urine was found to have primarily tri- and tetrasaccharide O-linked oligosaccharides and hCG samples from the urine of choriocarcinoma patients over 50% of the O-linked oligosaccharides were hexasaccharide in structure (13). A study in 1997 demonstrated that overall, the hCG in first trimester pregnancy urine had up to 19% hexasaccharide structures (mean = 15.6%) and choriocarcinoma urine hCG contained 48-100% hexasaccharides (mean = 74.2%), which demonstrates that the hyperglycosylated hCG is the principal hCG found in choriocarcionomas (1). In addition, greater fucose content on the N-linked oligosaccharides and greater sialic acid contents of hCG-H has been noted (30). More

recently, the predominance of biantennary oligosaccharides on serine 127, 132, and 138 of β subunit of hCG-H in choriocarcinoma and triantennary N-linked oligosaccharides on the α and β subunits of hCG-H from choriocarcinoma cases were noted to account for hCG and hCG-H differences (1,2,29,30). Several commercial immunoassays with the ability to detect hCG-H only have been developed (10,12). Birken et al. developed a site specific monoclonal antibody, B-152, against hyperglycosylated hCG-H (19).

Testicular malignancies, especially testicular choriocarcinoma, assume the cytotrophoblast-like physiology of gestational trophoblastic diseases and secrete large quantities of hCG that appear like the hCG in gestational choriocarcinoma (10, 11). It has been reported elsewhere that hyperglycosylated hCG accounts for all the hCG immunoreactivity in conditioned media of three choriocarcinoma cell lines including JAR, JEG-3, and BeWo choriocarcinoma cell lines (17). Testicular embryonal carcinomas have also been shown to produce hCG-H, including NTERA, a testicular embryonal testicular carcinoma cell line (15).

The effect of hCG-H on the growth and invasion of cytotrophoblasts in JEG-3, a gestational choriocarcinoma cell line and NTERA, a testicular embryonal carcinoma cell line has been demonstrated by Cole et al. They found that hCG-H increases the growth and invasive capability of these cell lines while regular hCG did not have this effect. The action of B152, an anti-hyperglycosylated hCG monoclonal antibody, was also investigated, and it was observed that B152 blocks growth of the JEG-3 and NTERA testicular cancer cell lines *in vitro* and *in vivo* using murine models (15). However, the amount of hCG-H produced by two testicular embryonal carcinoma cell lines, n2102Ep and NT2/D1, is unknown. The effect of B152 on the proliferation of these cell lines *in vitro* is also unknown.

We consider the cytotrophoblastic histologic similarities between nonseminomatous testicular malignancies and choriocarcinoma. In this study, we quantify hCG-H production by n2102Ep and NT2/D1. We also examine the effect of B152 on the proliferation of both cell lines in addition to JEG-3. The development of human monoclonal antibodies or humanized antibodies to block testicular germ cell malignancy growth would be clinically useful as a potential cure or adjuvant therapy for these cancers to prevent recurrence or new tumor growth.

Materials and Methods

Germ cell lines

The production of total hCG and hCG-H by two embryonal carcinoma cell lines, n2102Ep and NTERA 2.clone D1 (NT2/D1), was measured in this study. A previous study has demonstrated hCG-H production by JEG-3 choriocarcinoma cell line (ATCC, Manasses, Virginia), and this test was not repeated here (15). The activity of the anti-hyperglycosylated hCG (antibody B152) on all three cell lines were investigated. The testicular embryonal carcinoma cell lines were graciously provided by Peter W. Andrews, University of Sheffield, UK, in DMEM containing 4.5 g/L glucose, 2mM glutamine, and 10% fetal bovine serum.

hCG-H production of two testicular embryonal carcinoma cell lines

Culture medium was collected from a 95 percent confluent flask of n2102Ep and a 100% confluent flask of NT2/D1. Cells were cultured to confluency in RPMI-1640 medium with 10% fetal calf serum (FCS) (RPMI-10%). The spent culture fluid was used to test for hCG and hCG-H production. Total hCG production by the two testicular embryonal carcinoma cell lines, n2102Ep and NT2/D1, was measured using the Siemens Diagnostics, Inc. (formerly DPC, Los Angeles, CA) Immulite hCG assay on the Immulite automated immunoassay platform. This assay is calibrated in mIU/ml against the 3rd International Standard (17,31). This test has been

shown to equally recognize, on a molar basis, regular hCG, nicked hCG, hCG-H, and free β -subunit (31). hCG-H was measured using an in-house plate immunoassay based on the B152 capture antibody (results in ng/mL).

Action of antibody B152

The effect of multiple concentrations of anti-hyperglycosylated hCG monoclonal antibody, or B152, on the proliferation of three different cell lines n2102Ep, NT2/D1, and JEG-3 was investigated. Each cell line was seeded at 3000 cells per well in a dedicated 96-well covered cell culture microtitre plate (Becton Dickinson, Meylan Cedex, France). The cells were incubated for 24 hours in RPMI-10%. After the 24 hour incubation, the media was removed from each microtitre plate and 100 μ L of fresh media was placed in each of the 96 wells for each cell line. Next, 100 μ L of media containing one of 7 concentrations was added to the wells for a total of 6 replicates at each concentration. The 7 different concentrations of B152 were prepared from a 1mg/mL concentration of the antibody and RPMI-10%. These concentrations were 1.0 μ g/ml, 2.5 μ g/mL, 5.0 μ g/mL, 10.0 μ g/mL, 25 μ g/mL, 50 μ g/mL, 80 μ g/mL, and no additive as a control. Final B152 concentrations were as follows: 0.0 μ g/mL, 0.5 μ g/mL, 1.25 μ g/mL, 2.5 μ g/mL, 5.0 μ g/mL, 12.5 μ g/mL, 25 μ g/mL and 40 μ g/mL. All three cell lines were then incubated for 72 hours with the varying concentrations of B152 in RPMI-10%.

After 72 hours, the medium was removed from each microtitre plate and replaced with 200 μ L RPMI without phenol red. Cells were then incubated for one hour. The cell density was then determined with the published microtitre plate tetrazolium blue method (16). Tetrazolium bromide (Sigma Chemicals, St. Louis MO) was added to the RPMI without phenol red in each well and incubated for 3 hours. Next, the tetrazolium bromide media was removed and 200 μ L dimethylsulfoxide (Sigma Chemicals, St. Louis MO) was added to each well to dissolve the

formed formazan crystals. The cell proliferation or the absorbance of each well was read at 540 nm in a microtitre plate reader. The effect of B152 was determined following cell proliferation with B152 in sextuplet relative to the average result for 6 wells without B152. Cell proliferation values were determined by the following equation and are presented as the mean \pm SD.

(Microsoft Excel, Redmond, Washington)

$$\chi_2 = \frac{\Sigma (100w/\chi_1)}{n}$$

Where:

- χ_1 = The mean absorbance value resulting from the six control wells.
- χ_2 = The mean percentage cell number at each concentration of incubant.
- Σ = The sum of the percentage cell number of the six replicates at any one incubation concentration.
- w = The absorbance value of any give test well.
- n = The number of replicates (six) at any one concentration of incubant.

Bartholomew's test of increasing means was used to analyze the results.

Results

This study examined the effect of B152, a monoclonal antibody to hyperglycosylated hCG, on the proliferation of two embryonal carcinoma cell lines, n2102Ep and NT2/D1, and JEG-3, a choriocarcinoma cell line. The embryonal carcinoma cell lines were found to produce hCG-H. Over 72 hours, n2102Ep produced 0.02 mIU of total hCG per 10,000 cells and 0.002 mIU of hCG-H per 10,000 cells. The proportion of hCG-H produced by n2102Ep was 11.5%. Over the same time period, NT2/D1 produced 0.04 mIU of total hCG per 10,000 cells and 0.004 mIU of hCG-H per 10,000 cells. The proportion of hCG-H produced by NT2/D1 is 10.0% (Table 1).

Source	Total hCG, median (mIU/10,000 cells)	hCG-H, median (mIU/10,000 cells)	Proportion of hCG-H
2102Ep testicular embryonal carcinoma cells	0.02	0.002	11.5%
NT2/D1 testicular embryonal carcinoma cells	0.04	0.004	10.0%

Table 1. Production of regular hCG and hCG-H in culture fluid from two testicular embryonal carcinoma cell lines.

Next, all three cell lines, N2102Ep, NT2/D1, and JEG-3 were plated and exposed to increasing concentrations of B152 in replicates of six. The control group for each cell line was allowed to proliferate without the addition of B152. A significant decrease in the proliferation of n2102Ep, NT2/D1, and JEG-3 with increasing concentrations of B152 was observed using Bartholomew’s test of increasing means to determine significance ($P < 0.001$, 0.005 , and 0.001 respectively) for all three cell lines (Figure 1, Table 2).

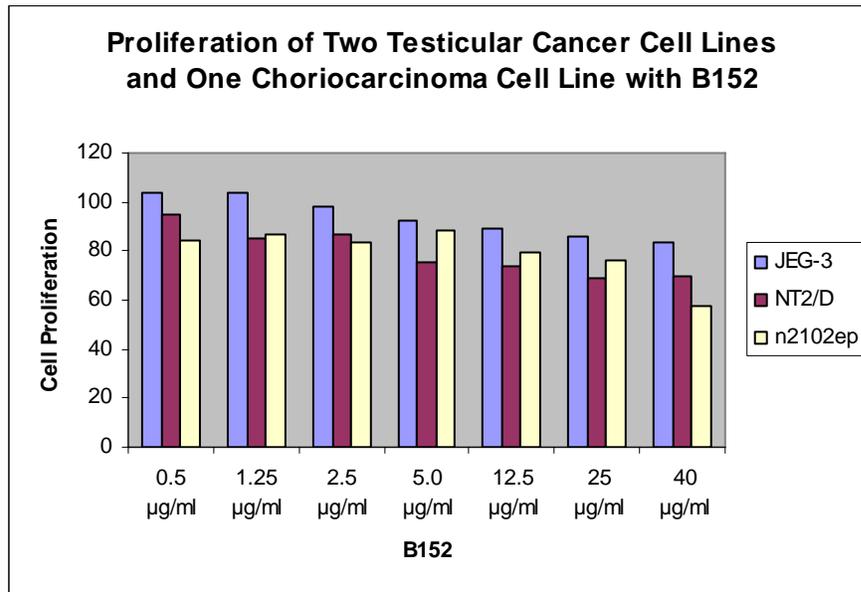


Figure 1. Proliferation of Two Testicular Embryonal Cancer Cell Lines and One Choriocarcinoma Cell Line with B152.

Cell lines	B152 (Anti-hCG-H)						
	0.5 µg/ml	1.25 µg/ml	2.5 µg/ml	5.0 µg/ml	12.5 µg/ml	25 µg/ml	40 µg/ml
n2102Ep testicular embryonal carcinoma ^a	84.21 ± 4.80%	86.43 ± 5.08%	83.57 ± 8.73%	88.1 ± 7.82%	79.1 ± 11.04%	76.15 ± 8.85%	57.76 ± 5.87%
NT2/D1 testicular embryonal carcinoma ^b	94.77 ± 14.18%	85.54 ± 10.11%	86.66 ± 17.37%	75.56 ± 10.49%	73.6 ± 10.56%	68.67 ± 12.24%	69.35 ± 12.92%
JEG-3 chorio-carcinoma ^c	104.00 ± 13.19%	103.65 ± 4.13%	98.19 ± 15.80%	92.35 ± 8.59%	89.13 ± 4.68%	85.59 ± 5.48%	83.12 ± 6.75%

Table 2: Effect of Monoclonal Antibody B152 (Anti-hCG-H) on Testicular Cancer Cell and Choriocarcinoma Cell Proliferation.

- a. Significant decrease observed in mean cell growth (Bartholomew’s Test), $p < 0.001$.
- b. Significant decrease observed in mean cell growth (Bartholomew’s Test), $p < 0.005$.
- c. Significant decrease observed in mean cell growth (Bartholomew’s Test), $p < 0.001$.

Discussion

Cole et al. previously demonstrated hCG-H production by choriocarcinoma cells and by NTERA, a testicular embryonal carcinoma cell line. They also observed the retardant effect of B152, a monoclonal antibody to hyperglycosylated hCG, on invasion, growth and tumorigenesis of choriocarcinoma and testicular embryonal carcinoma *in vivo* in a murine model and *in vitro*. Finally, they also demonstrated B152's effect on decreased cellular proliferation of JEG-3, a choriocarcinoma cell line, and NTERA, an embryonal carcinoma cell line (15). In this present study, we quantified total hCG and hCG-H production and the proportion of hCG-H produced by two embryonal carcinoma cell lines, n2102Ep and NT2/D1, and JEG-3, a choriocarcinoma cell line. We also examined the effect of B152 on all three cell lines.

Unlike regular hCG, hyperglycosylated hCG is secreted by cytotrophoblast cells in early pregnancy, gestational trophoblast diseases, and testicular malignancies. hCG-H functions as an autocrine and blocks TGF beta activity resulting in increased cytotrophoblast cell proliferation (23). Though it is known that testicular embryonal carcinomas produce hCG, whether these carcinomas produce the hyperglycosylated variant of hCG was unknown (25). We demonstrate that two testicular embryonal carcinoma cell lines, n2102Ep and NT2/D1, produce hCG-H (Table 1), and that B152 appears to inhibit hCG-H (Table 2). Based on these findings, it is inferred that hCG-H promotes cellular proliferation of n2102Ep and NT2/D1. Our findings confirm B152's inhibitory effect on the proliferation of JEG-3, a choriocarcinoma cell line (15). NT2/D1 is an NTERA derivative. Cell proliferation of all three cell lines appears to be significantly decreased with increasing concentrations of B152 (P value 0.001, 0.005, and <0.001, Table 2). This observed decreased was relative to n2102Ep cells not exposed to B152.

The limitation of this study is the control test. Our data suggests that B152 inhibits cellular proliferation of n2102Ep, but there may be a confounding effect from the addition of any antibody to the cell cultures. Further studies involving a better control such as inactive B152 or an IgG antibody may help elucidate B152's effect on these cells. Cell membrane invasion studies and *in vivo* studies would be the next steps to effectively determine B152's effect on the growth of n2102Ep and NT2/D1. This information would be useful in the development of human monoclonal antibodies to block testicular germ cell malignancy, which would be clinically useful as a potential cure or adjuvant therapy for the prevention of recurrence or new tumor growth.

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