Functional Correlation of Trophinin Expression with the Malignancy of Testicular Germ Cell Tumor

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ABSTRACT

Trophinin is a membrane protein that is potentially involved in human embryo implantation by mediating homophilic cell adhesion between trophoblastic cells and endometrial cells. Trophinin expression by maternal cells may be induced by the embryo that secretes human chorionic gonadotropin (hCG). Because the process of tumor metastasis resembles that of trophoblast invasion and proliferation during embryo implantation, we hypothesized that testicular cancers that synthesize hCG express trophinin thus becoming aggressive trophoblast-like cells. We screened paraffin-embedded orchietomy specimens of 158 patients with testicular germ cell tumor by immunohistochemistry using antitrophinin antibody. This screening identified trophinin-positive specimens with the frequencies of 91 (43%) in stage I, 14 of 24 (58%) in stage II, and 41 of 43 (95%) in stage III (P < 0.001). Thus, trophinin expression positively correlates with clinical stage. Remarkably, trophinin was found in all of the cases (33 of 33) with lung metastasis. The levels of serum hCG-β were significantly higher in the patients with trophinin-positive tumors than those with trophinin-negative tumors (P = 0.004). To determine whether trophinin promotes aggressiveness of the cell, trophinin-negative human seminoma cell line JKT-1 was stably transfected with a mammalian expression vector containing trophinin cDNA. In vitro assays revealed that trophinin-expressing JKT-1-Tro cells are more invasive than JKT-1-mock cells, whereas there are no differences between JKT-1-Tro and JKT-1-mock in their proliferation activity. Upon orthotopic inoculation to athymic nude mice, JKT-1-Tro cells exhibited i.p. metastases in all of the mice (n = 5), whereas JKT-1-mock produced no metastases (n = 5). These results suggest strongly that trophinin enhances invasiveness of the cells and promotes metastasis of testicular germ cell tumor.

INTRODUCTION

Testicular germ cell tumor remains the most common solid malignancy in young men between 15 and 35 years of age (1). Recent statistics suggest disturbing trends that incidence of testicular germ cell tumor is increasing, and the age at which testicular germ cell tumor develops is becoming younger (2, 3). With the introduction of cis-platinum chemotherapy in the late 1970s, the survival rate of patients with testicular germ cell tumor now exceeds 90% (4, 5). However, it is still difficult to treat advanced testicular germ cell tumor with multiple distant metastases.

In germ cell tumors, all of the patients of choriocarcinoma, 40–60% of embryonal carcinoma, and 5–10% of patients with pure seminoma show the elevation of human chorionic gonadotrophin (hCG) β in their sera (6). The hCG is a 38-kDa glycoprotein, which is composed of α and β polypeptide chains. The hCG is normally produced by trophoblastic cells in the placenta (7). The β subunit of hCG is one of the tumor markers of germ cell tumors, and this value correlates well with the patient populations having clinical stages I, II, and III testicular tumors (6).

Trophinin is a membrane protein that potentially mediates the initial adhesion between human embryo and uterine epithelial cells through a unique apical cell adhesion between two cell types, trophoblastic cells and endometrial epithelial cells (8–10). In humans, endometrium is under strict hormonal control and is generally not permissive to implantation. Trophinin is not expressed during proliferative and ovulation phases, whereas strong expression of trophinin within a restricted area of human endometrium was detected at early secretory phase or time of implantation window (8–10). Recent findings of ectopic pregnancy suggest strongly that trophinin expression by maternal cells is embryo-dependent. Thus, hCG-β secreted from the implanting embryo induces trophinin in maternal cells through juxatricrine manner (11).

The processes of human embryo implantation, which include rapid proliferation and invasion of trophoblasts, are often compared with the aggressive behaviors of malignant cancer cells (12). Recent studies have suggested that hCG expressed in trophoblast and various malignant tumors promotes cellular motility (6–7). These observations prompted us to investigate the possible role of hCG in testicular germ cell tumors. We describe here the expression of trophinin and hCG-β in testicular germ cell tumors and the effect of ectopic expression of trophinin on cellular motility, proliferation, and metastatic activities.

MATERIALS AND METHODS

Human Testicular Germ Cell Tumor Specimens. Testicular germ cell tumor specimens from 158 patients (76 seminoma and 82 nonseminomatous germ cell tumors) at various clinical stages were collected between 1981 and 2002. Informed consent was obtained from all of the patients whose specimens were used in this study. Pathological findings were recorded according to American Joint Committee on Cancer staging system (13).

Serum hCG-β Measurement. Before orchiectomy, blood sample was obtained by routine venipuncture from each patient and was subjected to serum hCG-β subunit measurement by ELISA.

Immunohistochemistry. Immunohistochemistry for trophinin, tastin, and bystin was performed as described previously (14), except Simple Stain Max PO kit and Simple Stain AEC solution (Nichirei Corp., Tokyo, Japan), according to manufacturer’s instructions. Anti-hCG antibody was obtained from Nichirei Corporation.

Cell Line and Cell Culture. JKT-1 is a human testicular seminoma cell line provided by Dr. Keigo Kinugawa (Department of Urology, Kawasaki Medical School, Kurashiki, Japan; Ref. 15). This cell line was maintained in α-MEM containing 10% fetal bovine serum in a humidified 5% CO2 atmosphere at 37°C.

Establishment of Stable Transfectant. JKT-1 cells, which do not express trophinin, were transfected with pcDNA1-trophinin (8) using LipofectAMINE (Life Technologies, Inc.) as described in the protocol provided by the supplier. After 2 weeks in G418 selection (400 µg/ml; Life Technologies, Inc.), 20 single colonies were examined for immunohistochemical detection of trophinin using anti-trophinin mouse monoclonal antibody and FITC-conjugated goat affinity-purified F(ab')2 fragment specific to mouse IgM (Cappel). Three stable transfecants expressing trophinin were established (JKT-1-Tro). Among
**Flow Cytometric Analysis.** JKT-1-Tro cells and mock-transfected cells were assessed by fluorescence-activated cell sorting analysis after incubation with antitrophinin antibody (mouse IgM; Ref. 14) followed by incubation with FITC-conjugated secondary antibodies. Analyses were carried out by FACSort flow cytometry using the CellQuest program (Becton Dickinson).

**Growth Rate of Cell Lines.** JKT-1-Tro cells and mock-transfected cells were seeded in 96-well plates at 10^5 cells/ml in α-MEM containing 10% fetal bovine serum and 400 μg/ml of G418 and cultured for various times. The number of living cells was measured each day using the Cell Counting kit (Wako Pure Chemical Industries, Tokyo, Japan). Triplicate cultures were used for each analysis.

**Motility and Invasion Assays.** A transwell cell culture chamber (Costar, Cambridge, MA) was used for in vitro motility and invasion assays with modifications (16). Briefly, the bottom of the upper chamber was sealed with a polyvinylpyrrolidone-free polycarbonate filter with a pore size of 8 nm. The lower face was covered with 50 μg/ml fibronectin (Wako Pure Chemical Industries) in α-MEM medium. Cells (1 × 10^3) were plated in the upper chamber and incubated in a humidified CO_2 incubator at 37°C for 5 h. The lower chamber was filled with serum-free α-MEM medium. Cells that did not migrate through the membrane were removed, and the cells that migrated to the lower face of the membrane were fixed with methanol followed by Giemsa staining. The number of cells on the lower face was counted under microscope. The mean number of 10 different fields was plotted. For the invasion assay, the upper face of the filter was covered with 100 μg/ml Matrigel (Collaborative Research, Bedford, MA), and the number of cells on the lower face was counted. These assays were carried out in triplicate. The SD of these values was always within 5%.

**Orthotopic Tumor Cell Inoculation.** BALB/c nude (nu/nu) mice, 6–8-week-old males obtained from CLEA Japan, Inc. (Tokyo, Japan), were used for orthotopic tumor cell injection. Mice were anesthetized with avertin, and intratesticular injection was performed. JKT-1-Tro cells (2 × 10^5) and mock-transfected JKT-1 cells were suspended in 100 μl of serum-free α-MEM and inoculated into the right testis. Three weeks after injection, mice were sacrificed, and testis and other organs having metastasis were removed and fixed in a buffered formalin solution.

**Adhesion of JKT-1 Cells to Endothelial Cells.** The human pulmonary microvascular endothelial cells (HMVEC-L) were obtained from the Sanko Junyaku Co., Ltd. (Tokyo, Japan) and were cultured in EGM-2M medium containing microvascular endothelial cell growth factors, antimicrobials, and 5% fetal bovine serum to confluence in 24-well culture plate. The wells were washed three times with PBS. JKT-1-Tro cells and JKT-1-mock cells were detached from the plate with trypsin and resuspended in α-MEM (1% fetal bovine serum) and added at 1 × 10^3 cells/ml on a monolayer of HMVEC-L. For the antibody treatment, JKT-1-Tro cells were incubated with monoclonal antibody against trophinin on ice for 30 min, washed with PBS, and added onto the HMVEC-L monolayer.
RESULTS

Expression of Trophinin by Patients with Testicular Tumors. Immunohistochemistry of testicular tumors revealed significant positive correlations between trophinin expression and clinical stage (Ref. 17; Table 1). Especially, all of the tumor specimens from patients with lung metastasis were positive for trophinin. Furthermore, serum hCG concentration was positively correlated with trophinin expression (Fig. 1), which was confirmed by immunohistochemistry (Fig. 2, C and D).

We screened 158 patients with testicular tumors. Among them, trophinin was found positive in 39 of 91 (43%) patients in stage I, 14 of 24 (58%) patients in stage II, and 41 of 43 (95%) patients in stage III ($P < 0.001$). Testicular germ cell tumors with stage III were positive strongly for trophinin. Serum hCG concentration was significantly higher in the trophinin-positive group than in the trophinin-negative group (Fig. 1). Remarkably, all of the specimens with lung metastasis were trophinin positive, regardless of seminoma or non-seminomatous germ cell tumor (Table 1). Thus, trophinin expression in the tumor correlates positively with distant metastasis and high levels of serum hCG. These observations suggest that the trophinin-positive testicular tumors are more aggressive than trophinin-negative tumors.

Properties of Trophinin-Positive Seminoma Cell Line, JKT-1-Tro. Because the function of trophinin in cell proliferation and invasion are not known, we investigated whether trophinin plays a role of malignant phenotype in testicular germ cell tumor. Human seminoma cell line JKT-1 does not express trophinin nor trophinin-associated cytoplasmic proteins tastin and bystin (data not shown). JKT-1 cells were transfected with a mammalian expression vector having trophinin cDNA. JKT-1-Tro cells and JKT-1-mock cells obtained by transfection of trophinin cDNA and vector without insert, respectively, were evaluated for their expression of trophinin using antitrophinin antibody by immunocytochemistry (Fig. 3A) and flow cytometry (Fig. 3B) analysis. Thus, two clones for JKT-1-Tro express trophinin on the cell surface. Note that JKT-1-mock cells (panel a) are negative for trophinin, whereas stable transfectant for trophinin, JKT-1-Tro-1 cells (panel b) are positively stained with antitrophinin antibody. Another stable transfectant, JKT-1-Tro-2 cells, were also positively stained with antitrophinin antibody with similar intensity (data not shown).

Motility and invasion activities of JKT-1 cells. Motility and invasion activities of JKT-1 transfectants were assayed by transmigration chambers. A, JKT-1-mock cells (panel a) and JKT-1-Tro-1 cells (panel b) migrated through a filter membrane from uncoated upper chamber to fibronectin coated lower chamber. Cell number count (panel c) showed the significant increase of motility of trophinin expressing JKT-1 cells ($P < 0.001$, Mann-Whitney U test). B, JKT-1-mock cells (panel a) and JKT-1-Tro-1 cells (panel b) migrated through Matrigel-coated filter membrane. Invasion potential was significantly increased in JKT-1-Tro cells (panel c) ($P < 0.001$, Mann-Whitney U test); bars, ±SD.
cell surface, whereas two clones for JKT-1-mock do not (Fig. 3B). Thus, clonal variations among JKT-1-Tro and those among JKT-1-mock are considered to be minimum.

Because malignancy is closely associated with cell proliferation activity, we determined cell numbers of JKT-1-Tro cells and JKT-1-mock cells. The results show no significant difference between JKT-1-Tro cells and JKT-1-mock cells in cell numbers during in vitro culture (data not shown), suggesting that acquisition of trophinin does not solely enhance cell proliferation activity of JKT-1 cells cultured in vitro.

Next, JKT-1-Tro cells and JKT-1-mock cells were subjected to motility and invasion assays using a transwell cell culture system. Fibronectin and Matrigel were coated on the lower and upper face of the transwell for testing motility and invasion, respectively. These assays revealed that JKT-1-Tro cells are significantly mobile (Fig. 4A) and invasive (Fig. 4B) compared with JKT-1-mock cells. The possibility for clonal deviations of these results is excluded, because the differences between JKT-1-Tro clones were not significant.

**Metastatic Potential of JKT-1-Tro Cells in Vivo in the Mouse.** Because clinical data (Table 1) indicated a strong correlation between trophinin-positive testicular tumors and lung metastasis, we tested whether JKT-1-Tro cells metastasize to the lung in the mouse. JKT-1-Tro cells and JKT-1-mock cells were inoculated in the right testis of nude mice. Three weeks after inoculation, mice were sacrificed to determine the tumor size and metastasis. JKT-1-Tro cells produced massive intra-abdominal metastasis in all of the mice (n = 5; A), whereas JKT-1-mock cells produced no metastasis (n = 5; A). No differences in the size of testes were seen between JKT-1-mock and JKT-1-Tro (black arrows). Note that JKT-1-Tro-1 produces numerous mesenteric metastases (open arrows in B). JKT-1 mock (C) or JKT-1-Tro-1 (D) produced no lung metastasis. JKT-1-Tro-2 cells showed also the same result as JKT-1-Tro-1 (data not shown). E, immunohistochemistry of metastasized tumor with antitrophinin antibody.
cell tumors, we compared the adhesion of JKT-1-Tro cells and JKT-1-mock cells to human lung microvascular endothelial cells by cell adhesion assay in vitro. As shown in Fig. 6, JKT-1-Tro cells were only slightly more adhesive to HMVEC-L than JKT-1-mock cells, whereas antitrophinin antibody had no effect on the adhesion between JKT-1-Tro cells and HMVEC-L (Fig. 6). These results suggest that trophinin is not the factor determining the adhesion of tumor cells to HMVEC-L.

**DISCUSSION**

Testicular germ cell tumor is one of the few neoplasms associated with accurate serum marker, hCG (6). The hCG is found in the placenta in pregnant women (18) but not in any tissue from healthy men. This allows careful follow-up in men with testicular germ cell tumor early in the course of the disease (19, 20). In germ cell tumors and the placenta, syncytiotrophoblastic cells are responsible for the production of hCG. Although both human and mouse produce testicular germ cell tumors, spontaneous trophoblastic transformation of cancer cells does not naturally occur in the mouse (21). Thus, in humans, trophoblastic transformation occurs in the breast, prostate, bladder, thyroid, colon, lung, and endometrial cancers (22–24).

Studies on embryo implantation have revealed significant differences of this process among mammals (25). Remarkably, ectopic pregnancy occurring at a rate as high as 1.4% in all of the pregnancies in humans (26, 27) does not occur naturally or experimentally in other animals, including nonhuman primates (28–30). As described below, trophoblastic transformation of tumors in humans may be explained by the uniqueness of the β subunit of hCG (11, 31).

The hCG is a 38-kDa glycoprotein hormone composed of α and β polypeptide chains (7). The hCG and its pituitary counterpart, lutropin, comprise a family of heterodimeric glycoprotein hormones, including follitropin and thyrotropin, that share a common α subunit but differ in their hormone-specific β subunit (7). The gene encoding CG-α is homologous to those encoding thyroid-stimulating hormones, of which the structures are evolutionarily conserved among wide variety of animals (32). By contrast, CG-β subunit is specific to primates, and human CG-β gene is diverged from the CG-β genes of nonhuman primates (31).

Trophinin mediates homophilic cell adhesion between human trophoblastic cells and endometrial epithelial cells at their respective apical cell surfaces (8, 9). Strong expression of trophinin was found at the human embryo implantation site (14). Trophinin is also expressed during ectopic pregnancies (11). Interestingly, trophinin expression by the fallopian tubal epithelium depends on the existence of implanting embryo. Thus, the maternal epithelia adjacent to the implantation site express trophinin strongly, whereas the maternal cells a few millimeters away from the implantation site barely express trophinin (11). Such spatially restricted trophinin expression suggests the existence of an embryo-derived factor that stimulates the maternal cells for trophinin expression. This embryonic factor may be CG-β, which is secreted from the trophectoderm of the blastocyst (33–35), because the fallopian tubal explants incubated with hCG showed the elevation of trophinin transcripts (11).

In this study, we found that testicular germ cell tumors often express trophinin (Table 1; Figs. 1 and 2). It is noteworthy that trophinin expression positively correlates with the malignancy of testicular tumor, particularly with the metastasis (Table 1). Our data also showed positive correlation between trophinin expression and hCG-β in the sera of the patients (Table 1; Fig. 1). Taken together, hCG-β expressed by human cancer cells might act on the cancer cells in an autocrine manner to induce trophinin expression.

In this study, we showed that ectopic expression of trophinin in JKT-1 cells enhances motility and invasiveness of the cells in vitro (Fig. 4). When JKT-1-Tro cells were inoculated into the mouse, they showed massive peritoneal metastasis (Fig. 5). Clinical data of the testicular germ cell tumors (Table 1) showed that testicular germ cell tumors metastasize to the lung (36). This suggested a possibility that trophinin enhances the lung metastasis. However, our experiments using JKT-1-Tro cells did not show the lung metastasis in the mouse (Fig. 5). Such an apparent discrepancy between the mouse experiments and clinical observations may be explained by the expression of tastin and bystin in the lung metastasized tumors (data not shown).

The mechanism underlying the lung metastasis of these tumors remains to be defined in the future study.

Because we identified trophinin, tastin, and bystin as proteins with potential involvement in embryo implantation (8–10), functions of these proteins other than cell adhesion has not been described. Because trophinin, tastin, and bystin are expressed in the human placenta during the periods of early stage of placental development (14), it is possible that these proteins are involved in the subsequent trophoblast invasion process triggered by the initial cell adhesion. Therefore, present study is the first to provide with a suggestion that trophinin plays a role in invasion. Future studies should define the mechanisms of how trophinin promotes cellular motility in embryo implantation and cancer metastasis. The information obtained by such studies will help in designing a therapy against trophoblastic neoplasm, including testicular germ cell tumor.

**ACKNOWLEDGMENTS**

We thank Dr. Elise Lamar for editing the manuscript and Aleli Morse for her secretarial assistance.
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*Cancer Res* 2004;64:4257-4262.

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