EXHIBIT W

Lodish Decl. in Support of Opposition to Roche's Motion for Summary Judgment of Invalidity for Double Patenting Over Claim 10 of the '016 Patent

World J Urol (1984) 2:2–5

World Journal of Urology

In Vitro Models of Human Testicular Germ-Cell Tumors*

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Summary. Murine teratocarcinoma models have been widely used in the study of several biological processes including early mammalian development and neoplasia. The relatively new human germ-cell tumor models have an important role in the study of human embryogenesis and neoplasia since human embryogenesis differs significantly from species such as mice and the histopathology of human testicular germ-cell tumors differs substantially from tumors obtained in highly inbred strains of mice. In this report we describe the use of human germ-cell lines in the study of cell differentiation; expression of early embryonic antigens; production of the major regulator of erythropoiesis, erythropoietin; and production of retrovirus-like particles.

Murine and human germ-cell tumors have been a source of fascination for many scientists over the past few decades. As one example, these tumors have provided the teratocarcinoma models which have proved to be a valuable model system for the study of early mammalian development and of neoplasia [9, 13, 18]. In addition, these tumors have been an excellent source of material for research in fields such as cell biology, immunology and virology.

The histological classification of germ-cell neoplasms has been a subject of much controversy, and several different classification systems have been proposed (see reviews by Nochomovitz et al. 1977 [12] and Mostofi 1977 [11]). The system proposed by the World Health Organization is widely accepted and classifies germ-cell tumors into seminoma, embryonal carcinoma, teratocarcinoma (embryonal carcinoma with teratoma), teratoma, choriocarcinoma, and yolk sac tumor [12]. It is thought that the embryonal carcinoma (EC) cell represents the malignant version of the primordial germ cell and, because of its pluripotent nature, has the capacity to differentiate along both intra-embryonic (somatic) and extra-embryonic lines. Differentiation along intra-embryonic lines produces a teratoma, which contains elements representative of the three germ layers (endoderm, ectoderm and mesoderm). Extra-embryonic differentiation is thought to produce choriocarcinoma (malignant version of the trophoblast) and yolk sac tumor (malignant version of the yolk sac). Thus, the EC cell would be the stem cell of all nonseminomatous germ-cell tumors (cf. article by Damjanov in this issue).

This concept of the stem cell is based on experimental work such as that done by Stevens, in which transplantation of genital ridges from fetal mice into the testes of mature strain 129 mice produced teratocarcinomas in the hosts [17]. Additional support for this concept was the demonstration that EC cells could participate in the development of a normal adult mouse. In such experiments, inoculation of EC cells into host blastocysts, which were then transferred to pseudopregnant females, led to some manipulated embryos developing to term; and the resulting animals contained tissues derived both from the EC cells and from host blastocysts that could be distinguished by genetic differences [10].

Mouse Teratocarcinoma Models[1]

The valuable mouse teratocarcinoma models were established by the pioneering work of Leroy C. Stevens and G. Barry Pierce in the 1950's and 1960's. These tumors may arise spontaneously or be artificially induced.

Spontaneous Tumors

Most strains of mice rarely or never develop spontaneous teratomas or teratocarcinomas. However, certain sublines such as 129/ter Sv have a 30% incidence of congenital testicular teratocarcinomas. These valuable sublines were developed by inbreeding strains that have a low frequency of such tumors.

* Supported in part by a grant from the Minnesota Medical Foundation

[1] See the article by Narayan et al. in this issue for further details

Table 1. Twenty human testicular germ-cell tumor cell lines established at the University of Minnesota[a]

Designation	(Type)	Virus production	In vitro differentiation	Markers		Other products
				AFP	hCG	
833K	(EC)	+	–	–	–	HLA, B_2M, SSEA-1, SSEA-3, ALP, PA
577M-RP	(UdC)	–	–	–	–	?
577M-Lu	(UdC)	–	–	–	–	?
577M-F		–	–	?	?	HLA, B_2M, PA
2044L	(EC)	+	–	–	–	?
1156Q	(EC)	+	–			HLA, B_2M, SSEA-3, SSEA-1, ALP, PA
2102E-Pr	(EC)	++[c]	~	+	–	HLA, B_2M, SSEA-1, SSEA-3, ALP, PA
2102E-RP	(EC)	++	?	?	?	?
2061H	(EC)	+	–	–	–	?
1218E	(EC)	+	–	–	–	HLA, B_2M, SSEA-3, ALP, PA
1255O	(EC)	+	–	?	?	?
1242B	(EC)	+		–	–	?
1428A	(EC)	+		?	?	?
1411H	(YS+EC)	–	–	+	–	Ep
1411H-RP	(YS+EC)	–	–	+	–	
1446S	(EC)	+		?	?	
1075L-Lu[b]	(EC+Chorio)	+ → –[d]	+			
1075L-HEp[b]	(EC+Chorio)	+ → –	+			
1777N-Pr[b]		+ → –	+	– → +[d]	– → +	
1777N-RP[b]	(EC-SSC)	+ → –	+	– → +	– → +	

[a] Abbreviations; RP = retroperitoneal metastasis; Lu = lung metastasis; F = forehead (subcutaneous) metastasis; Pr = primary tumor; HEp = liver metastasis; EC = embryonal carcinoma; UdC = undifferentiated carcinoma (malignant teratoma?); YS = yolk sac carcinoma; SSC = spindle cell sarcoma; HLA = human leukocyte (transplantation) antigen(s); B_2M = beta$_2$-microglobulin; ALP = placental-type alkaline phosphatase; SSEA-1 & -3 = stage-specific (murine) embryonic antigens; PA = plasminogen activator; EP = erythropoietin

[b] Lines established on feeder layers of human fibroblasts

[c] Produces two types of virions

[d] Lines are virus-positive and marker-negative in undifferentiated (EC) state; become virus-negative and marker-positive as they differentiate [4]

Induced Tumors

Murine teratocarcinomas may be induced in several ways. Teratomas and teratocarcinomas may be produced by transplanting genital ridges from fetuses into the testes of adult syngeneic mice [18]. This model is known as the Stevens model. A second and particularly useful model is obtained by transplanting embryos to extrauterine sites; e.g., the testis or beneath the kidney capsule, where they may develop into tumors that are histologically indistinguishable from teratomas and teratocarcinomas that arise spontaneously [19].

These well-established mouse teratocarcinoma models have been widely used to study numerous biological processes, including differentiation and its control, mechanisms of gene expression, cell-surface antigens, and aspects of early embryogenesis. In addition, some important concepts regarding oncogenesis have been proposed on the basis of the studies of these models. For example, the observation that primordial germ cells can be transformed into malignant EC cells supports the concept that tumor cells appear undifferentiated, not because they have dedifferentiated (as classic dogma holds), but rather because they arise from undifferentiated stem cells [14].

The Human Teratocarcinoma System

The human teratocarcinoma model has been studied to a lesser extent than the murine models, and its potential value as a model for studying aspects of human neoplasia and embryogenesis therefore is less well defined. It is known, however, that the findings in the mouse models cannot be extrapolated directly to humans, both because embryology differs significantly from that of mice and because the pathology of murine and human teratocarcinomas differ, partly because the model murine teratocarcinomas occur in highly inbred strains, whereas human tumors occur in an outbred population.

Studies of Human Germ-Cell Tumors In Vitro

The investigation of the human teratocarcinoma system in our laboratory began in 1976 with the establishment of a testicular germ-cell tumor line designated 833K-E [3]. Subsequently, 26 more such cell lines have been established; some properties of 20 of them are shown in Table 1. On occasion, there has been concomitant growth of lymphoblastoid cells (LC), which has led to the establishment of several LC lines. These lympho-

blastoid cells are polyclonal B lymphocytes that multiply indefinitely and express Epstein-Barr virus antigens. These cells are valuable controls in experiments with autologous tumor cell lines. The production of antibody by these LC lines is being investigated.

The cell lines are established directly from fresh tumor tissue, which is obtained aseptically and immediately placed in tissue culture medium. The specimen is minced into tiny fragments, which are placed in plastic tissue culture flasks containing a feeder layer such as human lung fibroblasts. This feeder layer enhances attachment and spread of cells from the tumor fragments. The flasks are incubated at 37°C and the culture medium changed every two days. When the tumor cells have formed a confluent monolayer, they are detached from the flasks by incubation for 7 min at 37°C with trypsin citrate and then subcultured.

Cells of most of our lines have properties of EC. However, a few lines represent malignant teratoma in vitro, and other lines are mixtures of yolk sac tumor, perhaps with EC elements, and EC with choriocarcinoma. These yolk sac tumor lines produce large quantities of alphafetoprotein (AFP), and those containing choriocarcinoma readily produce human chorionic gonadotropin (hCG).

Differentiation of EC Cells In Vitro

The propensity for differentiation of some EC cell lines from our laboratory has been well documented [4]. Differentiation of these malignant "stem cells" is monitored by the production of the oncodevelopmental markers AFP and hCG, distinct changes in cell morphology and growth rate, and changes in cell-surface antigens. Some EC cell lines exhibit morphological changes indicative of in vitro differentiation when seeded at low density, suggesting that cell-to-cell contact may be an important factor affecting cellular differentiation, as has been described for some of the murine EC cell lines.

The EC cells characteristically exhibit a large clear nucleus containing one or more dark nucleoli and have a narrow rim of cytoplasm. In contrast, differentiating cultures contain several cell types, including syncytia, multinuleated giant cells, and fibroblastoid and spindle-shaped cells. High levels of hCG and AFP are detected in the supernatant medium of cultures undergoing these morphological changes, and this is thought to reflect differentiation of EC cells into trophoblastic and yolk sac elements, respectively.

Cell-Surface Antigens

It is thought that various antigens expressed by early embryonic cells play a significant role in cell–cell recognition, which may be responsible for selecting the direction of differentiation of these cells [8]. These cell-surface molecules, which are called developmentally regulated antigens, are associated with the processes of both embryogenesis and differentiation, being expressed and suppressed at different stages [8]. Examples of such antigens are the stage-specific embryonic antigens SSEA-1 and SSEA-3. Both SSEA-1 and SSEA-3 have been detected on early stage mouse embryos [15, 16]. These antigens also have been found on the surfaces of both mouse and human EC cells. However, whereas murine EC cells express SSEA-1 but not SSEA-3 [16, 15], human EC cell lines from our laboratory express SSEA-3 but little or no SSEA-1 [1]. Furthermore, when the human cells undergo differentiation, SSEA-3 expression is reduced and SSEA-1 expression is increased [1].

The recent development of monoclonal antibodies to developmentally regulated antigens will be of value in identifying and understanding their normal function, which still is largely unclear. Also, SSEA-1 may provide information about cell-surface carbohydrate changes in cancer [7], and SSEA-3 may be useful in future radioimmunodetection of metastatic human teratocarcinoma [15].

Production of Retrovirus-Like Particles

Small numbers of particles with the morphology of retroviruses have been detected in cultures of all of our EC cell lines and in cultures of the Tera-1 EC cell line [5, 6]. These particles are morphologically identical to the retroviruses found in human placental tissue and are formed by budding from the cell surface. The number of particles produced is increased by treating EC cultures with dexamethasone and iododeoxyuridine.

Biochemical detection of the enzyme RNA-dependent DNA polymerase (reverse transcriptase: RT) is necessary for proving the presence of retrovirus. This enzyme transcribes the viral RNA genome into a DNA copy, which may then be integrated into the host genome. Very low levels of enzyme activity suggestive of RT were detected in assays using concentrates of 10 liters of supernatant medium from EC cultures.

Yolk sac tumor and malignant teratoma cell lines, which are differentiated derivatives of EC cells, do not produce these particles. Also, particle production ceases when EC cells begin to differentiate. These observations suggest that expression of the virus particles is developmentally regulated (Bronson et al., in press). There is no evidence that these particles are responsible for any pathological condition.

Production of Erythropoietin by a Human Yolk Sac Carcinoma Cell Line

We recently detected production of significant amounts of erythropoietin (Ep) by a cell line designated 1411H

[2], which is a yolk sac carcinoma cell line with, perhaps, elements of EC (N. Vogelzang, personal communication). This cell line secretes copious amounts of AFP, the fetal equivalent of albumin, which is produced by the embryonic yolk sac and fetal liver during normal mammalian ontogeny.

Ep, a glycoprotein hormone produced by the fetal liver and adult kidney, is the primary regulator of erythropoiesis in man. Certain anemias, particularly those caused by chronic renal disease, are responsive to Ep; but at present, no abundant source of this hormone is available, and this has prevented its use clinically. It is hoped that with new advances in genetic engineering, the Ep gene will be cloned and transferred to a different organism such as *E. coli*, as this would facilitate production of the hormone in quantities adequate for clinical use. Clearly, then, the production of EP by 1411H is of significant biological interest and may be of clinical value if the gene controlling Ep synthesis can be cloned and used for the manufacture of the hormone.

Conclusion

The mouse teratocarcinoma system has provided a valuable tool for the study of several biological processes, the most important of which are embryogenesis and neoplasia. The more recent and less well-studied human system has already generated important information in regard to these processes. We believe that the human model will complement the mouse model in that it will provide a source of material with which to study human cell differentiation and thus shed light on human embryogenesis and neoplasia and their relation. Human germ-cell lines also may provide a valuable source of genes for several important growth factors and hormones and, perhaps, a system for studying human retroviruses.

Acknowledgements. The authors thank Patti Lien for excellent secretarial assistance and Judith Gunn Bronson for editing the manuscript.

References

1. Andrews PW, Goodfellow PN, Shevinsky LH, Bronson DL, Knowles BB (1982) Cell-surface antigens of a clonal human embryonal carcinoma cell line: morphological and antigenic differentiation in culture. Int J Cancer 29:523–531
2. Ascensao JL, Gaylis F, Bronson D, Fraley E, Zanjani ED (1983) Inducible production of erythropoietin by a human yolk sac tumor cell line. Am Fed Clin Res 31:307A
3. Bronson DL, Andrews PW, Solter D, Cervenka J, Lange PH, Fraley EE (1980) Cell line derived from a metastasis of a human testicular germ cell tumor. Cancer Res 40:2500–2506
4. Bronson DL, Andrews PW, Vessella RL, Fraley EE (in press) In vitro differentiation of human embryonal carcinoma cells. Cold Spring Harbor Conference on Cell Proliferation, Vol. 10 in press
5. Bronson DL, Fraley EE, Fogh J, Kalter SS (1979) Induction of retrovirus particles in human testicular tumor (Tera-1) cell cultures: an electron microscopic study. J Natl Cancer Inst 63:337–339
6. Bronson DL, Ritzi DM, Fraley EE, Dalton AJ (1978) Morphologic evidence for retrovirus production by epithelial cells derived from a human testicular tumor metastasis. J Natl Cancer Inst 60:1305–1308
7. Fox N, Damjanov I, Knowles BB, Solter D (1983) Immunohistochemical localization of the mouse stage-specific embryonic antigen 1 in human tissues and tumors. Cancer Res 43:669–678
8. Hakomori S-I, Fukuda M, Nudelman E (1982) Role of cell surface carbohydrates in differentiation: behavior of lactosaminoglycan in glycolipids and glycoproteins. In: Muramatsu T, Gachelin G, Moscona AA, Ikawa Y (eds) Teratocarcinoma and embryonic interactions. Japan Scientific Societies Press, Tokyo, pp 179–199
9. Martin GR (1975) Teratocarcinomas as a model system for the study of embryogenesis and neoplasia. Cell 5:229–243
10. Mintz B, Illmensee K (1975) Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc Natl Acad Sci USA 72:3585–3589
11. Mostofi F[illegible] 1977) Epidemiology and pathology of tumors of human tes[illegible] Recent Results Cancer Res 60:176–195
12. Nochomovitz LE, De La Torre R FE, Rosai J (1977) Pathology of germ cell tumors of the testis. Urol Clin North Am 4:359–378
13. Pierce GB (1967) Teratocarcinoma: model for a developmental concept of cancer. Curr Top Dev Biol 2:223–246
14. Pierce GB (1974) The benign cells of malignant tumors. Dev Asp Carcinogen Immun 32:3–22
15. Shevinsky LH, Knowles BB, Damjanov I, Solter D (1982) Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. Cell 30:697–705
16. Solter D, Knowles BB (1978) Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci USA 75:5565–5569
17. Stevens LC (1964) Experimental production of testicular teratomas in mice. Proc Natl Acad Sci USA 52:654–661
18. Stevens LC (1967) The biology of teratomas. Adv Morphogen 6:1–31
19. Stevens LC (1970) The development of transplantable teratocarcinomas from intratesticular grafts of pre- and post-implantation mouse embryos. Dev Biol 21:364–382