J. **16**, 627 (1976); W. A. Kachadorian, J. B. Wade, C. C. Uiterwyk, V. A. DiScala, *J. Membr. Biol.* **30**, 381 (1977).
5. W. A. Kachadorian, S. D. Levine, J. B. Wade, V. A. DiScala, R. M. Hays, *J. Clin. Invest.* **59**, 576 (1977); W. A. Kachadorian, S. J. Ellis, J. Muller, *Am. J. Physiol.* **236**, F14 (1979).
6. S. D. Levine, W. A. Kachadorian, D. Schlondorff, *Clin. Res.* **27**, 422A (1979).
7. P. J. Bentley, *J. Endocrinol.* **17**, 201 (1958).
8. P. Eggena, *J. Gen. Physiol.* **59**, 519 (1972).
9. G. W. Snedecor and W. G. Cochran, *Statistical Methods* (Iowa State Univ. Press, Ames, 1969), pp. 91–119.
10. R. M. Hays, N. Franki, R. Soberman, *J. Clin. Invest.* **50**, 1016 (1971).
11. H. D. Price and T. E. Thompson, *J. Mol. Biol.* **41**, 443 (1969).
12. A. Finkelstein, *J. Gen. Physiol.* **68**, 137 (1976).
13. J. H. Wang, C. V. Robinson, I. S. Edelman, *J. Am. Chem. Soc.* **75**, 466 (1953).
14. L. Orci, A. Perrelet, F. Malaisse-Lagae, P. Vassalli, *C. R. Acad. Sci. Ser. D* **283**, 1509 (1976).
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Müllerian Duct Regression in the Embryo Correlated with Cytotoxic Activity Against Human Ovarian Cancer

Abstract. *A significant cytotoxicity index was obtained when human ovarian cancer cells in a microcytotoxicity assay were exposed during the S (DNA-synthesizing) phase of the cell cycle to purified fractions of testis exhibiting high Müllerian inhibiting substance bioactivity. The same effect was not observed when these fractions were tested against human glioblastoma or fibroblast lines. Most human ovarian cancers are said to resemble Müllerian tissues histologically. Müllerian inhibiting substance may thus deserve further study as a potential chemotherapeutic agent.*

Embryonic development is dependent on a series of intricate systemic and local signals that stimulate organ differentiation, or, conversely, cause dissolution or even self-destruction leading to disappearance of parts or all of an organ system. Müllerian inhibiting substance (MIS), a fetal testicular product (*1*), initiates regression of the Müllerian duct in the male embryo of many mammalian species. The Müllerian ducts in females develop into the uterus, fallopian tube, and upper vagina (*2*). The common epithelial carcinomas of the ovary resemble histologically the tissues derived from the Müllerian duct (*3*), that is, fallopian tube, endometrium, and endocervix; MIS may have an inhibitory effect that is specific for fallopian, endometrial, and cervical tumors as well as ovarian tumors. In this study, we tested the cytotoxic effects of partially purified (*4*) MIS-active fractions (*5*) of testes from newborn calves (*6*) against a human ovarian cancer in tissue culture (*7*).

The ovarian tumor was surgically removed from a 56-year-old woman in 1971 and has been serially subcultured since then. Histologically, the tumor is described as a papillary serous cystadenocarcinoma that is moderately well differentiated. The epithelioid line doubles every 28 hours, has a near diploid karyotype, and can produce tumors with a histology similar to the original tumor when 10^4 or more cells are heterotransplanted into the hamster cheek pouch (*7*, *8*). This line has been serially subcultured in our laboratory in stationary monolayers at 37°C in Eagle's minimum essential medium (MEM) containing 15 percent fetal calf serum, 1 percent penicillin (10,000 unit/ml), and streptomycin (10,000 μg/ml). As control lines we used a human fibroblast strain derived from the foreskin of a 1-year-old undergoing circumcision, and a glioblastoma (non-Müllerian duct tumor) line (*9*). The control lines are grown in stationary monolayers at 37°C in F10 nutrient medium containing 10 percent fetal calf serum and 1 percent penicillin and streptomycin. All lines are free of mycoplasma (*10*).

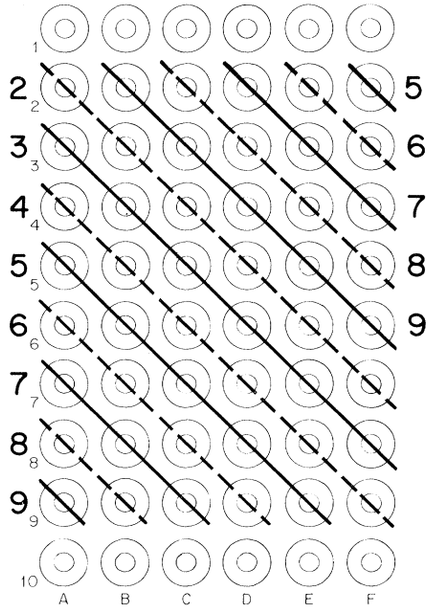

Fig. 1. Evaporation was minimized by applying experimental and controlled fractions on the microtest plates in the template illustrated and by surrounding the plates with moist gauze in a small plastic food box with the lid slightly ajar at 37°C in a 5 percent CO_2 humidified incubator. Rows 1 and 10 were eliminated.

By methods adapted from those of Wood and Morton (*11*), monolayers of the cell lines, after they approached confluency (3 days after a 1:2 subculture), were washed with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution, dispersed with 0.25 percent trypsin-EDTA (Gibco), and counted in a hemocytometer. Appropriate dilutions for each cell line were made with either MEM or F10 containing 20 percent fetal calf serum, and 300 to 400 cells were delivered to each well of a Falcon 3034 microtest plate in a 0.01-ml volume with a six-barrel Teresaki syringe. Plates were surrounded by moistened gauze, placed in a plastic box with the lid ajar (*12*), and incubated at 37°C in a humidified incubator with an atmosphere containing 5 percent CO_2 and 95 percent air. The following morning medium was blotted from the wells and replaced with new medium (0.01 ml per well). Testis fractions and controls were then added in 0.01-ml portions (total volume, 0.02 ml per well) according to the pattern shown in Fig. 1 (*12*). After a 24-hour incubation period, the plates were washed and stained with Giemsa, and the adherent cells were counted on a projection screen. Six replicate wells were used for each test fraction. The microtest plates contained phosphate-buffered saline (PBS) as a negative control, fractions from newborn calf heart (no Müllerian duct regression) as a tissue negative control, and fractions from newborn calf testes that were either inactive (biochemical negative controls) or active (test substance) in the organ culture assay. The counts of the 18 PBS control replicate wells were averaged and compared with the average of the six replicates of each fraction tested, and a cytotoxicity index [CI = (control well counts − test well counts)/control well counts] was calculated separately for each plate. A CI greater than 0.25 differed significantly from the controls ($P < .01$, Student's t-test). Müllerian duct regression activity was simultaneously determined for each test fraction, and the activity in an organ culture assay was correlated with the cytotoxicity assay on the human ovarian cancer cells.

Newborn calf testes were diced rapidly in an automatic tissue chopper, suspended in a 1*M* guanidine hydrochloride

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solution containing 5 m*M* benzamidine at 4°C, and sequentially purified (Table 1) (*4*). Multiple-step dialysis and concentration back to an equivalent weight of the starting material (*4*) was performed at each step, and all fractions were tested for biological activity in the organ culture assay for Müllerian duct regression. Aliquots of the biologically active and inactive fractions were stored at −80°C or −196°C for subsequent microcytotoxicity assay.

The urogenital ridge was dissected from a 14-day-old female rat embryo and transferred to agar-coated stainless steel grids in Falcon 3010 organ culture dishes. Specimens were incubated for 72 hours at 37°C in 5 percent CO_2 and 95 percent air over 2 ml of culture medium [CMRL 1066 containing 10 percent fetal calf serum, 1 percent penicillin (10,000 unit/ml), and streptomycin (10,000 μg/ml)], or containing a 1:1 mixture of culture medium and purified fractions from calf testis, heart extracts, or PBS. The incubated tissue was then coated with a mixture of 2 percent agar and albumin at 44°C and fixed and embedded in paraffin. Serial sections (8 μm) were stained with hematoxylin and eosin for viewing by light microscopy. Sections from the cephalic end of the Müllerian duct were assigned a coded number and graded for regression on a scale of 0 to 5 by two independent observers (Table 1). Grade 0 refers to no regression; grade 1, minimal; grade 2, mild; grade 3, moderate; grade 4, severe; and grade 5, complete regression, based on characteristic morphologic changes in the epithelial cells and surrounding mensenchyme (*5*, *13*).

As shown in Table 1, a significant CI (0.37, $P < .01$) against the human ovarian carcinoma was obtained when the biologically active fraction 11, obtained by density gradient sedimentation of the guanidine extract, was applied to the cells in the microtest wells. Biologically active fraction B, obtained by column chromatography of fraction 11, had a significant cytotoxic index (CI = 0.45, $P < .01$), whereas gel chromatography fractions A and C, which were inactive in the organ culture assay, had an insignificant CI (0.24). A significant cytotoxic effect was not obtained when similar fractions from newborn calf heart were tested against the human ovarian carcinoma cells (HOC-21). Similarly, none of these fractions from nontesticular tissue demonstrated Müllerian duct regression in the organ culture assay. When gel chromatography fraction B from calf testis was tested in the microtest system against a human fibroblast line or a glioblastoma line, no significant cytotoxic effect was observed (CI = 0.01 and −0.11, respectively). A cytotoxic effect was not observed when biologically active fractions were applied against the human ovarian cancer line which had been subcultured for more than 3 days earlier. This effect also was not present when the plates were sparsely (less than 50 cells per well) or densely (greater than 100 cells per well) seeded.

Table 1. Newborn calf testis or heart fragments were extracted with guanidine, subjected to density gradient sedimentation (DGS) in $CsCl_2$ (1.5 g/ml), ultrafiltration (Amicon PM30), and then gel chromatography (GC) (fractions A, B, and C) in a 200- by 2.6-cm column packed with Bio-Rad A-5M Bio-Gel. Dialyzed and concentrated fractions were then assayed for Müllerian duct regression. Fractions with positive and negative biological activity were mixed with medium and added to wells of the microtest plates in which cells of appropriate density were synchronized in the S phrase of the cell cycle.

Sample	Ovarian carcinoma		Skin fibroblasts		Glioblastoma		Organ culture regression grades
	Average number of cells per well	CI	Average number of cells per well	CI	Average number of cells per well	CI	
			Testis				
DGS fraction 11 (1:1 dilution)	54.0	0.37*	60.0	−0.01			2 to 3+
CMRL	91.2	−0.06	68.3	−0.15			0
PBS	83.7		61.2				0
DGS fraction 11 (1:3 dilution)	65.3	0.24	54.3	0.09			2 to 3+
CMRL	91.8	−0.07	70.7	−0.19			0
PBS	84.8		58.3				0
GC fraction A	52.0	0.24					0
GC fraction B	37.7	0.45*					2+
GC fraction C	51.6	0.24					0
PBS	66.5						0
			Heart				
GC fraction B	66.0	0.03					0
			Testis				
GC fraction B	68.3	0.32*	75.5	0.01			2+
PBS	100.5		71.5				0
			Heart				
GC fraction B	85.0	0.16	82.2	−0.08			0
			Testis				
DGS fraction 11					104.0	0.05	3 to 4+
GC fraction B					116.0	−0.05	0
			Heart				
GC fraction B					119.0	−0.08	0
PBS					108.8		0
			Testis				
GC fraction B					122.2	−0.11	2 to 4+

*$P < .01$.

Thus fractions isolated from newborn calf testes that demonstrated Müllerian duct regression in the organ culture assay of the 14-day embryonic rat Müllerian duct also demonstrated a cytotoxic effect against a human ovarian carcinoma line, presumably of Müllerian duct nature. This same effect against the human ovarian cancer line was not observed with fractions lacking MIS biological activity in the organ culture system. Biologically active fractions had no effect against the non-Müllerian human glioblastoma or against a human fibroblast strain.

The cytotoxic response elicited by testis fractions that cause Müllerian duct regression in the embryo indicates that this tumor is, like embryonic tissue, responsive to fractions with MIS activity. The response also indicates that the interaction of these fractions with the tumor has led to loss of cell adherence and possibly cell death. Whether this phenomenon represents cell death, as implied by the term "cytotoxicity," or simply cell detachment, was not addressed in this study.

During fetal life, receptors to MIS, like receptors to androgens (*14*), are constitutive; male and female Müllerian duct cells can respond equally well to the testicular substance (*15*). However, it is not known if hormone-receptor interaction occurs after fetal life. Picon (*15*) demonstrated that the female rat Müllerian duct in the embryo older than 16 days does not regress when cocultured with fetal testis. It is not yet known whether MIS after a specific critical period of fetal de-

velopment (i) binds but does not initiate regression, (ii) binds but the interaction initiates a totally different cellular response, or (iii) does not bind at all. It could be that the receptor is exclusively fetal, but can be reactivated in a tumor originating from cells that had constitutive receptors to MIS during fetal life. Fetal enzymes, isoenzymes, antigens, proteins, and hormones, such as alpha fetoprotein (*16*), carcinoembryonic antigen (*17*), and galactosyltransferase (*18*), are known to occur spontaneously in adult tumors. Müllerian duct tumors, occurring in the cervix, endometrium, fallopian tube, and vagina, as well as tumors of Müllerian duct nature (in the ovary) may have receptors for MIS, and the receptor-ligand complex may initiate cellular regression.

The epithelial morphology of earlier Müllerian duct structures predominates in ovarian tumors (*3*). For example, in the well-differentiated types, serous tumors are characterized by papillary ciliated cells of fallopian tube character; mucinous tumors have epithelium that resembles the endocervix, and endometrioid carcinomas have epithelium resembling the lining of endometrial glands. Clear cell tumors of the ovary (*19*), like those of the vagina (*20*), have been shown to be related to an endometrial type of epithelium. Most of the more poorly differentiated adenocarcinomas and undifferentiated carcinomas are thought to arise from the same coelomic surface epithelium.

The cytotoxic effect was not observed unless synchrony of the cell cycle and the density of cell seeding were carefully controlled. Noncoercive partial synchrony of the cell cycle was obtained by subculture with a 1:2 division 3 days prior to and again at the time of harvesting and seeding the microtest plates, in each case just before confluency was reached. It has been shown (*21*) that DNA synthesis (S phase), as measured by thymidine uptake, is maximal just before confluency. Test fractions were added 20 hours later, coincident again with the S phase of the cell cycle, and allowed to incubate for a subsequent 24 hours. Preliminary experiments demonstrated that cells subcultured more than 5 days before seeding, when thymidine uptake falls to negligible levels (*21*), failed to respond to biologically active MIS fractions. Presumably, cells in the S phase are more responsive to MIS. This finding correlates with previous observations with the electron microscope of active, euchromatic nuclei occurring in all Müllerian duct epithelial cells during the initial phases of regression (*13*).

Cells seeded at densities of greater than 100 cells per well failed to respond to MIS. The effect of cell density on MIS responsiveness may be related (i) to diminished surface receptors for the cytotoxic material, since a dense population might cover receptor sites that would become more exposed if the cells were allowed to spread more fully on a monolayer, or (ii) to the uncoupling of DNA synthesis usually seen with cell adherence and spreading, as observed by Folkman and Moscona (*22*).

The finding that a human ovarian cancer (HOC-21) can respond to fractions that cause Müllerian duct regression in the embryo implies the presence of MIS receptors on the tumor and provides an opportunity for small quantities of MIS to be detected.

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References and Notes

1. A. Jost, *C. R. Soc. Biol.* **140**, 463 (1946); *ibid.*, p. 460; *ibid.* **141**, 135 (1947); N. Josso, *C. R. Acad. Sci.* **274**, 3573 (1972).
2. C. D. Edman, A. J. Winters, J. C. Porter, P. C. MacDonald, *Obstet. Gynecol.* **49**, 208 (1977).
3. R. E. Scully, *Hum. Pathol.* **1**, 73 (1970).
4. D. A. Swann, P. K. Donahoe, Y. Ito, Y. Morikawa, W. H. Hendren, *Dev. Biol.* **69**, 73 (1979).
5. R. Picon, *Arch. Anat. Microsc. Morphol. Exp.* **58**, 1 (1969); P. K. Donahoe, Y. Ito, W. H. Hendren, *J. Surg. Res.* **23**, 141 (1977).
6. P. K. Donahoe, Y. Ito, J. M. Price, W. H. Hendren, *Biol. Reprod.* **16**, 238 (1977).
7. T. Yamada, *Keio J. Med.* **23**, 57 (1974).
8. On the advice of the Cell Source Information Bank of the American Type Culture Collection, Rockville, Md., we sought this line which T. Yamada gave to our laboratory in June 1978.
9. The glioblastoma was explanted in 1973 and carried in culture by P. L. Kornblith [P. L. Kornblith, F. C. Dohan, W. C. Wood, B. O. Whitman, *Cancer* **33**, 1512 (1974)].
10. S. Madoff and W. N. Pachas, in *Rapid Diagnostic Methods in Medical Microbiology*, C. Garber, Ed. (Williams & Wilkins, Baltimore, 1970), pp. 195–217.
11. W. C. Wood and D. L. Morton, *Science* **170**, 1318 (1970).
12. R. L. Martuza, M. B. Moore, F. C. Dohan, Jr., M. E. Proffitt, *Transplantation* **21**, 271 (1976).
13. J. M. Price, P. K. Donahoe, Y. Ito, W. H. Hendren, *Am. J. Anat.* **149**, 353 (1977).
14. S. Ohno, *Cell* **7**, 15 (1976).
15. R. Picon, *C. R. Acad. Sci.* **21**, 237 (1970); *Arch. Anat. Microsk. Morphol. Exp.* **56**, 281 (1967).
16. G. I. Abelev, I. V. Assecritoua, N. A. Kraevshy, S. D. Perova, N. I. Perevodchikova, *Int. J. Cancer* **2**, 551 (1967).
17. P. Gold, M. Gold, S. Freedman, *Cancer Res.* **28**, 1331 (1968).
18. D. K. Podolsky, M. W. Weiser, K. J. Isselbacher, A. M. Cohen, *N. Engl. J. Med.* **299**, 703 (1978).
19. R. E. Scully and J. F. Barlow, *Cancer* **20**, 1405 (1967).
20. A. L. Herbst and R. E. Scully, *ibid.* **25**, 745 (1970).
21. A. Maciera-Coelho, in *Tissue Culture, Methods and Applications*, P. F. Kraus and M. K. Patterson, Eds. (Academic Press, New York, 1973), p. 412.
22. M. J. Folkman and A. Moscona, *Nature (London)* **273**, 345 (1978).
23. We are grateful for the helpful suggestions of W. C. Wood, R. Trelstad, and R. E. Scully. This work was supported by NCI grant CA-17393, the American Cancer Society, a junior faculty fellowship award to P.K.D., and the Pediatric Surgical Research Fund, Massachusetts General Hospital.

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Trigeminal Ganglion Infection by Thymidine Kinase–Negative Mutants of Herpes Simplex Virus

Abstract. *The incidence of trigeminal ganglion infection after corneal inoculation of guinea pigs with thymidine kinase-negative mutants of herpes simplex virus was markedly reduced compared to infection after inoculation of thymidine kinase-positive virus. Thymidine kinase-negative herpes simplex virus replicated well in ocular tissues in which dividing or potentially dividing cells were present, but not in trigeminal ganglion infection of nondividing neurons. Thymidine kinase-positive virus, however, replicated well in ocular tissues as well as in trigeminal ganglion. These results suggest that thymidine kinase expression of herpes simplex virus may be important in infections of sensory ganglia.*

Infection of the trigeminal ganglion of guinea pigs with thymidine kinase-negative (TK^-) mutants of herpes simplex virus (HSV) was studied to further examine the mechanisms of latent HSV infection of sensory ganglia. Latent infection of sensory ganglia appears to be an infection of neurons (*1*, *2*), that is, cells that do not replicate beyond the neonatal period. HSV codes for a virus-specific TK (deoxypyrimidine kinase) (*3*), which is expressed in infected cell cultures (*4*), in latent in vivo infection (*5*), and in HSV-transformed cells (*6*). Since viral TK expression is important for virus replication in nondividing cells (*3*), it seemed that HSV TK might be important in the pathogenesis of in vivo infections in nonreplicating sensory ganglion neurons.

To compare TK^- and TK^+ HSV infections, we performed experiments using standard TK^+ HSV type 1 (HSV-1) strain KOS, and a TK^- mutant derived from the TK^+ parental stock. Arabinofuranosylthymine (ara-T) was used to select for the TK^- phenotype since the

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