Mullerian Inhibiting Substance Inhibits Growth of a Human Ovarian Cancer in Nude Mice

PATRICIA K. DONAHOE, ARLAN F. FULLER, JR., ROBERT E. SCULLY, STEPHEN R. GUY, GERALD P. BUDZIK

Mullerian inhibiting substance (MIS) was investigated for its ability to inhibit growth of a human ovarian cancer in nude mice. Biologically active preparations from newborn calf testes, obtained after sequential ion exchange chromatography, delayed or prevented growth of a human ovarian cancer (HOC-21) when 2×10^6 cells were preincubated with them prior to subcutaneous injection of the tumor cells into Balb/C homozygous nude mice. Preincubation of a human colon carcinoma cells (SW-48) with similar preparations of MIS failed to inhibit growth of the tumor cells in nude mice. Human serous carcinomas are thought to arise from the ovarian surface epithelium, a derivative of the coelomic epithelium of the urogenital ridge, which invaginates to form the mullerian duct early in embryonic life. The neoplastic cells of serous tumors simulate morphologically the lining cells of the fallopian tube, which are derivatives of mullerian duct epithelium. This study provides physiologic confirmation of the mullerian nature of this type of tumor and suggests that MIS may ultimately prove to be effective in its therapy.

From the Pediatric Surgical Research Laboratory and the Departments of Surgery, Gynecology and Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

MULLERIAN INHIBITING SUBSTANCE (MIS) is known to cause regression in the male mammalian embryo of the mullerian duct, the analge of the fallopian tube, the uterus, and upper vagina.[1-3] We, along with others, have attempted to characterize this fetal regressor[4-7] and have investigated partially purified preparations of it for their ability to inhibit a malignant tumor of mullerian type. The substance has been demonstrated to be cytotoxic to a human ovarian serous cystadenocarcinoma cell line (HOC-21) in monolayer culture[8] and to inhibit colony growth of the same cell line in soft agar.[9] After successfully heterotransplanting this tumor into nude mice,[9] we have investigated the ability of MIS to inhibit its growth *in vivo*. This report describes an attempt to prevent or delay its appearance in nude mice after these cells have been preincubated with MIS.

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Reprint requests: Patricia K. Donahoe, M.D., Division of Pediatric Surgery, Massachusetts General Hospital, Boston, Massachusetts 02114.

Methods

Animals and Environment

Athymic, hairless male and female mice (Nu/Nu), four to six weeks of age, from the Charles River Laboratories (Balb/C or CD1) or Harlan Industries (Swiss) were kept in sterile, covered cages in a draft-free, filtered and ventilated nude mouse facility at 75°F and 45–50% humidity. The cages were supplied with sterile bedding and food, and water with added multivitamins. The mice were observed for a week prior to the experiments, and were handled with mask and glove technique at all times.

Preparation of Cell Lines for Inoculation

The human ovarian cancer cell line (HOC-21) doubles every 28 hours in tissue culture. Its previous diploid number of 46 XX noticed in a karyotype performed in 1974, changed to a modal number of approximately 70 in 1981 after continuous serial subculture. The histologic appearance of the tumor, however, has remained similar to that of the original tumor removed in 1971,[10] if 10^4 or more cells are heterotransplanted into the hamster cheek pouch or 10^5 or more cells are transplanted into homozygous nude mice[9] (Fig. 1A). Since 1978, this cell line has been subcultured twice weekly in our laboratory in stationary monolayers at 37 C in Eagle's Minimal Essential Media (MEM) containing 15% fetal calf serum, 1% penicillin (10,000 units/ml) and streptomycin (10,000 ug/ml).

A human carcinoma cell line (SW-48) was established from poorly differentiated colonic adenocarcinoma by

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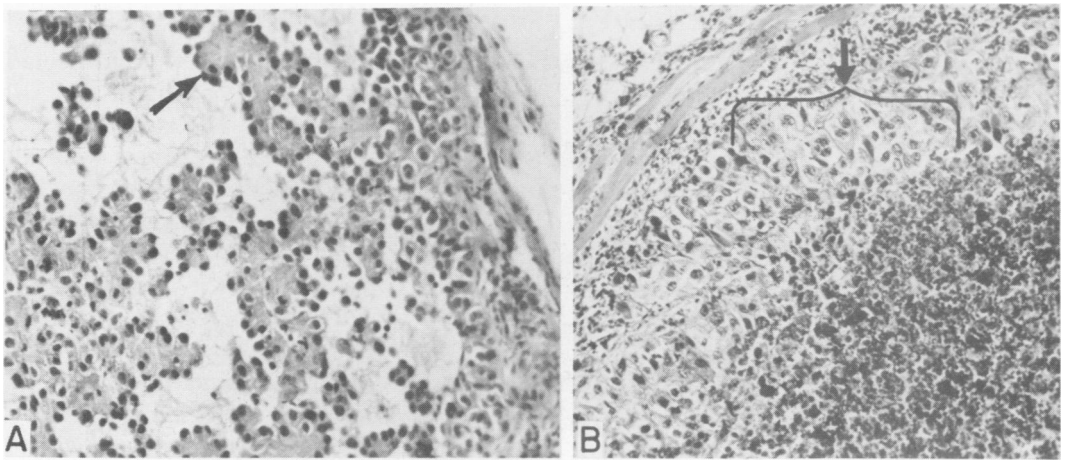

FIG. 1. (A) HOC-21 cell line after injection into nude mice. The original papillary pattern seen in 1971 persists (220×). (B) Tumor nodule palpated two days after injection of 2 × 10^6 HOC-21 cells. Central necrosis and a peripheral zone of viable tumor are seen (150×).

Dr. Albert Leibovitz and associates at the Scott and White Clinic, Temple, Texas,[11] and obtained from Dr. Alfred Cohen, Massachusetts General Hospital. The cells of this tumor have a modal chromosome number of 47 and produce small amounts of carcinoembryonic antigen. Subcutaneous heterotransplantation of 6 to 10 × 10^6 cells in the dorsum of the nude mouse has produced tumors of similar histologic appearance.

The tumor cells were harvested when they were synchronized in the log phase of growth after multiple three-day cultures.[12] Previous studies with the HOC-21 cell line revealed maximum cytotoxicity by MIS in microcytotoxicity[8] and soft agar colony inhibition[9] during the S-phase of growth. Cells were washed twice in Hank's basic salt solution (Gibco), without magnesium or calcium, for three minutes, and trypsinized for five minutes at 37 C with trypsin-EDTA (1:250) (Gibco). Cell clumps were disaggregated to create a single cell suspension by passage through a #25 gauge needle. The cells were counted in a hemocytometer chamber, and viability (>95%) was assessed by trypan blue exclusion.[13]

The appropriate cell number (1 to 5 × 10^6) for a given group of mice was aliquoted into sterile 15 ml tubes and the contents were centrifuged at 1,500 rpm for ten minutes. The supernatant was decanted, and the cells were washed twice with plain MEM and resuspended in enriched CMRL-1066 (Connaught Medical Research Laboratory) containing either 1) an MIS preparation; 2) heat inactivated MIS; 3) a heart preparation; 4) phosphate buffered saline (PBS), 5) Doxorubicin (Adriamycin R, Adria Laboratories, Inc., Columbus, OH) 0.1 ug/ml, or cis-diaminedichloroplatinum (Platinol ®, Bristol Laboratories, Syracuse, N.Y. 13201) 0.04 ug/ml. Cells, varying from 1 × 10^6 to 5 × 10^6/0.5 ml were incubated by gentle manual agitation with each of these substances for one hour at 37 C prior to injection. The injection of 0.5 ml was done with a #25 gauge needle in the subcutaneous tissue of the middorsal right flank.[14] Selected animals were reinjected at 24 and 48 hours at the site of the original inoculation.

Preparations of Fractions with MIS Biologic Activity

Mullerian inhibiting substance fractions were prepared by methods described in detail elsewhere.[7] Briefly, 40 newborn calf testes were minced and incubated in serum free Ham's Nutrient Media F-10 (Gibco) at 37 C for 45 minutes; 5 mM benzamidine was subsequently added. The tissue residue was removed by centrifugation and the supernatant was concentrated to 40 ml. Thus, the amount of MIS released by one testis was concentrated to 1 ml (1 testis/ml). After rapid re-equilibration to 0.05 M NaCl, 10 mM sodium phosphate (pH 8.0) by Sephadex G-25 (Pharmacia) chromatography, the media was fractionated on DEAE Bio-Gel A (Bio-Rad). The biologically active unbound fraction (DEAE-1) was concentrated by ultrafiltration, adjusted to pH 6.0, and further fractionated on CM Bio-Gel A (Bio-Rad). The unbound fraction (CM-1) was biologically active after concentration back to 1 testis/ml (Fig. 2A). This method results in a 50-fold purification with a protein concentration of 7 mg/ml, as determined by the Lowry method.[15] Purification of 8,000-fold has been accomplished, but the less pure, fractions have been used for the present studies because of their ease of preparation. Biologically inactive CM-1 prepared from bovine heart tissue, phosphate buffered saline (PBS), and bioactive CM-1 inactivated by heating at 100 C for ten minutes were selected as controls for MIS (Fig. 2B).

Biologic Activity

All the fractions were evaluated in a semiquantitative organ culture assay[16] adapted from Picon,[17] which uses 14-day urogenital ridges of female rat embryos.

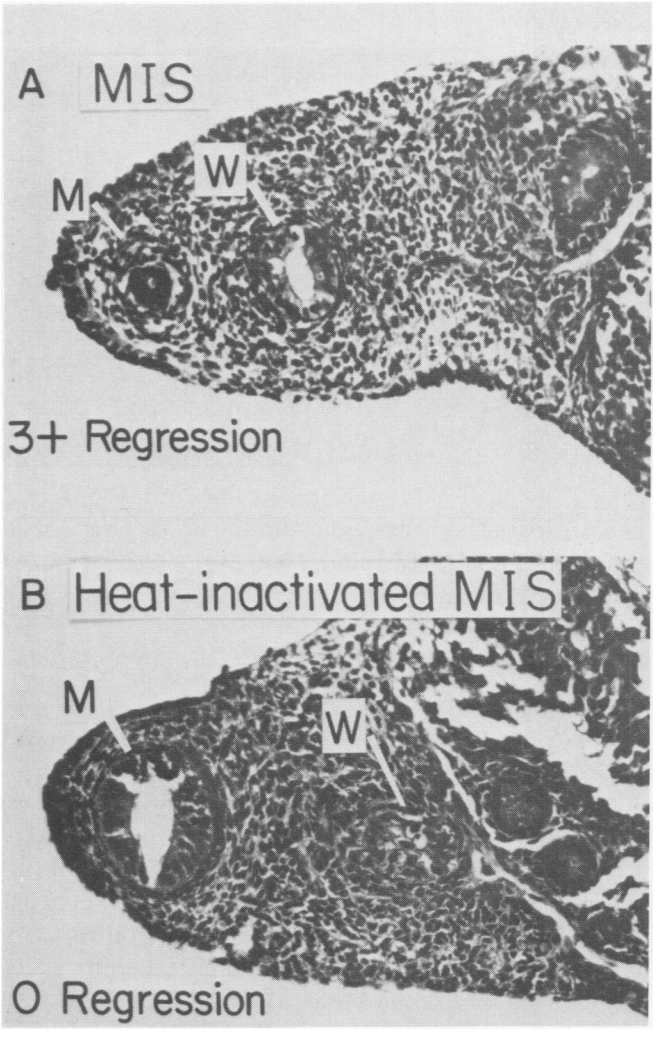

FIG. 2. (A) Mullerian duct (M) after three days incubation in organ culture with a partially purified preparation of MIS shows 3+ regression. (B) The mullerian duct (M) is intact (0 regression) after incubation with heat inactivated MIS. W = Wolffian duct (220×).

The specimens of urogenital ridge were incubated for 72 hours at 37 C in 5% CO_2 over 0.35 ml of culture media mixed 1:1 with the fractions to be assayed or with PBS. After incubation, the tissue was fixed and embedded in paraffin. Serial sections were stained with H & E and viewed with the light microscope. Sections from the cephalic end of the mullerian duct were assigned a coded number and graded for regression from 0 to 5 (complete) by two independent observers (Figs. 2A and B). The grading was based on characteristic morphologic changes in the mullerian duct epithelial cells and surrounding mesenchyme.[16–20] Fractions that were both positive and negative for MIS biologic activity were incubated with human ovarian cancer cells prior to their injection into the nude mice.

Observations of Tumor Growth

The animals were inspected for tumor development daily and the length and width of each tumor was measured. The earliest day of tumor appearance was noticed. The animals were killed at selected intervals throughout and at the conclusion of the study, and the presence or absence of tumor was confirmed by histologic examination. The proportion of animals free of tumor in each group was plotted against time from inoculation using Kaplan-Meier life table calculations, and the significance of the delay in tumor appearance was determined by the log rank test of Mantell on a programmable calculator.[21,22] Tumor-free MIS-treated animals were compared with tumor free control animals by the Fisher Exact Test; $p < 0.05$ was considered statistically significant.

Results

The tumors grew equally well in Swiss, Balb/C and CD1 nude mice. The Balb/C strain, however, survived repeated handling without the development of a wasting disease.[23,24] The experiments were done in either Balb/C or Swiss mice. Tumor grew at similar rates in Balb/C males, Balb/C females and gonadectomized Balb/C males, except in cages where the males were cannibalistic. The unbitten dominant male in these cages exhibited tumor growth at a normal rate. Because of the observation of this "pecking" phenomenon, female mice were used for the definitive experiments.

Tumors grew readily in control animals, invading locally but not metastasizing. Nodules of 3–4 mm in diameter could be palpated at 48 hours (Fig. 1B). Subsequently either central necrosis and tumor regression occurred, or the tumors grew logarithmically. The pattern of growth depended on the cell number of the inoculum. Those animals that received 1 to 1.5×10^6 cells had regression of their tumors, but the tumors growing in animals receiving more than 2×10^6 cells persisted and continued onward to log growth. Histologically confirmed nodules, 3–4 mm in diameter, should have a total volume of 14–32 mm^3. If an individual cell 20 u in diameter has a volume of approximately 4×10^{-6} mm^3, then 3.5 to 8.4×10^6 HOC-21 cells were calculated to be present in the nodule. Therefore, if 2×10^6 cells were injected, this number of cells must have resulted from approximately two cell divisions; such a conclusion is consistent with the kinetics of the HOC-21 tumor observed in tissue culture.[10]

In a preliminary experiment (Fig. 3) 5×10^6 HOC 21 cells were injected into Balb/C nude mice after pretreatment of the cells with 0.02 to 0.5 ml of MIS prep-

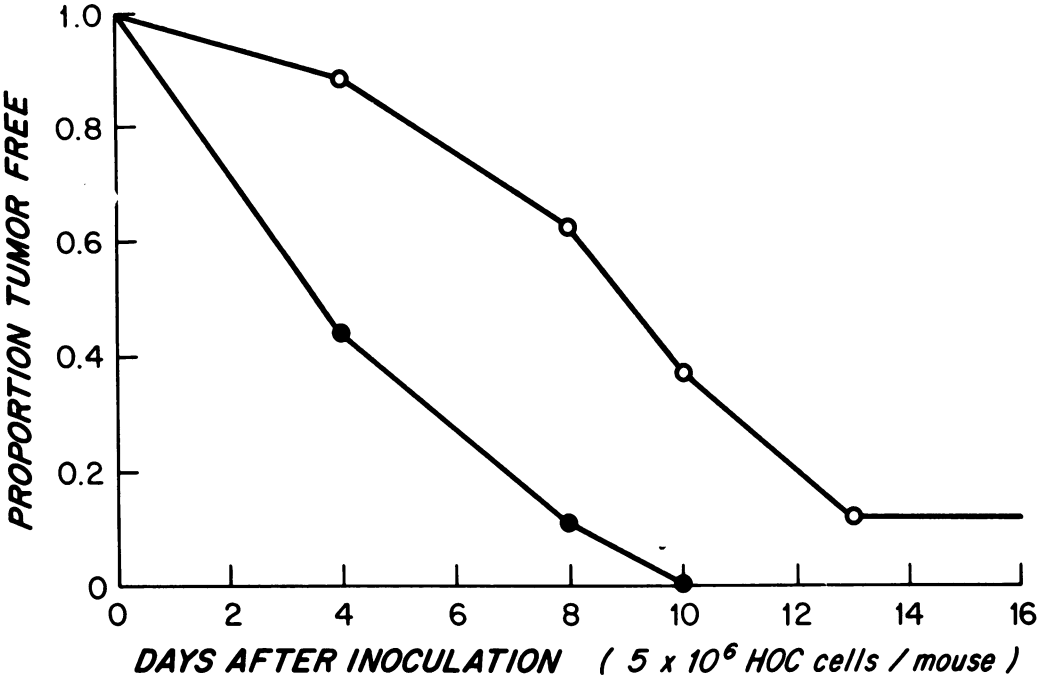

FIG. 3. Incubation of 5 × 10^6 HOC-21 cells with biologically active CM-1 (MIS) (open circles) prolonged the tumor free interval in Balb/C nude mice compared to incubation with heat inactivated MIS and PBS (solid circles).

aration per animal. A statistically significant delay in appearance of tumor was observed when pretreatment with MIS (n = 8) was compared with pretreatment with heat inactivated MIS or PBS (n = 9). A cell inoculum of 2 × 10^6 or 1.5 × 10^6 produced tumors in all the control mice whereas an inoculum of 1 × 10^6 produced tumors in only 60%.

In the inoculum used in the experiments in Figure 4 (n = 29), 2 × 10^6 cells were used. This study explored the effects of varying doses of MIS on tumor growth with five mice in each group. An MIS dose response relation was observed both when the time of appearance of the tumor and the tumor-free survival of the animals were evaluated (Figs. 4 and 5). Pretreatment with

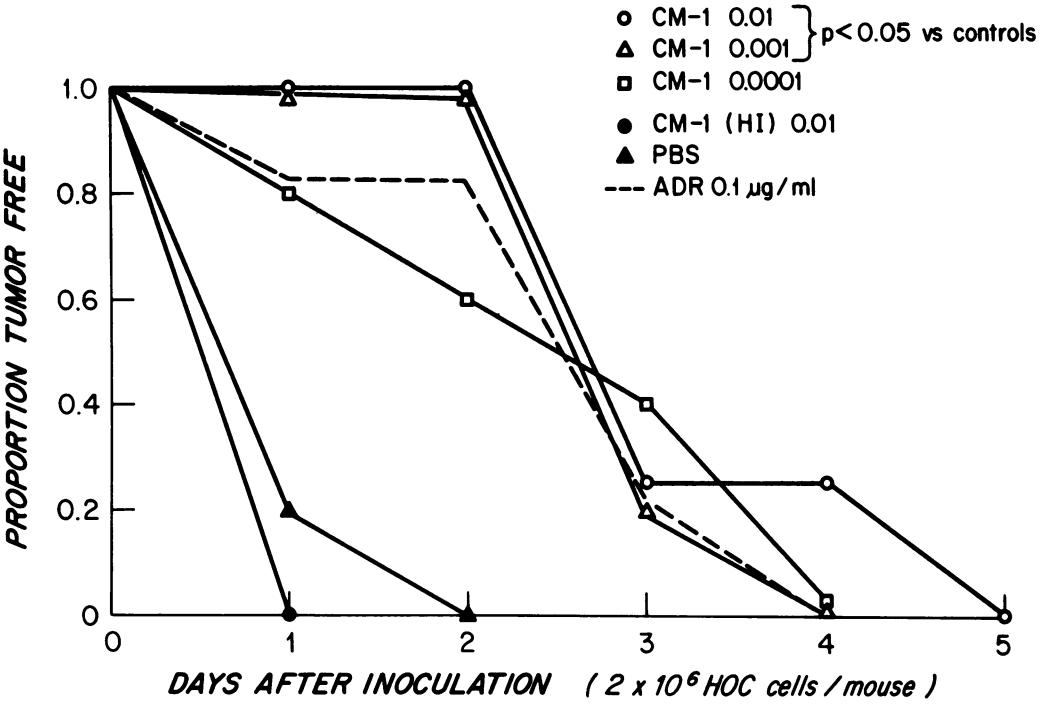

FIG. 4. Increasing concentrations of CM-1 (MIS) prolonged the tumor free interval ($p < 0.05$) (open circles) in Swiss nude mice. A similar prolongation is seen after pretreatment of the cells with Adriamycin. Heat inactivated MIS (solid circles) did not prolong the tumor free interval in comparison to PBS. Each group tested consisted of five mice except at the highest dose where the number of mice was four. 2 × 10^6 cells were injected.

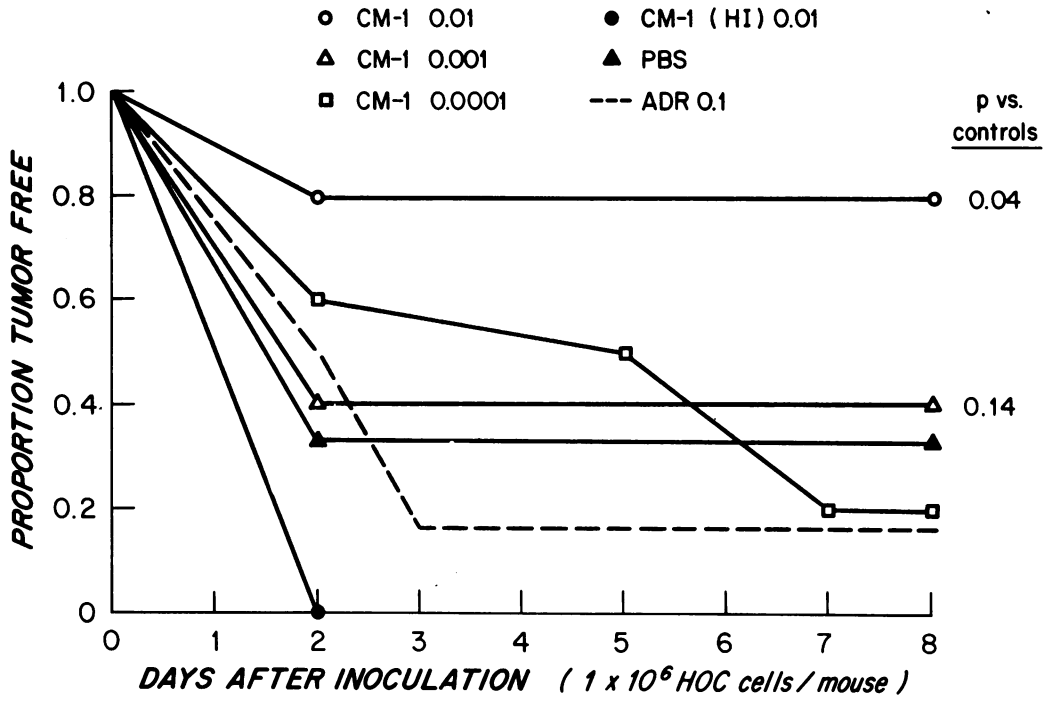

FIG. 5. Pretreatment of 1×10^6 HOC-21 cells with increasing concentrations of CM-1 (MIS) prolonged the tumor free interval in a dose dependent manner in Swiss nude mice. The highest concentrations of CM-1 at this cell number resulted in 80% tumor free survival ($p = 0.04$). In each group $n = 5$, except for the PBS treated group where $n = 6$.

Adriamycin also delayed the appearance of the ovarian tumor (Figs. 4 and 5). A biologically inactive preparation from bovine heart, purified in the same manner as the CM-1 preparation of MIS from bovine testes, did not prolong the tumor free survival (Fig. 6), but produced a curve of time to tumor appearance identical to that of the heat inactivated CM-1 (Figs. 4–6), or PBS. Injection of 0.001 ml MIS in 0.5 cc at the original inoculation site 24–48 hours later, resulted in a proportion of tumor free animals equivalent to that achieved by a single pretreatment of tumor cells with 0.01 ml of MIS (Fig. 6). The tumor nodules were sampled histologically for confirmation at the end of the experiment in all the animals (Fig. 1A), and at two days (Fig. 1B), three days, and five days after injection in selected control animals. The histologic appearance was similar to that seen in the original tumor.[10]

An inoculum of 5×10^6 human colon carcinoma

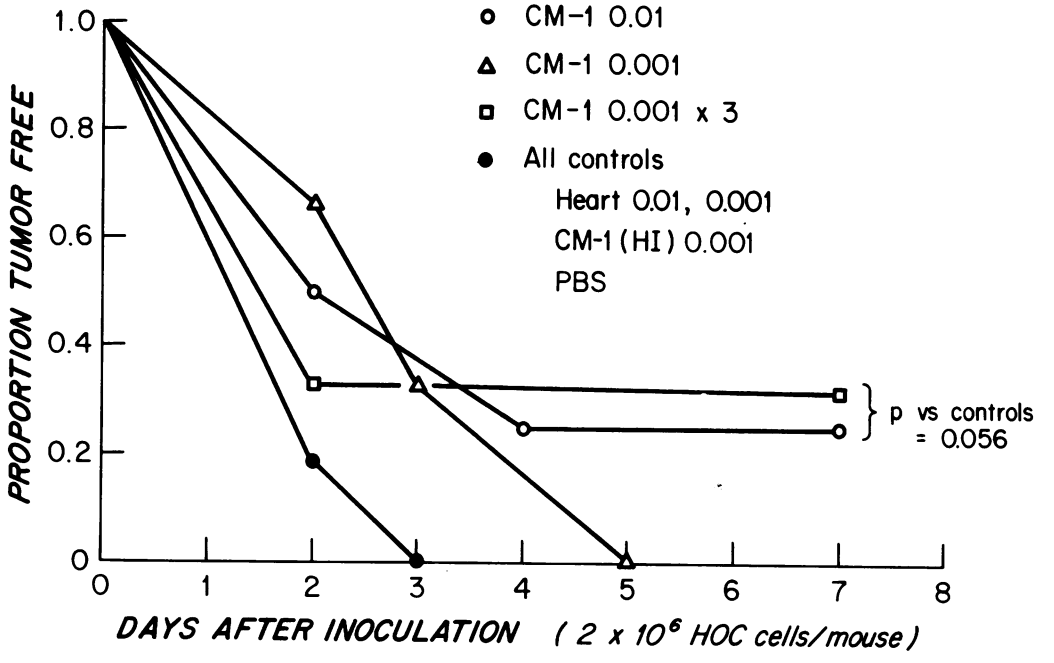

FIG. 6. The proportion of tumor free mice after treatment with 0.01 ml as a single dose, or three doses of 0.001 ml CM-1 (MIS) was increased at a rate approaching statistical significance. Curves for the heart preparation, heat inactivated CM-1 and PBS treated animals were identical. Each group consisted of three or four animals.

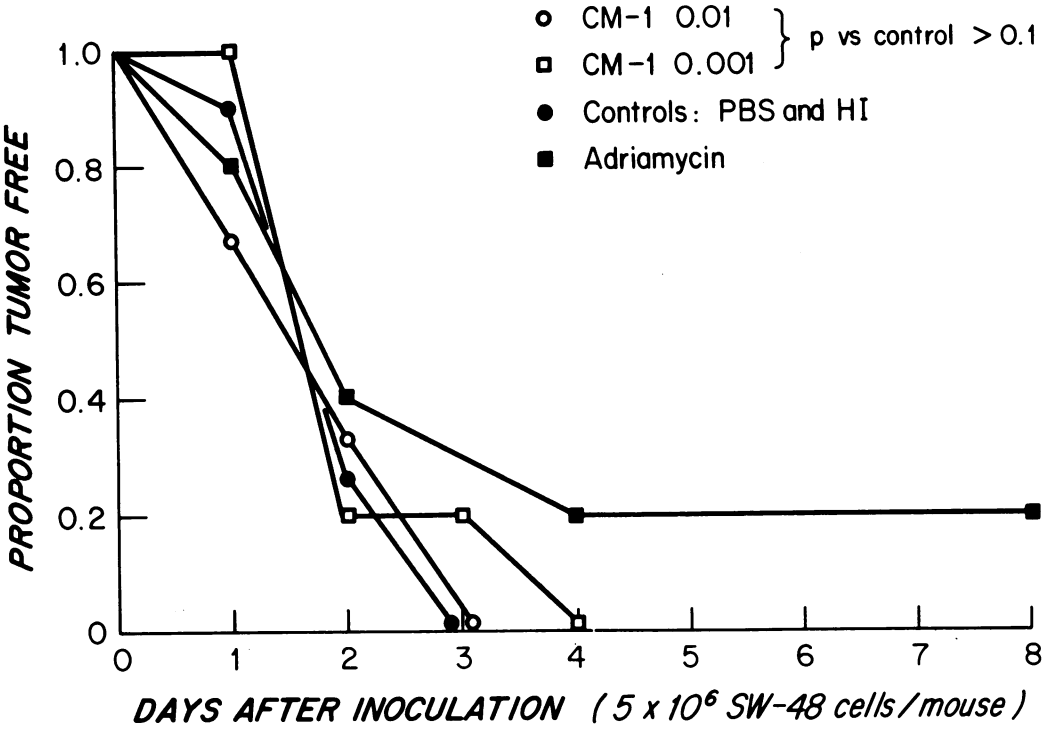

FIG. 7. Pretreatment of colon carcinoma cells (5×10^6) with MIS did not delay tumor appearance when compared with PBS or heat inactivated MIS. In each group, n = 6.

cells (SW-48) was required to produce a palpable tumor in all the control animals within 5 days. 1×10^6 cells produced a tumor in only 40%, and 2×10^6 cells in only 60% of the animals injected. Pretreatment of the SW-48 cells with MIS resulted in no delay in the appearance of the tumor in comparison to that observed with the use of PBS or heat inactivated MIS (Fig. 7).

Discussion

Athymic, hairless, homozygous nude mice (Nu/Nu) are deficient in thymus derived lymphocytes, and therefore lack the ability to mount a cell mediated immune response. These animals are capable of accepting xenogenic organ grafts as well as tumor transplants. Human tumors can be heterotransplanted into nude mice, providing an *in vivo* system for testing tumor susceptibility to chemotherapeutic agents.[25–29] Since there are no syngeneic transplantable ovarian common epithelial tumors or epithelial tumors of mullerian duct derivatives in small animals, the availability of the HOC-21 nude mouse system has provided an excellent model for investigation of the effects of MIS *in vivo*. A palpable nodule of tumor is evident two days

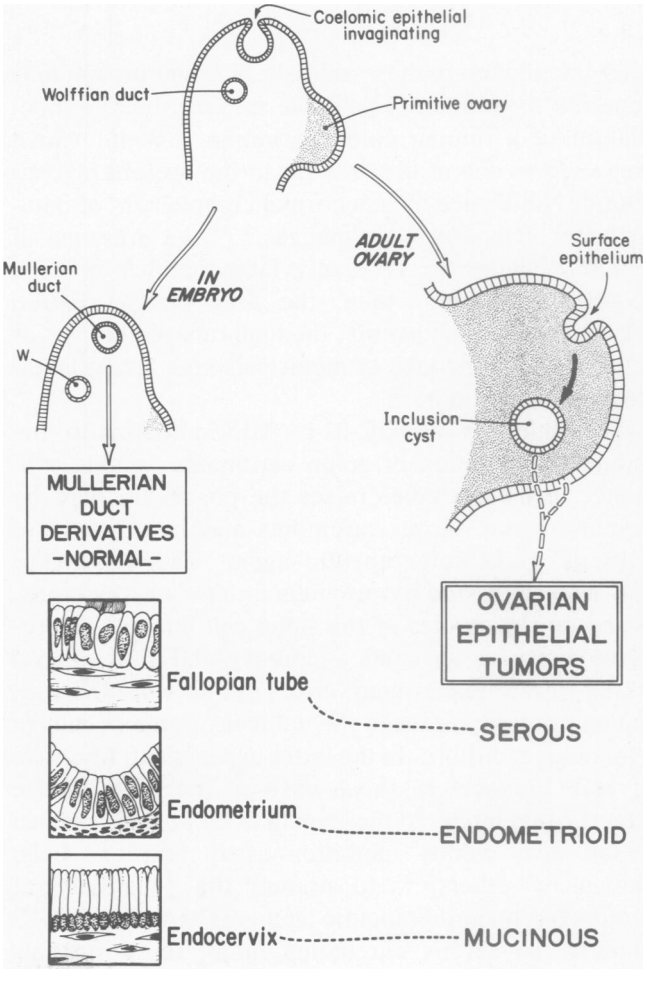

FIG. 8. Proposed schema suggesting parallelism between the development of the mullerian duct from coelomic epithelium and the formation of ovarian epithelial tumors from the surface (coelomic) epithelium.

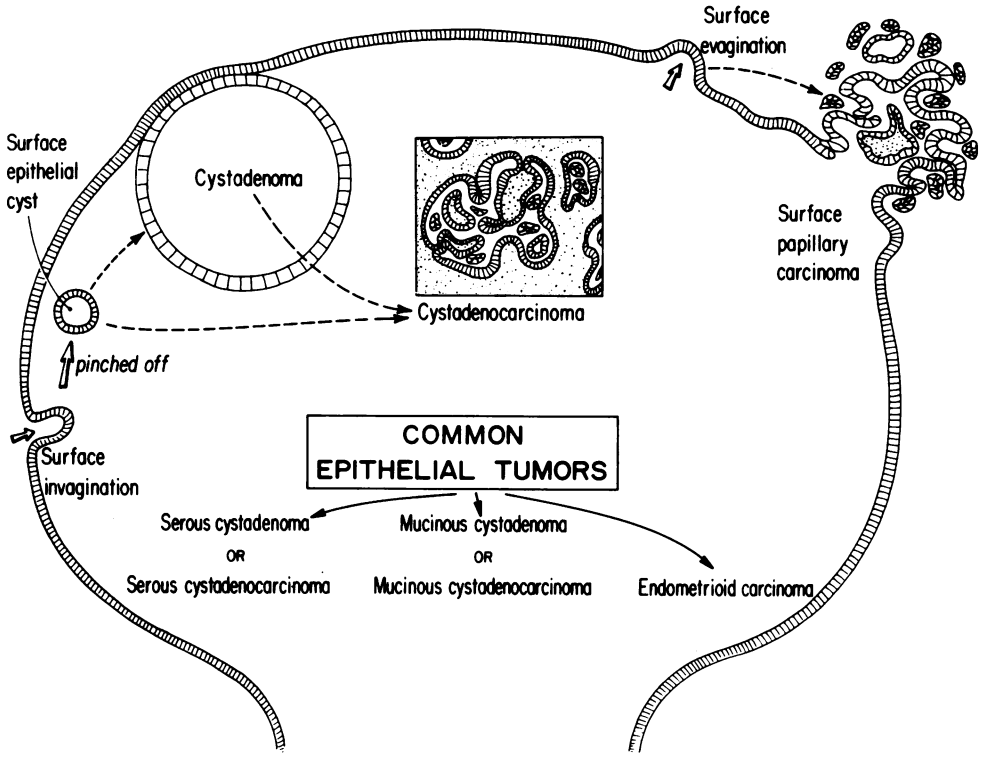

FIG. 9. Proposed histogenesis of common epithelial ovarian tumors from surface (coelomic) epithelium.

after inoculation in these animals. A larger inoculum is required to produce a palpable tumor after the inoculation of a human colon carinoma, SW-48, which was used as one of the controls in the present investigation. Nude mice have a normal complement of natural killer cells and macrophages,[30,31] the presence of which explains the relatively large number of cells required for tumor take, the long plateau before logarithmic growth occurs, the high rate of necrosis or rejection, and the lack of metastasis after transplantation of these tumors.[32]

The inhibition of HOC-21 by MIS in contrast to the failure of inhibition of colon carcinoma by this substance in these studies raises the possibility that the human ovarian serous carcinoma may be sensitive to MIS as a chemotherapeutic agent. This possibility was also suggested by previous *in vitro* observations, based on the studies of the same cell line in a microcytotoxicity assay[8] and a colony inhibition assay.[9] In the former experiment, crude extracts of MIS with biologic activity reduced the number of tumors cells in a monolayer culture. In the latter experiment, fractions of MIS identical to those used in the nude mouse experiments inhibited the growth of clonogenic cells in a soft agar colony inhibition assay that has been shown by others[33,34] to predict the sensitivity of tumors to chemotherapeutic agents. The fact that MIS inhibits the serous carcinoma in all three systems suggests the possibility that this substance has anticancer properties.

The serous carcinoma accounts for most of the human ovarian cancers encountered in the Western world. Several pathologic observations indicate that this tumor originates from the surface epithelium of the ovary and differentiates toward a mullerian type of epithelium, specifically that of the fallopian tube (Fig. 8). In the best differentiated areas of a serous carcinoma, one may encounter the typical ciliated epithelium of benign serous tumors, which mimics the lining of the fallopian tube. In less differentiated regions a papillary pattern that resembles the plication of the fallopian tube mucosa is typically maintained. When serous carcinomas are entirely or partly exophytic, they clearly arise from the surface epithelium. More often they are cystic, however, apparently originating from surface epithelial inclusion cysts within the ovarian stroma (Fig. 9). The evidence for the latter origin consists of the vague delimitation between inclusion cysts and small serous cystadenomas, set at an arbitrary diameter of 1 cm,[35] and the origin of some serous carcinomas within serous cystadenomas.

Surface epithelial inclusion cysts are seen mostly in older women, but are encountered occasionally in young women in the reproductive age group and rarely in prepubertal children. Although the mechanism of formation of these cysts has not been fully elucidated

they have been observed to arise as a result of inclusion of the surface epithelium into the underlying cortical stroma, possibly related to proliferative activity of both epithelial and stromal elements, and the formation of adhesions on the ovarian surface. Radisavljevic[36] has ascribed the origin of these cysts to ovulation, with incorporation of the surface epithelium into the ovarian cortex during repair at the site of rupture of the follicle. Although this explanation is attractive in view of epidemiologic evidence that the frequency of ovarian carcinoma is related directly to the frequency of ovulation,[37] the topographic association of inclusion cysts with sites of ovulation has rarely been recorded by pathologists. Although the surface epithelium of the ovary is not a derivative of mullerian duct epithelium, it is not surprising that it has a mullerian potential on undergoing neoplasia, since it descends from the coelomic epithelium, which covers the urogenital ridge and gives rise to the mullerian duct in close proximity to the developing ovary (Fig. 8).

Although the mullerian nature of serous tumors of the ovary has a sound morphological basis, the growth inhibition of a serous carcinoma in the nude mouse by pretreatment of their cells with MIS suggests that these tumors have at least one distinctive physiological property of mullerian epithelium as well. The specificity of this inhibition for mullerian-type neoplasia by MIS is suggested by its failure to inhibit the growth of a human colonic carcinoma under similar conditions, but additional studies on larger numbers of tumors of mullerian nature or origin and other cancers are indicated to confirm the specific nature of this phenomenon.

The rapid succession of complex metabolic events that results in the full development of an animal from a single cell is under the influence of exceedingly accurate control mechanisms. Study of these mechanisms may uncover methods of controlling growth of various cancers, many of which have characteristics similar to those of embryonic and fetal tissues. Mullerian Inhibiting Substance is a natural biologic modifier known to inhibit or cause regression of a specific organ system during embryonic development. The speculation that it might serve as a specific cancer modulator or therapeutic agent is reasonable in view of the results of the present investigation.

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Discussion

Dr. M. Judah Folkman: During the development of the embryo hundreds of growth factors and inhibitors are switched on for brief periods of time, after which they fall silent, and remained unexpressed throughout adult life.

Dr. Donahoe and her associates have isolated and purified one of these inhibitory factors, the one that is secreted by the embryonic testis for the single purpose of preventing the development of any female genital tract tissue in the male. This is the müllerian inhibiting substance.

What Dr. Donahoe has reported to us this morning is an even more important and novel step, that is, the demonstration that this substance can inhibit the growth of human ovarian tumor cells *in vitro*. Why is this so important?

The answer is that, aside from its immediate potential practical value as a new approach for ovarian cancer, especially ascites, it also suggests to us that theoretically a whole new field of chemotherapy alongside conventional chemotherapy may be possible in the future for other tumors—a field of agents, highly specific, yet not immunologic, in which each antitumor agent is derived from some as yet undiscovered embryonic inhibitor.

This is, of course, high speculation, but any scientific achievement always starts that way. This speculation in particular is based on the experience that once you find one compound as a natural product, as Dr. Donahoe has, it is rarely the only one in existence, because nature usually makes many more like it, a whole class of such compounds, like the antibiotics.

Dr. E. Thomas Boles, Jr. (Columbus, Ohio): Most of our knowledge in the past of steroid biochemistry has been directed toward intersex disorders rather than on neoplasms. It is interesting, however, that a particularly interesting form of intersex, the male pseudohermaphroditic state, and particularly those that are lumped under the term androgen resistant disorders, both the complete and incomplete forms, do have an increased incidence of gonadal tumors. These, of course, are patients who are genetically, and from the standpoint of their gonads, males, but have a distinct tendency, usually in puberty, toward gonadal tumors.

We know that in such patients, probably because of the negative feedback mechanism involving the pituitary and the hypothalamus, and the effect of LH on the testis, there is an increased level of testosterone in the serum of these patients. I would like to ask Dr. Donahoe if she has any knowledge concerning MRF or the müllerian inhibiting substance, as she calls it, under such circumstances.

Dr. Patricia K. Donahoe (Closing discussion): Gonadal tumors occur in patients with dysgenetic gonads. Thyroid tumors occur as a result of high TSH levels. High gonadotropin levels caused by poor negative feedbacks from dysgenetic gonads may be tumorogenic in patients with dysgenetic gonads, although there is no direct experimental evidence to substantiate this hypothesis.

(slide) Future directions for müllerian inhibiting substance research include 1) purification; 2) development of a monoclonal antibody to müllerian inhibiting substance; 3) development of a radioimmunoassay for müllerian inhibiting substance; 4) application of recombinant DNA techniques to study the müllerian inhibiting substance mRNA and DNA; 5) study of the matrix biochemistry of the müllerian duct; 6) study of the interaction of the müllerian inhibiting substance glycoprotein with steroid hormones, and 7) study of müllerian inhibiting substance as a chemotherapeutic agent.