Direct Cloning of Human Ovarian Carcinoma Cells in Agar

Anne W. Hamburger, Sydney E. Salmon, Mary B. Kim, Jeff M. Trent, Barbara J. Soehnlen, David S. Alberts, and H. Jack Schmidt

ABSTRACT

We have recently developed an in vitro assay for human tumor stem cells that permits cloning of human ovarian adenocarcinoma cells in soft agar. Tumor colonies grew from both effusions and biopsies from 85% of 31 ovarian cancer patients. The cloning efficiency did not vary with the histology of the tumor. Growth was induced with medium conditioned by the adherent spleen cells of mineral oil-primed BALB/c mice. Up to 2000 colonies appeared after 10 to 14 days in culture, yielding a plating efficiency of 0.001 to 1%. Cells from nonmalignant effusions did not form colonies under these conditions. The number of tumor colonies was proportional to the number of cells plated between concentrations of 10⁴ to 10⁶ cells/dish.

Morphological and histochemical criteria showed that the colonies consisted of cells with the same characteristics as those of the original tumor. Results of cytogenetic studies were also consistent with a malignant origin for the tumor colonies with marked hyperdiploidy in colonies from four patients and hypodiploidy in a fifth patient.

[¹H]Thymidine and hydroxyurea suicide indices provided evidence that in most cases a high proportion of ovarian tumor colony-forming cells were actively in transit through the cell cycle. Removal of phagocytic macrophages with carbonyl iron markedly reduced the plating efficiency, and 2-mercaptoethanol could only partially substitute for macrophages.

The assay appears useful for screening differential cytotoxic effects of specific anticancer drugs (such as cis-platinum) against the tumor stem cells from various ovarian cancer patients.

INTRODUCTION

We have recently demonstrated the ability to clone human tumor stem cells in soft agar (8, 9). In vitro colony-forming assays for stem cells from transplantable murine myelomas have been predictive of therapeutic response in vivo (19, 20). In applied studies of the bioassay, a range of in vitro sensitivities to various chemotherapeutic drugs has been observed. We are currently testing the predictive value of our assay.

The ability to clone ovarian cancer cells in soft agar could have major importance. Epithelial tumors of the ovary are now the most common fatal gynecological cancer in the United States. Their incidence has tripled in the last 40 years, yet the 5-year survival has not changed appreciably during that period (29). Despite this the growth of ovarian tumors in vitro has not been extensively pursued. Only a few groups have developed the ovarian cell lines that are essential to study the kinetic, biochemical, and immunocytological properties of these tumors (7, 10, 12, 30).

We now report on the application of our bioassay method to the study of ovarian carcinoma cells. This paper describes the growth of the cells in vitro, their morphology, karyology, and the factors controlling their growth.

MATERIALS AND METHODS

Patient Studies. Patients with well-documented ovarian carcinomas were selected for this study. The international system for clinical staging of ovarian cancer was utilized (26). Patients with ovarian cancer all had epithelial type cancers.

Collection of Cells. Malignant effusions were collected in heparinized vacuum bottles (100 units/ml). After centrifugation at 150 × g for 10 min, the cells were collected and washed twice in HBSS* (Grand Island Biological Co., Grand Island, N. Y.) with 10% heat-inactivated FCS (Flow Laboratories, Rockville, Md.). Tumor nodules obtained immediately after surgery were mechanically dissociated under aseptic conditions in a laminar flow hood. Tumors were minced with a scalpel and then teased apart with needles. Cells were filtered through sterile gauze to remove cell clumps passed through 25-gauge needles, and then washed by centrifugation as described previously. The viable nucleated cell counts determined in a hemocytometer with trypan blue were routinely more than 90% if samples were obtained within 2 hr of surgery. Only samples with more than 90% viability were studied. Differential counts were performed on slides prepared with a cytocentrifuge and stained by the Papanicoulau (7) and/or Wright-Giemsa methods (28).

Culture Assay for Ovarian Colony-Forming Cells. Cells were cultured as described by Hamburger and Salmon (9). One ml under layers containing 0.25 ml of Millipore-filtered medium conditioned by the adherent spleen cells of mineral oil-primed BALB/c mice in 0.5% agar were prepared in 35-mm plastic Petri dishes. Cells to be tested were suspended in 0.3% agar in enriched CMRL 1086 medium (Grand Island Biological Co.) with 15% horse serum (Flow Laboratories). In this standard assay system, 2-ME was added to a final

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2 To whom requests for reprints should be addressed.
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*The abbreviations used are: HBSS, Hank's balanced salt solution; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; PAS, periodic acid-Schiff, [₁H]dTd, [₁H]thymidine.
concentration of 50 μM. However, the requirement for 2-ME for ovarian carcinoma was specifically studied as an experimental variable and was found not to be necessary. Each culture received $2 \times 10^6$ cells in 1 ml of agar-medium mixture. Cultures were incubated at 37°C in a 7.5% CO₂-humidified atmosphere.

**Scoring of Cultures.** Cultures were examined with a Nikon MS inverted-phase microscope at ×100 and ×200. Final colony counts were made 10 to 14 days after plating. Aggregates of 30 or more cells were considered colonies. Individual colonies were removed from the dishes with a fine capillary pipet and were suspended in a drop of heat-inactivated FCS and deposited on slides that were air-dried for 3 to 4 hr. Cells were stained routinely with Wright-Giemsa, Papanicoulau, and methyl green pyronin stains (9) for morphology and for peroxidase, nonspecific esterase, oil red O, and PAS reactivity (16). Cells picked from colonies were compared to the tumor cells in the original cell suspension with the help of a clinical pathologist.

**Determination of Percentage of Cells in DNA Synthesis by [³H]dThd or Hydroxyurea Suicide.** The [³H]dThd suicide method was used to measure the proportion of ovarian colony-forming cells in the DNA synthetic (S) phase of the cell cycle (13). Samples of $2 \times 10^6$ cells suspended in HBSS and 10% heat-inactivated FCS were added to 1.0 ml of HBSS containing 40 μCi of [³H]dThd (23 Ci/mmol, Amer-sham/Searle, Arlington Heights, Ill.). Control samples were added to HBSS. Cell suspensions were incubated for 30 min at 37°C and washed twice with 20 ml of cold HBSS containing 100 μg/ml of unlabeled dThd and 10% FCS. Each suicide and control suspension was cultured in 4 replicate plates at a concentration of 5 × $10^5$ cells/plate. Alternatively, $2 \times 10^6$ cells in 1 ml of HBSS and 10% heat-inactivated FCS were incubated with 76 μg of hydroxyurea for 1 hr at 37°C. Cells were then washed twice in HBSS and plated as described.

**Drug Sensitivity Testing.** Single cell suspensions prepared as described previously for initial plating were adjusted to a final concentration of $1 \times 10^6$ cells/ml in HBSS and 10% FCS in the desired concentration of cis-platinum diamine dihydrochloride (National Cancer Institute). Cells were incubated with drugs for 1 hr at 37°C. Cells were then centrifuged at 150 × g for 10 min, washed twice in HBSS and prepared for culture. For quantitative comparison of in vitro curves, a “sensitivity index” was defined as the area under linear-survival-concentration curves to an upper boundary concentration. For cis-platinum, the boundary concentration was 0.06 μg/ml.

**Removal of Phagocytic Cells.** One $\times 10^7$ cells obtained from effusions were incubated in 15 ml of McCoy's Medium 5A with 10% heat-inactivated FCS and 80 mg of dry-heat-sterilized carbonyl iron (Tridom Fluka, Hauppauge, N. Y.) in 250-ml Falcon flasks. After incubation at 37°C for 45 min in a shaking water bath, the flasks were placed flat down on a magnet, and supernatant cells were carefully decanted and kept for subsequent study. This step was repeated as often as necessary (generally 4 to 5 times) to remove all the iron powder and iron-laden phagocytic cells. The cells in the supernatant were considered nonphagocytic. Cells were washed twice in HBSS and 10% heat-inactivated FCS. This procedure removed between 30 and 60% of cells.

**Cytogenetic Analysis.** Agar cultures were incubated at 37°C in the presence of 2.5 ml of Ham's Medium F-12 (Grand Island Biological Co.) containing 0.1 μM colchicine. Four to 15 hr later, cultures were removed from the incubator, and the overlying medium was vigorously agitated to dislodge colonies from the agar. The medium was removed and centrifuged for 5 min at 150 × g. The supernatant was carefully removed, and the pelleted colonies were resuspended in 0.075 m KCl for 10 min at 37°C. They were then recentrifuged for 5 min, and the supernatant was discarded. Five ml of fresh, cold fixative (3:1 absolute methanol to glacial acetic acid) was added, and the suspension was agitated with a vortex. They were then washed twice more with fixative and stored at −9°C.

Slides were prepared by placing 2 to 3 drops of the colony suspension onto precleaned slides that had been dipped in 50% acetic acid. A sharp burst of air was then delivered to each slide to further disperse the cells. Slides were then air-dried for a minimum of 48 hr. Metaphase spreads were stained with 3% Giemsa (Gurr's R-66; Reboz, Washington, D. C.) for 5 to 7 min. G banding was performed with the technique of Sun et al. (25).

**RESULTS**

**Development and Identification of Colonies.** Cell doublings were usually observed within 24 hr of plating and clusters of 8 to 20 cells appeared within 3 to 8 days. Individual cells appeared to increase in size within 24 hr of plating. Colonies (collections of 30 or more cells) appeared 10 to 14 days after plating. Cell lysis generally occurred 21 days after plating. Cultures were not refed, and the average life of a culture was 3 weeks. No attempt was made to subculture the colonies. During the first 5 days of incubation, there was a progressive increase in the number of cells that commenced proliferation. Colonies consisted of 30 to several hundred large (30 μM) round cells. Many cells were vacuolated with a characteristic signet ring appearance. Cells in large colonies were tightly packed without the free cell layer at the periphery that is characteristic of macrophage colonies. The morphology of the colonies did not vary with histological type (Fig. 1).

The number of colonies ranged up to 2000/plate, yielding a maximum plating efficiency of up to 1%. A linear relationship was obtained between the number of nucleated cells plated and the number of colonies found after 14 days (Chart 1). This line back extrapolated through zero, indicating origin of the colonies from a single clonogenic cell. Origin of colonies from single cells could also be observed directly by serial inverted-phase microscopy.

When cells from individual colonies were plucked from agar and stained by the Wright-Giemsa and Papanicoulau methods, they appeared to have the same morphological characteristics as did tumor cells in the original suspension. In general, cells from colonies grown from patients with serous adenocarcinoma were oval with pale-staining cytoplasm. The nucleus was pleomorphic with one or more nucleoli. Multinucleated cells were occasionally present. Cells usually contained a moderate amount of PAS-positive material and occasional oil red O- granules (Fig. 2b).

Mucinous adenocarcinoma cells were larger, polyhedral,
All patients but one were in relapse from treatment with cinous, endometrioid, or undifferentiated types (Table 3). Serous adenocarcinoma and 100% of patients with mucinous adenocarcinoma and 100% of patients with mucinous, endometrioid, or undifferentiated types (Table 3).

Effect of Histological Type on Cell Growth. Ovarian colony growth has been achieved from 75% of patients with serous adenocarcinoma and 100% of patients with mucinous, endometrioid, or undifferentiated types (Table 3). All patients but one were in relapse from treatment with multiple drugs or radiotherapy.

Cells from nonmalignant effusions (consisting of macrophages, lymphocytes, mature granulocytes, and mesothelial cells) obtained from patients in cardiac failure failed to support cell growth as well as did BALB/c spleen cell-conditioned media (Table 1).

Table 1
Effect of substitution of alternative feeder layers on ovarian colony growth

<table>
<thead>
<tr>
<th>Substituted material</th>
<th>Control (oil-primed conditioned media)</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>306 ± 35b</td>
<td>320 ± 40</td>
</tr>
<tr>
<td>Effusion</td>
<td>2549 ± 39</td>
<td>833 ± 15</td>
</tr>
<tr>
<td>Heat</td>
<td>420 ± 30</td>
<td>300 ± 50</td>
</tr>
<tr>
<td>Pronase</td>
<td>380 ± 19</td>
<td>350 ± 30</td>
</tr>
<tr>
<td>CD-1 conditioned media</td>
<td>580 ± 27</td>
<td>320 ± 40</td>
</tr>
<tr>
<td>DBA/2 conditioned media</td>
<td>580 ± 27</td>
<td>240 ± 36</td>
</tr>
<tr>
<td>BALB/c conditioned media</td>
<td>580 ± 27</td>
<td>109 ± 9</td>
</tr>
<tr>
<td>MA-184 conditioned media</td>
<td>420 ± 50</td>
<td>0</td>
</tr>
<tr>
<td>WI-38 conditioned media</td>
<td>420 ± 50</td>
<td>0</td>
</tr>
</tbody>
</table>

a See "Materials and Methods."
b Mean ± S.E.

Table 2
Effect of 2-ME on ovarian colony growth

<table>
<thead>
<tr>
<th>No. of colonies/200,000 cells</th>
<th>± 2-ME</th>
<th>− 2-ME</th>
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<tr>
<td>Trial</td>
<td>2-ME</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>280 ± 14a</td>
<td>306 ± 48</td>
</tr>
<tr>
<td>2</td>
<td>183 ± 16</td>
<td>195 ± 21</td>
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<tr>
<td>3</td>
<td>91 ± 1</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>4</td>
<td>100 ± 11</td>
<td>146 ± 35</td>
</tr>
</tbody>
</table>

a Mean ± S.E.

Table 3
Effect of tumor type on ovarian colony growth

<table>
<thead>
<tr>
<th>No. of successful cases/total no. of cases</th>
<th>Tumor type</th>
<th>Effusion</th>
<th>Solid tumor</th>
<th>% successful</th>
<th>Colony range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous adenocarcinoma</td>
<td>10/15</td>
<td>5/5</td>
<td>75</td>
<td>127–500</td>
<td></td>
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<tr>
<td>Mucinous adenocarcinoma</td>
<td>3/3</td>
<td>1/1</td>
<td>100</td>
<td>12–2549</td>
<td></td>
</tr>
<tr>
<td>Endometrial adenocarcinoma</td>
<td>2/2</td>
<td>100</td>
<td>144–5000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td>5/5</td>
<td>100</td>
<td>20–220</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
form colonies at concentrations of $5 \times 10^3$ to $5 \times 10^6$ cells/plate.

**Proliferative State of Ovarian Colony-forming Cells.** Incubation of cells with high specific activity $[^3H]dThd$ reduced colony formation to as little as 35% of control. No suicide effect was seen in 2 patients with undifferentiated cancer. Hydroxyurea, also known to kill cells in the S phase of the cell cycle, reduced colony formation to about the same levels as did $[^3H]dThd$ (Table 4).

**Drug Sensitivity Assay.** Survival plots of ovarian colony-forming cells from 11 patients whose cells were tested with cis-platinum diamine dihydrochloride are shown in Chart 2. The wide difference observed in the plots indicates a spectrum of sensitivity to the drug. The dose of cis-platinum required to reduce colony formation to 50% of control ranged from 0.02 to 0.06 $\mu$g/ml for 4 patients. A subsequent plateau effect, wherein increasing lethality with increasing dosage did not occur, was frequently observed and suggested the presence of a subpopulation of cells resistant to cis-platinum. Unit areas under the survival concentration curves for cis-platinum charts (to a defined upper boundary of 0.06 $\mu$g/ml) ranged from 2.16 in the most sensitive patient studied to 7.13 in the cells from the patient with the highest degree of in vitro resistance. Resistant colonies within the population manifested continued growth on serial observation and did not differ in morphology from sensitive colonies.

**Effect of Depletion of Phagocytic Cells.** The number of ovarian colonies seen after depletion of phagocytic macrophages was decreased in every case. The addition of 50 $\mu$M 2-ME only partially restored ovarian cell growth (Table 5).

**Cytogenetics.** Results presented are derived from the cytogenetic analysis of 11 cultures from 5 patients. Approximately 300 metaphases were observed, and over 100 spreads were analyzed for chromosome number and recognizable chromosome aberrations.

The number of chromosomes per cell ranged from 38 to 200. Three patients had a modal count near 58 to 60, one had a modal count at 68 to 69, and one had a modal count of 42.

When cultures were harvested with our procedure, most colonies remained intact. Thus, the mitotic figures observed could be scored as occurring within or outside a colony. Seventy % of the mitotic figures occurred within a colony, 30% near or outside a colony. In cultures of 4 of the 5 patients, diploid metaphases were found primarily outside the colony, while hyper- or hypodiploid metaphases were found within the colony. In 3 patients, all of the mitoses within a colony were hyperdiploid. In one patient (modal count, 42), hypodiploid figures were found most often within colonies. In the final patient, all mitoses were outside the colonies, thus not allowing comparison. Double-minute bodies, or dot chromosomes, were found in cultures from 2 patients. One case contained multiple double minute chromosomes in approximately 20% of the cells, the second in approximately 5%.

**DISCUSSION**

These studies have demonstrated that tumor cells from patients with epithelial-type ovarian cancer can form colonies in soft agar. The growth pattern did not vary significantly with the histological type of the tumor.

The use of a battery of morphological and histochemical stains indicated that cells picked from colonies had the same features as did the original tumor cells. However, routine staining techniques are often inadequate for the recognition of malignant cells in pleural and peritoneal effusions (23). Even the use of histochemical stains for ovarian tumor cells is not completely specific (12, 15). The development and use of an ovarian tumor-specific antisem, such as that raised by loachimef a/. (12) and Bhattacharya and Barlow (3), would be of use in confirming the malignant nature of the colony cells.

However, the good coincidence observed between properties of cultured cells and cells in the original suspension and our cytogenetic observations suggested that the colony cells were derived from clonogenic neoplastic cells from the original tumor.

Cytogenetic analysis revealed a wide range of both chromosome number and modal count in cultured cells. This is consistent with the few published cytogenetic analyses of cultured or primary ovarian ascitic and solid tumors (1, 2, 14, 27). The finding of double-minute bodies (2, 6) in cultures from 2 patients is also of interest. Double-minute bodies were originally observed in cultured cells from

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**Table 4**

<table>
<thead>
<tr>
<th>Stage at diagnosis</th>
<th>Estimated tumor bulk</th>
<th>Source of tissue</th>
<th>Control (No. of colonies/500,000 cells) $[^3H]dThd$</th>
<th>Hydroxyurea % survival of colony-forming cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>&gt;1 kg</td>
<td>Tumor nodule</td>
<td>54 ± 6 $^a$</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>&gt;1 kg</td>
<td>Tumor nodule</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>&gt;2 kg</td>
<td>Ascites</td>
<td>500 ± 19</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>&gt;1 kg</td>
<td>Tumor nodule</td>
<td>183 ± 16</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>&lt;1 kg</td>
<td>Ascites</td>
<td>233 ± 40</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>&gt;1 kg</td>
<td>Ascites</td>
<td>47 ± 12</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>&gt;1 kg</td>
<td>Ascites</td>
<td>20 ± 5.7</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>&gt;1 kg</td>
<td>Ascites</td>
<td>120 ± 23</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.E.
patients with neuroblastoma. Since this initial finding, they have been reported in nonneural tumors. Significantly, double minute bodies have only been found in malignant tissue. The fact that cells from nonmalignant effusions do not grow in our system at concentrations of $2 \times 10^8$ cells/plate provides further supportive evidence that our culture system has some optimization for the growth of tumor colonies.

Plating efficiency, although low, was in the same range as that observed for granulocyte or erythroid colony-forming cells or stem cells from transplantable mouse myeloma. Therefore, our assay should prove useful in determining factors governing the proliferation of ovarian tumor cells in the same manner that soft agar assays have proven useful for elucidation of granulocyte and erythroid progenitor behavior (4, 24).

The results reported here also indicate that macrophages were needed for proliferation of ovarian tumor cells derived from effusions. A number of authors have noted the stimulatory effect of macrophages on malignant cell growth (5, 11, 18). Nathan and Terry (18) have demonstrated that DNA synthesis of many (although not all) mouse lymphomas was stimulated by normal mouse peritoneal macrophages. Similarly, Namba and Hanoka (17) reported that phagocytic cells or a glycoprotein released by them was required for stimulation of lymphoma cell DNA synthesis by normal macrophages was only evident when lymphoma lines could be stimulated by normal mouse peritoneal macrophages. Similar stimulation of L1210 leukemic cells in the dialyzed plate provides further supportive evidence that our culture system has some optimization for the growth of tumor colonies.

2-ME only partially restored ovarian cell growth in the absence of macrophages. Nathan and Terry (18) found that stimulation of lymphoma cell DNA synthesis by normal macrophages was only evident when lymphoma lines could be equally stimulated by 2-ME.

Whether the stimulatory effect seen here was due to direct contact between macrophages and tumor cells or to a macrophage-derived factor is not known. Interestingly, autologous effusions, which presumably contain macrophage-derived factors, could not support ovarian tumor cell growth in the absence of medium conditioned by macrophages from BALB/c mouse spleens. However, malignant effusions probably contain a complex mixture of stimulatory and inhibitory substances derived from many cell types, including activated lymphoid cells as well as macrophages and tumor cells. We speculate that the reason that ovarian cancer often fails to spread outside peritoneal or pleural cavities may be related to the dependence of tumor cells on macrophages in such exudates for cell growth (21).

In addition to its use in studying the biological properties of ovarian tumor cells, this assay should prove to be of clinical importance. For example, in vitro tumor colony-forming assays of transplantable mouse myeloma have indicated that each subline had a different pattern of drug sensitivity that predicted that which could be observed in vivo (19). The drug sensitivity study reported here indicates a wide spectrum of sensitivities to the experimental agent cis-platinum.

Additionally, the presence of a viable, resistant subpopulation of cells could be inferred from serial observation of surviving colonies. Clonal proliferation of such inherently resistant subpopulations could be responsible for the development of cellular resistance and overt relapse that eventually occurs in most patients with advanced-stage ovarian cancer. Comparative study of the properties of sensitive and resistant colonies might help to clarify resistance mechanisms that are of clinical importance and provide opportunity to devise techniques to overcome specific types of resistance.

Our initial studies (22) correlating in vitro and in vivo sensitivity of myeloma and ovarian tumor colonies to various standard anticancer drugs have suggested that such assay results frequently correspond with clinical sensitivity or resistance. Careful prospective clinical testing of this drug assay technique appears warranted to determine whether it will have potential utility in screening useful new agents and selecting the most effective drugs for individual patients with ovarian cancer.

REFERENCES

Cloning of Ovarian Cells


Fig. 1. Phase-contrast view of a typical ovarian cancer colony from an 11-day-old culture grown from a patient with serous adenocarcinoma in relapse. A pseudoacinar pattern was apparent. × 435.

Fig. 2. a, three cells plucked from an ovarian tumor colony from a patient with a mucinous adenocarcinoma of the ovary. The cytoplasm of the cells were strongly PAS positive, × 2680. b, four cells plucked from an ovarian tumor colony from a patient with a serous adenocarcinoma of the ovary. The cytoplasm of these cells stained lightly with PAS. × 2680.
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