Evidence for a Direct Growth-stimulating Effect of Estradiol on Human MCF-7 Cells in Vivo

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ABSTRACT

The MCF-7 continuous line of human breast cancer cells requires that athymic nude mice receive supplemental estrogen so that inocula can produce progressively growing tumors. Although these cells contain a typical estrogen receptor complex, the lack of consistent growth stimulation induced by estrogens added to in vitro culture systems has raised the question as to whether this class of hormones acts directly upon the cells or induces a second message produced in other tissues. The present experiments were designed to test the effect of estradiol on the growth of these cells in vivo by exposing them directly to the hormone prior to its absorption into the hepatic portal circulation and subsequent metabolic inactivation. Tumor fragments that were placed next to an estradiol-containing pellet in the spleen grew to produce grossly evident tumor masses, whereas those in the subcutis of the same animals did not, although some minute residua did remain. In the splenic tumors, the mitotic index of the MCF-7 cells immediately adjacent to the estrogen pellets was 2.4 times that of cells on the other side of the same tumor and 3.5 times that of those in the minute s.c. residua. We interpret these data as indicating that in vivo estradiol is acting directly upon the MCF-7 cells to increase their rate of proliferation rather than to initiate the production of a second message to be released into the circulation.

Additionally, it was found that s.c. tumors that were decreasing in volume subsequent to withdrawal of systemic estrogen still contained dividing neoplastic cells but with a lower frequency than that seen in progressively growing tumors stimulated with estradiol. This finding indicates that MCF-7 cells can proliferate in vivo in the absence of a substantial amount of estrogen but only at a rate insufficient to sustain progressive tumor growth.

INTRODUCTION

It has been found routinely that the continuous culture line of human breast cancer cells, MCF-7, requires supplemental estrogen administration in order to sustain progressive growth in athymic nude mice (17, 20). The effect of adding estrogen to the culture medium on the rate of proliferation of these cells in vitro has varied from laboratory to laboratory. Lippman et al. (13) first reported a significant stimulatory effect that has been noted by few others (1, 14). Most investigators have failed in their attempts to reproduce such an effect (11, 16, 18), and this has led to the suggestion that the in vivo stimulatory action of estrogen is indirect (16, 18), possibly mediated through the elaboration in distant tissues of a second messenger (9, 19).

In the present study, we attempt to demonstrate that the in vivo growth stimulatory effect of estradiol on MCF-7 cells in nude mice is, in fact, an action exerted by the hormone directly upon the neoplastic cells. The design of the experiments, used successfully in the mouse Leydig cell tumor model (6, 8), depends on the original experiments of Biskind and Biskind (2), who demonstrated the capacity of the liver to inactivate the estrogenic effects of biologically active steroidal estrogens introduced into the hepatic portal circulation. Thus, estrogen sources placed in the spleen should produce a localized sphere of active hormone due to diffusion into the surrounding extracellular fluid yet fail to produce a systemic stimulation because the venous effluent would be cleared of biologically active hormone during its first passage through the liver. The effects of estrogen withdrawal on growing tumor masses in nude mice are also investigated in order to buttress some of the observations indicating that estradiol does exert its proliferation-promoting effects directly upon the MCF-7 cells.

MATERIALS AND METHODS

The animals used were from the colony of congenitally athymic nu/nu mice on a BALB/c background maintained at the Michigan Cancer Foundation. Castrated males, 6 to 8 weeks old, were used throughout in order to avoid the development of pyometra commonly associated with the chronic administration of estrogen to females of this genetic constitution. They were housed in laminar-flow units in a room that had a 12-hr light-dark photoperiodicity and was humidity and temperature controlled. Cages, bedding, food, and water were sterilized, and the latter 2 were available at all times. Estrogen was administered as a 7.5-mg fused 17β-estradiol:cholesterol (15:85 w/w) pellet 1/8 inch in diameter (22). In previous experiments (6, 8), normal and neoplastic testicular tissue has been placed subcapsularly in the spleen using a 17-gauge spinal needle as a trocar. When this procedure were done with small bits of growing MCF-7 tumor and the explants were placed into the subcutis of estrogenized nu/nu mice, only 1 of 20 grafts produced a growing tumor mass. Therefore, for the experiments reported here, tumor fragments, usually about 1 mm in diameter, were positioned appropriately using a fine thumb forceps. These fragments were prepared from excised tumors growing as second- or third-generation transfers in estrogenized nude mice by careful mincing in sterile minimal essential medium using crossed scalpels blades.

In brief, the surgical procedure consisted of a basal anesthetic with Nembutal, maintaining relaxation with open-drop ether. A 1-cm midline skin incision was made just caudal to the xiphoid, the abdominal cavity was entered by incising along the linea alba, and the caudal half of the spleen was delivered through the incision. The tip of the spleen was grasped with a blunt curved-thumb forceps, and a small incision was made through the pancreatic surface of the capsule 4 to 6 mm from the tip. A small pocket was then produced by blunt dissection between the capsule and the splenic pulp. When desired, a hormone pellet was inserted into the pocket followed by the placement of a tumor fragment; otherwise, only a fragment of tumor was placed in the pocket. No attempt was made to suture close the incision in the splenic capsule. The spleen was dropped back into the abdomen, and the incision in the abdominal wall was sutured with 5-0 absorbable gut. By blunt dissection through the same skin incision, a tumor fragment of similar size was placed in the subcutis of the right thorax, and when systemic estrogenization was...
desired, an estradiol:cholesterol pellet was similarly placed in the left thorax. The skin was then closed with metal clips.

The animals were observed at weekly intervals, and all s.c. tumor masses were measured in 2 perpendicular diameters. All animals were necropsied, most between 75 and 105 days postsurgery, when the s.c. masses were measured in 2 perpendicular diameters. All animals were necropsied, and the abdominal contents were scrutinized under × 7 to ascertain if adhesions had formed between the spleen and the parietal peritoneum and to look for tumor deposits. Similarly, the areas where the s.c. grafts had been placed were searched for residua. Tissues were fixed in 10 ml formalin, 45 ml 80% methanol, 45 ml 80% ethanol, and 5 ml glacial acetic acid, dehydrated, mounted in paraffin, sectioned at 5 μm, and stained with hematoxylin:eosin. Mi² determinations were made under oil immersion evaluating 20 to 25 contiguous but nonoverlapping fields per tumor. Only cells lacking a nuclear membrane and containing clearly identifiable chromosomes were considered as being in mitosis. The data were subjected to χ² analysis.

For the tumor regression studies, tumor volumes were calculated from the 2 perpendicular diameters using the formula

\[ \text{Volume} = d_1 \times d_2 \times d_3 \times 0.5236 \]

where \( d_1 \) was the larger diameter and \( d_2 \) the smaller, since the lesser of the diameters approximated more closely the height of the tumor masses. To validate this procedure, 26 progressively growing tumors in estrogenized nu/nu mice were measured, the animals sacrificed, and the tumors were removed and weighed. The mean of the calculated volumes in cu mm + by tumor weight in mg was 1.075 ± 0.268 (S.D.). Although this variation was quite large, the S.E. of 26 or more tumors would only be in the range of 5%, and no consistent difference in this ratio was found between those tumors weighing less than 100 and those exceeding 200 mg. The geometrical mean of the calculated tumor volumes was used in the study to be reported to lessen the impact of the few large tumors present, and the data were analyzed statistically using the Fisher exact test.

RESULTS

Animals Bearing s.c. 17β-Estradiol Pellets. As recorded in Table 1, 60% of the animals systemically estrogenized developed progressively growing s.c. tumor masses. A less than 100% incidence might have been expected from the small size of the tumor fragments used throughout these experiments, thus increasing the probability of implanting fragments containing an insufficient number of viable neoplastic cells. The success rate probably could have been increased through the use of supravital staining techniques to ensure that viable bits of tumor were always being implanted (4, 10).

At necropsy, tumor masses were recovered from the splenic-pancreatic tissues of 48% of these mice, an incidence not statistically significantly lower than that of growing s.c. implants. From the location of the tumors, it was obvious that a considerable number of the grafted fragments had been extruded from the confines of the spleen to rest either between the spleen and pancreas or entirely within the pancreatic tissue. However, no tumor masses were found in the abdominal cavity outside the area of the spleen and pancreas, i.e., no fragments or free cells had floated about the peritoneal cavity and established visible proliferating foci at the distance from the locale of fragment placement. In one mouse with a growing intrasplenic graft that was extending beyond the spleen capsule, enlarged celiac lymph nodes that had been almost entirely replaced by growing tumor were found, and in other animals, sections of intrapancreatic tumor masses occasionally revealed emboli of tumor cells within lymphatic channels at a distance from the main tumor masses.

Animals with 17β-Estradiol Pellets Placed in the Spleen. At necropsy, no instances were encountered in which vascular adhesions had formed between the spleen and the belly wall that would allow estradiol to enter the peripheral circulation before passage through the liver. As shown in Table 1, 28.6% of the mice had intraabdominal tumor masses that were evident grossly. This incidence was significantly less (p < 0.02) than that observed with the s.c. implantation of fragments of similar size into systemically estrogenized recipients and most probably resulted from failure of the grafts to be retained in close approximation to the estrogen source for a sufficient period after the conclusion of surgery. All 10 of the tumors observed were in close approximation to an estradiol capsule (Figs. 1 and 3). All but one were between 3 and 7 mm in largest diameter and extended beyond the confines of the splenic capsule. The tumor in a single animal exceeded 10 mm in diameter, was entirely outside the spleen and the hormone pellet, which was in contact with the surface of the tumor, and was separated from the free peritoneal space over most of its surface only by a thin connective-tissue membrane. In this situation, the peritoneal fluid would contain a high concentration of estradiol allowing diffusion into the tumor mass over its entire surface, thus permitting it to attain a large size. Since the great majority of peritoneal fluid is reabsorbed through the visceral peritoneum, systemic effects of the estrogen would be minimal, and in this animal the s.c. graft was not evident at the time of necropsy.

Histologically, the tumor masses next to the intrasplenic estradiol pellets were similar to those growing progressively in the subcutis of systemically estrogenized animals. Connective tissue elements were minimal, and the neoplastic cells were large, exhibiting a considerable degree of pleomorphism. As in the intraabdominal tumors of systemically estrogenized animals, a few tumor emboli were seen in lymphatic tissue close to pancreatic extensions of the tumor masses. No enlarged celiac nodes were noted, but these structures were not taken routinely for histological examination.

Careful inspection under × 7 of the tissues in the area of the s.c. grafts failed to reveal evidence of residual tumor in the 20 animals of 5 separate experiments. However, in 8 of 15 mice in the other 3 experiments small circumscribed pearl white bits of tissue were observed. The largest of these was 1.7 mm in diameter, the rest significantly smaller and readily discernible only with magnification. On histological examination, all revealed neoplastic cells embedded in a fibrous connective tissue matrix (Figs. 2 and 4) with an occasional mitotic figure recognizable in the tumor cells. The frequency of these residuals in the latter experiments did not differ statistically from the incidence of progressively growing s.c. tumors in the systemically estrogenized animals in the same experiments, suggesting that the tumor fragments obtained from these 3 donor tumors could maintain themselves but could not grow progressively in the absence of a significantly increased stimulation by estrogenic hormones.

Evaluation of Mitotic Activity. As indicated earlier, histological examination of the small s.c. residuals occurring in 8 animals bearing intrasplenic estradiol pellets revealed the presence of mitotic figures in tumor cells. It was important, therefore, to compare the rate of cell division in these apparently static nodules with that of the expanding s.c. masses in animals that were systemically estrogenized. Five of the residual nodules were...
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Table 1  
Summary of growth of MCF-7 grafts in athymic nude mice depending on location of grafts and placement of estradiol:cholesterol pellet

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>s.c. grafts</th>
<th>Intrasplenic grafts</th>
<th>p s.c. vs. intrasplenic grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>No s.c. residuals</td>
<td>Systemic estradiol (17β-estradiol)</td>
<td>14</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Intrasplenic estradiol</td>
<td>20</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>No estradiol</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>With s.c. residuals</td>
<td>Systemic estradiol</td>
<td>16*</td>
<td>12*</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Intrasplenic estradiol</td>
<td>15</td>
<td>0*</td>
<td>15</td>
</tr>
<tr>
<td>Combined</td>
<td>Systemic estradiol</td>
<td>30</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Intrasplenic estradiol</td>
<td>35</td>
<td>0</td>
<td>35</td>
</tr>
</tbody>
</table>

* NS, not significant.

Table 2  
Comparison of the MI of progressively enlarging s.c. MCF-7 grafts in systemically estrogenized athymic nude mice and s.c. tumor residua in mice bearing i.s. estradiol:cholesterol pellets

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pellet location</th>
<th>No. of tumors</th>
<th>No. of cells counted</th>
<th>MI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>C107</td>
<td>s.c.</td>
<td>4</td>
<td>4736</td>
<td>0.0150</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>i.s.</td>
<td>4</td>
<td>7406</td>
<td>0.0046</td>
<td>0.21</td>
</tr>
<tr>
<td>C95</td>
<td>s.c.</td>
<td>1</td>
<td>1606</td>
<td>0.0199</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>i.s.</td>
<td>1</td>
<td>720</td>
<td>0.0069</td>
<td>0.026</td>
</tr>
<tr>
<td>Combined</td>
<td>s.c.</td>
<td>5</td>
<td>6402</td>
<td>0.0162</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>i.s.</td>
<td>5</td>
<td>8126</td>
<td>0.0048</td>
<td>0.023</td>
</tr>
</tbody>
</table>

* i.s., intrasplenic.

Table 3  
Comparison of the MI of MCF-7 tumor cells next to an i.s. estradiol:cholesterol pellet with those in the same tumor mass opposite the pellet and with those in the s.c. residual of the same animal

<table>
<thead>
<tr>
<th>Animal</th>
<th>Location of tumor cells</th>
<th>No. of cells counted</th>
<th>MI</th>
<th>p of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C95-5</td>
<td>s.c.</td>
<td>720</td>
<td>0.0089</td>
<td>0.0102</td>
</tr>
<tr>
<td></td>
<td>i.s.</td>
<td>1203</td>
<td>0.0224</td>
<td>0.0096</td>
</tr>
<tr>
<td></td>
<td>Next to pellet i.s.</td>
<td>1270</td>
<td>0.0094</td>
<td>0.0028</td>
</tr>
<tr>
<td></td>
<td>Opposite pellet i.s.</td>
<td>2080</td>
<td>0.0082</td>
<td>0.0045</td>
</tr>
<tr>
<td>C107-7</td>
<td>i.s.</td>
<td>1992</td>
<td>0.0181</td>
<td>0.0060</td>
</tr>
<tr>
<td></td>
<td>Next to pellet i.s.</td>
<td>2419</td>
<td>0.0067</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Opposite pellet i.s.</td>
<td>2132</td>
<td>0.0028</td>
<td>0.0014</td>
</tr>
<tr>
<td>C107-6</td>
<td>s.c.</td>
<td>4154</td>
<td>0.0057</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>i.s.</td>
<td>4114</td>
<td>0.0202</td>
<td>0.0001</td>
</tr>
<tr>
<td>Combined</td>
<td>s.c.</td>
<td>4832</td>
<td>0.0084*</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>i.s.</td>
<td>5137</td>
<td>0.0084*</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* i.s., intrasplenic.

large enough to present an adequate number of cells in a single cross-section to obtain a reasonable evaluation of the MI. An equal number of progressively growing tumors was selected from systemically estrogenized hosts in the same experiments for comparison, and the data are presented in Table 2. It is obvious that the percentage of cells undergoing mitosis was significantly less in the small residuals than in the tumors that were growing progressively in systemically estrogenized animals. In both situations, cells with pyknotic nuclei and bare nuclear fragments were numerous, indicating a high rate of cell death. The centers of the growing tumors were routinely composed of connective tissue containing but few recognizable tumor cells.

These data suggested a method of analysis that could be of value in establishing more firmly the gross tumor observations indicating that estradiol exerts its growth-promoting effects by increasing directly the rate of proliferation of MCF-7 cells. According to the thesis upon which these experiments were designed, the effective stimulation from the intrasplenic estradiol pellets depended on diffusion of the estrogen through the extracellular fluid in the immediate vicinity prior to its entry into the hepatic portal circulation. One would not expect this sphere of diffusion to be very large (Ref. 6; Table 3) so that in a tumor >3 mm in diameter those neoplastic cells near the pellet should reside in a milieu of higher estrogen concentration than would those at the opposite pole of the tumor mass. Three animals in which the hormone pellet remained within the spleen had both a small residual s.c. nodule and a considerably larger tumor mass at a distance from the estrogen source. The centers of the growing tumors were routinely composed of connective tissue containing but few recognizable tumor cells.

compared to that seen in the s.c. residua was not statistically significant with the number of cells available for comparison. These data would indicate that the major growth of the intrabdominal tumors was occurring in an area close to the estradiol-containing pellets as a direct result of the high concentration of hormone in this region.
Mitotic Activity in Regressing Tumors. The presence of dividing cells in the residual s.c. nodules of some of the mice bearing pelleted estradiol in the hepatic portal drainage system raised the question as to whether these foci of MCF-7 cells had been maintained as a result of incomplete inactivation of the estrogen in its first passage through the liver or if a low level of proliferation can proceed in the absence of significant amounts of circulating estrogen. To test this, 16 estrogenized castrate male mice were given bilateral implants in the s.c. tissues of the thorax with MCF-7 fragments of slightly larger size, 1 to 2 mm in diameter, from 2 separate donor tumors. Thirty-one of these grafts resulted in progressively growing tumors that were readily measurable 26 days later. Growth was allowed to proceed for another 21 days, at which time the estrogen pellets were removed. As recorded in Chart 1, the size of the tumors decreased slowly over the next 77 days, at which time the calculated mean tumor volume was 41% of that at the time of pellet removal \((p = 0.0005)\). Representative tumors were excised for histological evaluation. Microscopic examination revealed a marked increase in fibrous connective tissue relative to the number of neoplastic epithelial cells present within each tumor mass. The fibrocytes did not appear to be actively proliferating at this time, so that the stroma consisted primarily of dense strands of adult collagen. Additionally, in widely dispersed foci, collections of lymphocytes were evident, but these were not surrounding the clumps of viable-appearing tumor cells. The neoplastic cells themselves were smaller, with considerably less cytoplasm than were those in progressively growing tumors under the influence of estrogen stimulation. Mitotic activity was still evident in this neoplastic epithelium, and as shown in Table 4, the MI in 6 tumors exhibiting different degrees of regression was similar to that found in the nongrowing s.c. residua in animals carrying an intrasplenic estradiol pellet. Ten tumors were observed for an additional 18 weeks, i.e., a total period of 7 months in the absence of estradiol stimulation, without further diminution in size or instances of regrowth.

DISCUSSION

In these experiments, tumor growth was sustained only when MCF-7 cells were in a milieu containing estrogenically active steroid. It is difficult to formulate a scenario in which the estradiol was causing the production by host cells of some active sub-

stance that then entered the circulation to stimulate proliferation of MCF-7 cells. It is unlikely that hepatic production of such a factor \((12)\) is involved in this growth process. As is evident in Fig. 3, very little tissue separated the intrasplenic estradiol:cholesterol pellets from the most actively growing portion of the adjoining tumor masses. This thin tissue membrane was composed of a row of large macrophagic cells supported by a very thin layer of fibrous connective tissue. It seems logical to conclude, therefore, that the growth-promoting effects of the estrogen resulted from an action of estradiol directly on the MCF-7 cells mediated through the estrogen receptor system they contain \((3, 5)\), although the possible importance of the stroma in this response is not to be overlooked. The greater MI found in tumor tissues residing in a milieu of higher estradiol concentrations indicates that the resultant increase in tumor mass was effected primarily through an increased rate of tumor cell proliferation, although some diminution in the microscopically apparent high rate of tumor cell death cannot be ruled out. That estrogens stimulate directly growth of MCF-7 cells in vivo does not alter the fact that the growth of cells in the presence of estrogen can be enhanced by other hormones, e.g., those of pancreatic and hypophysal origin \((15, 16, 21)\). It is well to point out, however, that administered estrogen has been shown to produce a rate of growth of this tumor implanted in hypophysectomized athymic mice quite comparable to that seen in ovariec-
tomized mice receiving the same amount of estradiol \((16)\).

In the experiments reported here, both a decrease in and stasis of tumor size occurred in the face of active tumor cell proliferation. This is in contrast to the situation recently detailed in estrogen-induced Leydig cell tumors in mice \((7)\). In that model, regression of estrogen-dependent tumors following hormone deprivation was associated with a disappearance of mitotic activity and led to a state of true dormancy in which nonproliferating neoplastic cells remained at the tumor site to be reactivated quickly when estrogen treatment was reinstituted. Which situation pertains in cases of breast cancer in patients where tumor regressions have been produced in response to hormone withdrawal has not been established. The sustained regression of progressively enlarging MCF-7 tumors following removal of the estrogen source recorded here was not observed in an earlier report from another laboratory \((17)\). This could well represent variations in the cell population of MCF-7 cells maintained in different laboratories as suggested by the variance reported in their in vitro response to added estradiol.

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**Table 4**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>% of decrease in calculated volume</th>
<th>No. of cells counted</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C117-8, Left</td>
<td>0</td>
<td>2890</td>
<td>0.0080</td>
</tr>
<tr>
<td>C118-5, Left</td>
<td>16</td>
<td>1314</td>
<td>0.0061</td>
</tr>
<tr>
<td>C118-4, Left</td>
<td>54</td>
<td>1533</td>
<td>0.0033</td>
</tr>
<tr>
<td>C117-9, Left</td>
<td>56</td>
<td>1172</td>
<td>0.0051</td>
</tr>
<tr>
<td>C117-14, Right</td>
<td>59</td>
<td>1432</td>
<td>0.0042</td>
</tr>
<tr>
<td>C117-12, Left</td>
<td>82</td>
<td>1421</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

**Chart 1.** Growth of 31 MCF-7 tumor grafts under the influence of estradiol stimulation for 67 days and their regression over 77 days after estrogen withdrawal. \(\oplus\) estradiol pellets removed.
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Fig. 1. Section through an intrasplenic estradiol:cholesterol pellet (EP) with actively proliferating MCF-7 tumor (Tu), at one end extending through the splenic (Sp)
capsule and protruding into pancreatic tissues (Pa), × 30.

Fig. 2. A s.c. MCF-7 residuum in the same animal, C107-7, illustrated in Fig. 1. This was the largest of 8 s.c. residua found in animals with an intrasplenic
estradiol:cholesterol pellet. × 30.

Fig. 3. Higher magnification of tumor illustrated in Fig. 1. The clear area to the right is space occupied by the estradiol:cholesterol pellet (EP) before being dissolved
during histological preparation. Note the rim of macrophagic cells (Ma) supported by a thin layer of fibrous connective tissue separating the pellet from actively proliferating
MCF-7 cells. Mi, mitotic figure, × 500.

Fig. 4. Higher magnification of residual tumor shown in Fig. 2. Note relative larger amounts of connective tissue stroma and lesser amounts of cytoplasm about the
tumor nuclei as compared to those in Fig. 3. A mitotic figure (Mi) is shown quite clearly. × 500.
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