Growth Potentials of Precancer of the Cervix Uteri

in Vitro and in Cortisone-treated Hamsters*

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WITH THE TECHNICAL ASSISTANCE OF ANNE-MARIE MALMBERG

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SUMMARY

In a series of 22 cases of invasive carcinoma, nine cases of carcinoma in situ, and seven cases of dysplasia of the uterine cervix, the pathological epithelium and corresponding normal epithelium were studied in vitro and after transplantation to the cheek pouch of cortisone-treated hamsters.

Regardless of the histological malignancy grade, the invasive cancer epithelium revealed a greater growth potential than the corresponding normal epithelium in vitro as well as in hamster.

Carcinoma in situ differed from invasive carcinoma in having a mean growth rate which was no higher than that of the normal epithelium from the same patients in vitro and very probably in hamsters. Very probably carcinoma in situ had a lower growth rate than invasive carcinoma when cultivated for prolonged periods in vitro and in hamsters. In hamsters, and essentially in vitro, the dysplasias agreed with carcinoma in situ, even if they deviated less from the invasive cancers.

The morphological differences between cancer and the precancerous changes are described.

These findings seem to indicate that carcinoma in situ continues to grow slowly because of inherent epithelial qualities.

That the prodromal phase of invasive carcinoma of the uterine cervix may proceed through various stages of epithelial atypia—dysplasia and carcinoma in situ—is agreed. However, it remains unsettled whether carcinoma in situ is a true carcinoma, despite the increasingly widespread adoption of a rigorous morphological classification (5, 8, 27, 38, 42, 49, 57, 68). Arguments purporting to prove that the lesion will eventually assume invasive properties have been advanced (2, 18, 19, 22, 30, 31, 32, 38, 55, 60), as have authenticated cases in which the lesion has remained stationary for years or healed spontaneously (7, 9, 11, 33, 35, 36, 37, 41, 50, 51, 61, 69). Clinical follow-ups alone are unlikely to supply a definite answer: the cytological smear does not reliably differentiate carcinoma in situ from mild atypias (10, 34, 46, 56, 57, 71), and many a lesion has simply been excised with the biopsy specimen (1, 29, 30, 62).

Attempts to estimate the growth potentials of carcinoma in situ and of cervical dysplasias have been made by explanting the abnormal epithelium to another milieu where a possible environmental resistance of the original host would be eliminated. However, although such trials in vitro have yielded inconclusive results (cf. below), precancerous tissue transplants have failed to grow in the anterior chamber of a heterologous eye (20, 25, 26). Against this background we deemed it interesting to study the growth in vitro and in cortisone-treated golden hamsters of epithelial transplants from a series of patients with invasive carcinoma, carcinoma in situ, and dysplasias of the cervix.

MATERIALS AND METHODS

Specimens were obtained from an original series of 47 patients with lesions of the uterine cervix.
However, only those cases were accepted in which growth was shown by at least two uninfected explants in vitro or transplants to hamster of both abnormal and normal tissue. Hence, six patients with carcinoma, one with carcinoma in situ, and two with dysplasia were subsequently rejected. The remainder comprised 22 cases of carcinoma (fourteen grown in vitro, eighteen in hamster), nine cases of carcinoma in situ (nine grown in vitro, seven in hamster), and seven cases of dysplasia (seven grown in vitro, six in hamster).

A punch-biopsy specimen of abnormal tissue and another of normal portio tissue were obtained from each patient. The specimens were immediately washed in Hanks BSS, containing 67 µg streptomycin sulfate and 64 µg benzyl-penicillin.

**TABLE 1**

**FLUID MEDIUM, MODIFIED FROM EAGLE (4)**

<table>
<thead>
<tr>
<th>Component</th>
<th>(ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>40</td>
</tr>
<tr>
<td>Hanks BSS</td>
<td>90</td>
</tr>
<tr>
<td>Antibiotics (streptomycin sulfate, 20 mg/ml + benzyl-penicillin, 4.8 mg/ml)</td>
<td>2</td>
</tr>
<tr>
<td>Vitamin stock solution, Eagle</td>
<td>1.5</td>
</tr>
<tr>
<td>Amino acids stock solution, Eagle</td>
<td>1.5</td>
</tr>
<tr>
<td>L-Glutamine, 900 mM stock solution, Eagle</td>
<td>1.5</td>
</tr>
<tr>
<td>Sodium bicarbonate, 1.4%, to pH 7.5</td>
<td>17 (approx.)</td>
</tr>
</tbody>
</table>

Human cord serum was used whenever available. From time to time pooled human cord and human adult serum were substituted, and occasionally human adult serum only, pooled from at least two donors, had to be accepted. Great care was taken to ensure that the same patient's abnormal and normal tissues received identical media.


per ml. and divided into pieces of ca. 50—75 mg each. Every piece was divided in two, one-half being examined histologically and the other used for the growth experiments.

The rigid criteria recommended by Wheeler and Hertig (70) and Peale (49) were applied in the histological classification of carcinoma in situ. Thus, only cases with obvious confinement of the lesion to natural surfaces but with complete loss of stratification and cellular polarity throughout the entire thickness of the epithelium, crowding of cells with large hyperchromatic nuclei, pleomorphia, and mitoses were accepted (Fig. 1). Borderline cases (especially such with surface stratification or partly lacking hyperchromasia) and slighter epithelial atypias were denoted dysplasias (Fig. 2).

Tissue cultivation technique.—The tissue pieces were freed from manifest connective tissue and cut into approximately 2 cu. mm. cubes. About 50 cubes per biopsy specimen were explanted in roller tubes, both on rat collagen (Ehrmann and Gey [6]), and with chicken plasma and chicken EE 50 on glass. Two ml. of fluid medium (Table 1) was added, and the tubes were kept immobile at 37°C. The medium was changed whenever the pH fell to 7.0.

Epithelial growth rate was estimated as follows. Every 2 or 3 days during 30 days the explants were examined, and the number of cells per colony were assessed by comparison with a set of nine reference colonies with known cell counts of from 36 to 47,000 in logarithmic progression. The cell number specified for each biopsy is the average of the numbers for the two colonies which on that particular occasion were largest.

The reference colonies had been selected from a series of cancer explants fixed after increasing lengths of time. The surface area of each explant's colony was found by measuring two perpendicular axes in colony and tissue, both elliptic by assumption. The total number of cells in the monolayer colony was computed from cell counts in four apparently representative, high-power fields of known area. This may seem rather a crude method, yet the scale intervals were small compared with the usually very large interindividual variation. Moreover, when the same patient's abnormal and normal epithelium differed little, the cell counts for the colonies in question were obtained in the same way as the reference colonies. The method was found to have a practical upper limit of some 50,000 cells. In such tubes a good deal of the glass surface was usually occupied, and the colonies were coalescent or splitting up. The rare tubes in which fibroblasts interfered with epithelial growth were discarded.

Morphological observations by phase-contrast microscopy were made on a separate series of explants on rat collagen in Carrel flasks.

Transplantation to hamsters.—Altogether 115 golden hamsters weighing 40—60 gm. were used for 31 patients. The transplant—three or four pieces of about 1 cu. mm.—was introduced into the cheek pouch with a trocar No. 16, the operative technic and cortisone treatment being that recommended by Handler (28). Transplants from each patient were distributed thus: abnormal tissue to both sides of one or two hamsters, normal tissue to both sides of one or two hamsters, and abnormal tissue to the left and normal tissue to the right cheek pouch of up to three hamsters.

The size of the growing nodules was measured every 2 or 3 days under Nembutal anesthesia. When the nodules no longer showed signs of growth, the cheek pouch containing the transplant was excised, fixed in formalin, and examined histologically.
RESULTS

Tissue Cultures

The results of cultivation on rat collagen and in chicken plasma were fundamentally similar. However, since 72 per cent of the explants on rat collagen grew somewhat faster and more regularly than those in chicken plasma (cf. Ehrmann and Gey [6]), the following will be confined to cultures on rat collagen.

After one or two hamster passages 36 transplants were explanted in vitro. Verifying previous observations (12, 14, 66), 22 of them grew faster than before the hamster passage. The relation between the same patient's abnormal and normal epithelium was maintained.

Carcinoma.—The mean growth rate of abnormal epithelium was higher than that of the same patient's normal epithelium (Chart 1, Table 2). In similarity to Fjelde (12), we noted no correlation between the histological malignant grade of the carcinoma and its rate of growth.

Morphologically, the carcinomatous epithelium was characterized by the well known pleomorphism; most of the normal epithelia maintained monomorphic cytology throughout the first month (Figs. 3, 4).

Carcinoma in situ.—Abnormal and normal epithelia from the same patients had the same mean growth rates. The mean growth curves are coincident during the 1st week, but thereafter normal epithelium tends to take the lead (Chart 2, Tables 2 and 3). The dissimilarity between carcinoma in situ and carcinoma in this respect is clearly illustrated by application of the $\chi^2$ test. Thus, after 7 days in vitro the abnormal epithelium was more abundant than the same patient's normal epithelium in two of nine carcinoma-in situ cases and in twelve of fourteen carcinoma cases ($P < 0.01$).

<table>
<thead>
<tr>
<th>癌 cases</th>
<th>C. in situ cases</th>
<th>Dysplasia cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>C 2</td>
<td>4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>C 3</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>C 4</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>C 5</td>
<td>3.3</td>
<td>1.8</td>
</tr>
<tr>
<td>C 6</td>
<td>4.4</td>
<td>1.8</td>
</tr>
<tr>
<td>C 7</td>
<td>2.9</td>
<td>1.0</td>
</tr>
<tr>
<td>C 8</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>C 9</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>C10</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>C11</td>
<td>1.6</td>
<td>0.7</td>
</tr>
<tr>
<td>C12</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>C13</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>C14</td>
<td>3.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* See Materials and Methods, tissue cultivation technics, for method of estimation of cell number.
and after 30 days the same applied to none of eight carcinoma-in-situ cases and to ten of fourteen carcinoma cases (P ≈ 0.002).

Morphologically, the carcinoma-in-situ epithelium in vitro closely resembled the normal epithelium, although that from occasional patients exhibited a characteristic pointed cell form and slight pleomorphia (Figs. 5–8).

Dysplasia.—The abnormal epithelium in this group had a growth rate and morphology similar to those in carcinoma in situ, especially after a long time in vitro. No convincing mean growth rate difference could be found between dysplastic and normal epithelium from the same patients.

After 30 days the dysplastic epithelium was more abundant than the same patient's normal epithelium in but one of seven cases against, as mentioned, ten of fourteen carcinoma cases (χ² test: P < 0.01). The corresponding difference after shorter periods was insignificant, and individual variations were large (Table 2).

**ABSOLUTE GROWTH RATES—INTER-GROUP DIFFERENCES**

During the first weeks in vitro the normal epithelium from patients with carcinoma in situ if anything tended to grow faster than the normal epithelium from patients with carcinoma (Table 2: Logarithms of cell numbers differ by 0.58 ± 0.39, P < 0.2). The reason might be age (23), the average age for the two groups being 39 years and 47 years, respectively.

The growth potential of the carcinomatous epithelium was nevertheless greater than that of the carcinoma-in-situ epithelium. Although the growth rate difference was merely hinted after 7 days in vitro (Table 2: Wilcoxon [72] and χ² tests yield P ≈ 0.05), it had become significant after 30 days (Table 3: 1.88 ± 0.71, P < 0.014). After the same length of time the carcinomatous epithelium was more abundant on the average than the abnormal epithelium from the pooled carcinoma in situ and dysplasias group (Table 3: 1.76 ± 0.62, P < 0.01).

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**TABLE 3**

**LOGARITHM OF NUMBER OF CELLS ON THE 30TH DAY IN VITRO***

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Cancer cases</th>
<th>Ca. in situ cases</th>
<th>Dysplasia cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1</td>
<td>2.4</td>
<td>0</td>
<td>2.4</td>
</tr>
<tr>
<td>C 2</td>
<td>4.7</td>
<td>0</td>
<td>4.7</td>
</tr>
<tr>
<td>C 3</td>
<td>4.7</td>
<td>4.5</td>
<td>0.2</td>
</tr>
<tr>
<td>C 4</td>
<td>4.7</td>
<td>1.2</td>
<td>3.5</td>
</tr>
<tr>
<td>C 5</td>
<td>3.0</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>C 6</td>
<td>4.0</td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>C 7</td>
<td>4.0</td>
<td>8.1</td>
<td>4.1</td>
</tr>
<tr>
<td>C 8</td>
<td>3.9</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>C 9</td>
<td>0.2</td>
<td>1.6</td>
<td>—1.4</td>
</tr>
<tr>
<td>C 10</td>
<td>0.9</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>C 11</td>
<td>1.6</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>C 12</td>
<td>4.7</td>
<td>4.7</td>
<td>0</td>
</tr>
<tr>
<td>C 13</td>
<td>3.2</td>
<td>3.6</td>
<td>—0.4</td>
</tr>
<tr>
<td>C 14</td>
<td>4.7</td>
<td>0</td>
<td>4.7</td>
</tr>
<tr>
<td>Mean</td>
<td>3.84</td>
<td>1.94</td>
<td>1.90</td>
</tr>
<tr>
<td>± st. err.</td>
<td>±0.41</td>
<td>±0.50</td>
<td>±0.50</td>
</tr>
</tbody>
</table>

* See footnote *, Table 2.
† Lost on the 10th day.

* Difference between pathological epithelium of cancer and pathological epithelium of cancer in situ cases: 1.88 ± 0.71.
Difference between pathological epithelium of cancer cases and pathological epithelium of combined cancer in situ and dysplasia cases: 1.73 ± 0.62.
Transplants to Hamster

Among 230 cheek pouches with transplants, a single nodule had grown in 199, two or more nodules of similar volume in twelve, two or more nodules of very unequal volume in three, and apparently none at all in sixteen. Because the larger nodules from the same biopsy specimen usually had similar growth rates but any smaller ones very unequal growth rates, we considered it appropriate to express the growth rate of a specimen in terms of the largest nodule's volume divided by the time to reach maximum.

We were unable to discern any differences between the results of transplantation of the same patient's abnormal and normal tissues to the same hamster and the results of transplantation of abnormal and normal tissues to separate hamsters.

Carcinoma.—The carcinomatous epithelium grew faster than the corresponding normal epithelium from twelve of eighteen patients, the growths of carcinomatous and normal epithelia from the remaining six patients being nearly equal. The mean growth to maximum was 4.6 cu mm/day for carcinoma and 1.2 cu mm/day for normal epithelium ($\chi^2$ test for difference: $P = 0.09$).

The growth rates of the same patient's explants in vitro and transplants to hamster seemed uncorrelated.

Carcinoma in situ and dysplasias.—These groups were indistinguishable in hamster. The mean growth to maximum for the pooled groups was 1.6 cu mm/day for the abnormal epithelium compared with 1.5 cu mm/day for the normal epithelium, the greater growth rate being shown by abnormal epithelium from six and by normal epithelium from seven of the thirteen patients. The growth rates in hamster of the same patient's abnormal and normal epithelia were faintly correlated ($\chi^2$ test: $P < 0.1$), but neither of them seemed individually correlated with the corresponding growth rates in vitro.

In hamsters, too, there was an indicated (though statistically unverifiable) mean growth rate difference between normal epithelium from patients with carcinoma (1.2 cu mm/day; average age, 47) and normal epithelium from patients with carcinoma in situ or dysplasia (1.5 cu mm/day; average age, 38). Abnormal epithelium from patients with carcinoma grew faster than abnormal epithelium from patients with carcinoma in situ or dysplasia (eight of eighteen versus one of thirteen with growth exceeding 1.8 cu mm/day; $\chi^2$ test: $P < 0.05$).

Morphologically, the carcinomatous transplants, at the time of maximum volume, took the form of a large, solid epithelial mass, as a rule clearly pleomorphic and with considerable central necrosis. Sometimes the borderline towards the host's connective tissue was unsharp, but manifest invasion was lacking. Transplants of normal, dysplastic, and carcinoma-in situ epithelia formed cysts, as a rule small and covered with sparse epithelium which was monomorphic or only slightly pleomorphic. The borderline was always distinct (Figs. 9–12).

The growth times from implantation to maximum volume ranged from 4 to 16 days and were apparently dispersed normally about a mean of 8.5 days, a little on the short side compared with other workers' findings (13, 28, 40, 47, 48, 65). Having checked our procedure—wherein we had the pleasure of collaborating with Handler—we can only explain our short time to maximum in terms of the characteristics of our hamster strain (from Mr. Have, SØsum, Denmark).

The mean time from implantation to maximum volume of the transplants was almost the same for abnormal and normal epithelium from the same patients and remarkably independent of the type of abnormality. Hence, the time to maximum would seem to be determined mainly by the host's characteristics.

Discussion

Our finding that cervical carcinoma, regardless of its histologic malignancy grade, grows faster in vitro than the same patient's normal epithelium, merely bears out previous reports (12, 14, 15, 16, 17, 43, 45). That this difference applies to corresponding transplants in cortisone-treated hamster, too, is also in agreement with previous experiences of the same technic though with different cell types (44, 47, 65, 66). Even in continuous strains, as shown by Foley and Handler (13), cells of malignant origin maintain greater growth potential than do those of benign origin when implanted in the cheek pouch of hamster.

Carcinoma in situ differed sharply from invasive carcinoma in having the same mean growth rate as the normal epithelium from the same patients in vitro, and very probably in cortisone-treated hamsters also. The growth rate of carcinoma in situ very probably was lower than that of invasive carcinoma, when cultivated for prolonged periods in vitro as well as in cortisone-treated hamsters. Though deviating less markedly from carcinoma, dysplasias exhibited a behavior resembling that of carcinoma in situ in hamsters and, with minor aberrations, in vitro.

Glatthaar (18, 19, 21) and Bromelow et al. (2, 3), using cover-glass methods, found no difference between invasive carcinoma and carcinoma in situ. However, they estimated the absolute growth rate
after very short observation times and apparently used abnormal and normal epithelia from different patients. Notably, too, although Glatthaar could see no growth whatsoever of normal portio epithelium, Bromelow et al. reported equal growth rates for embryonic and adult, normal portio epithelium. In these circumstances their findings are difficult to evaluate. Conceivably, one of the prerequisites for the appearance of perceptible differences between carcinoma and carcinoma in situ is a relatively long observation period.

Grand (24) used similar methods but longer observation times (4–5 weeks) for parallel studies on cytological smears and tissue cultures. In tissue cultures with a matching cytological smear showing “cells of the precancer type,” Grand found marked activity of tissue growth that was midway between normal epithelium and carcinoma. Similarly, epithelium cytologically judged as carcinoma in situ grew in vitro only slightly more slowly than did clinical carcinoma. However, if allowance is made for the facts that his classifications were based on smear patterns—whose agreement with the histological stages seem hardly exact (7, 34, 46, 71)—and that he chose normal tissue from other than the pathological cases, then Grand’s findings coincide relatively well with Gey’s and ours.

Gey (14) appears to be the only previous worker who has compared the growths in vitro of cervical carcinoma in situ and of invasive carcinoma with that of normal epithelium from the same patients. Attempting to establish continuous strains of normal and abnormal portio epithelium in roller tubes, he set up initial cultures in clotted substrate (40 per cent chicken plasma, 10 per cent bovine embryo extract, 50 per cent human cord serum; fluid phase, 50 per cent human placental serum) with explants from a series of cancers and also from three cases of intraepithelial carcinoma of the cervix, two of them with early invasion. In the latter three cases both normal and abnormal epithelium reportedly had the same growth response, slower than that of most invasive cancers, for 129 to 244 days with one or two transfers. Our findings are compatible with Gey’s, also insofar as many of our invasive carcinomas maintained their considerable growth potential for prolonged periods whereas but one of our carcinoma-in-situ epithelia kept on growing for more than 4 months.

A source of error in such trials has been debated in connection with Bromelow’s (2) work—namely, unequal amounts of epithelium in the primary explants. Any differences might give carcinoma an advantage over other tissues and, perhaps, normal tissue an advantage over carcinoma in situ or dysplasia, lesions often occupying only a small fraction of the sample. This source of error, however, cannot have had great importance. When classifying our carcinoma in situ and dysplasia cases into cases with great or sparse pathological epithelium in the histological slides, we found no variation related to the growth rate; both categories also had the same correlation with the growth of normal epithelium from the same patients.

Where carcinoma in situ transforms to invasive cancer, this usually takes place only after a prolonged, clinically stationary phase (9, 70). The transformation period itself is perhaps relatively short, because those few cases of carcinoma in situ with early invasive changes which have been subjected to detailed histological description as a rule showed a great range of the process and almost always multiple foci of invasion (7, 11, 64). The chance of catching a case in the progressive phase during an investigation such as ours may therefore be small. In just one case—not included in the data for this paper, since the normal epithelium was infected and failed to grow—we have seen carcinoma in situ grow relatively fast and continuously after as long as 6 months in vitro. It is questionable, however, whether this case would represent carcinoma in situ of an especially proliferative phase or type. Histologically, clinically, or cytologically it did not deviate from the rest. Finally, Gey’s results indicate that carcinoma in situ at the time of early invasion still maintains a low growth potential in vitro. Fisher and Boyes are therefore probably right when on histological grounds they assume that “the first invasive foci are also restrained” (11).

It is therefore probable that carcinoma in situ continues to grow slowly because of inherent qualities of the epithelium rather than because of any host resistance.

ACKNOWLEDGMENTS

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Fig. 7.—Case S 8. Carcinoma in situ from a woman of 37. Tissue culture after 12 days on rat collagen in a Carrel flask: characteristic, pointed, slightly irregular epithelium in loose sheet. Phase contrast, X100.

Fig. 8.—Normal epithelium of the portio from the same patient as in Fig. 7. Tissue culture after 12 days on rat collagen in a Carrel flask: regular epithelium in dense sheet. Phase contrast. X100.

Fig. 9.—Case C 15. Moderately differentiated invasive epidermoid carcinoma of the cervix in a woman of 36, 12 days after implantation in the cheek pouch of a hamster: great epithelial mass with central necrosis. van Gieson, X25.

Fig. 10.—Detail of Fig. 9. Pleomorphic epithelium with unsharp borderline. van Gieson, X100.

Fig. 11.—Case D 2. Slight dysplasia of the portio in a woman of 46, 10 days after implantation in the cheek pouch of a hamster: scanty epithelium has formed small cysts. van Gieson, X25.

Fig. 12.—Detail of Fig. 11. The scanty epithelium is fairly uniform with distinct borderline. van Gieson, X100.
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