Growth-promoting Activity of Acid Mucopolysaccharides on a Strain of Human Mammary Carcinoma Cells*

Luciano Ozzello, Etienne Y. Lasfargues, and Margaret R. Murray

(Departments of Surgery and Microbiology, College of Physicians and Surgeons, Columbia University, New York, N.Y.)

SUMMARY

A strain of pure epithelium from a human duct cell carcinoma of the breast (BT 20) can be maintained in good condition when the medium is supplemented by umbilical cord extract (CE).

CE can be entirely replaced by hyaluronate, partially by chondroitinsulfate C and not at all by the enzymatic digests of hyaluronate or by chick embryo extract. A polyvinylpyrrolidone of high molecular weight comparable to that of hyaluronate (K-90; av. mol. wt., 360,000) can partially replace CE in the maintenance of BT 20 cells.

It is suggested that the action of acid mucopolysaccharides on BT 20 cells is not of a nutritional, but of a physical or physico-chemical, nature and is exerted at the cell membrane. Polymerization in the neighborhood of mol. wt. 350,000 is most favorable, or essential, for this action, which is probably concerned with water and electrolyte exchanges between cells and medium and/or reciprocal electrical influences between cell surfaces and polyelectrolytes. A parallelism is suggested between the growth requirements of these human mammary carcinoma cells and the existence of large amounts of acid mucopolysaccharides in the ground substance of mammary carcinoma.

In March, 1958, a strain of neoplastic epithelial cells (BT 20) was isolated from a human mammary carcinoma (11). This strain has been characterized by a requirement for saline extract of human umbilical cord (CE) as a supplement to the standard culture medium.

Three chief considerations led us to expect that the virtue of the cord extract for this tissue might reside in its acid mucopolysaccharide content:

1. Wharton's jelly (mucoid connective tissue of umbilical cord) contains large amounts of hyaluronic acid and smaller amounts of chondroitin-sulfate C (18).

2. The stromal ground substance of human tumors is rich in acid mucopolysaccharides, which in breast carcinoma appear to be represented chiefly by hyaluronate and chondroitinsulfate C (14).

3. It has been reported (10) that saline extract of umbilical cord enhances for a limited time the growth of embryonic chick fibroblasts, through the action of hyaluronate.

The present experiments were undertaken to test the above hypothesis and to characterize chemically as far as possible the active factor in cord extract which contributes to the growth of this mammary carcinoma strain.

MATERIALS AND METHODS

The BT 20 strain was derived from an "infiltrating duct cell carcinoma of no special type" obtained at operation from a human female mammary gland. The strain is now 19 months old and in its 24th passage.

The cells were grown on glass in stationary tubes and were transferred at 3-week intervals (11) with the following feeding solution, which hereafter will be referred to as "standard feeding solution":

Received for publication November 24, 1959.

* This investigation has been supported by Grant No. C-5850 of the National Cancer Institute, U.S. Public Health Service. Presented in part at the 50th Annual Meeting of the American Association for Cancer Research, Atlantic City, April 10-12, 1959.

600
Eagle’s medium (without glutamine) 27.5%
Amniotic fluid 27.5%
Human placental serum 25.0%
Cord extract (CE) 10.0%
Antibiotic solution (Penicillin 10,000 U, Neomycin 2,500 γ/ml) 10.0%

The cord extract was prepared as previously described by Lasfargues et al. (10). The method consisted essentially in homogenizing human umbilical cords in an equal volume of balanced saline solution (BSS) and centrifuging at 4,000 r.p.m. for 30 minutes. The supernatant represented the cord extract, which was added to the culture medium in the optimal proportion of 10 per cent.

Controls consisted of:
1. Tubes maintained on the standard feeding solution, containing 10 per cent CE.
2. Tubes from whose feeding solution CE was simply omitted.
3. Tubes in whose feeding solution CE was replaced with embryonic extract (EE, extract made from equal parts of pulp of 9-day chick embryos and BSS).

Experimental materials used to replace the cord extract in the standard feeding solution, are listed below:
1. Hyaluronate (av. mol. wt., 350,000)
2. Tetrasaccharides from hyaluronate digests (mol. wt., 750)
3. N-acetylglucosamine (mol. wt., 180)
4. Glucuronolactone (mol. wt., 195)
5. Chondroitin sulfate C (av. mol. wt., 40,000)
6. Three polyvinylpyrrolidones (PVP): PVP K-30 (Plasdone C, av. mol. wt., 40,000), PVP K-60 (av. mol. wt., 160,000), PVP K-90 (av. mol. wt., 360,000).

For the acid mucopolysaccharides and their enzymatic digests, we are indebted to Dr. K. Meyer of the Departments of Biochemistry and Medicine in whose laboratory they were extracted and purified from human umbilical cords and from human neoplasms. The polyvinylpyrrolidones were prepared by the General Aniline and Film Corporation and were kindly supplied to us by Antara Chemicals, New York.

Growth measurement.—Control cultures in medium containing CE grew in epithelial sheets which, by the 5th day after transfer, lined the walls of the tube almost continuously. They multiplied rapidly, showing a very constant growth increment and growth pattern up to 3 weeks, by which time the cell population had increased to such an extent that it could be transferred into six new tubes.

Taking this as the norm, standard or optimal growth was graded as ++++ (Fig. 1); complete failure of growth was graded 0. Intermediate degrees in rate of multiplication and morphological achievement were given intermediate + grades. The observations and estimates were made by one observer and checked by another.

RESULTS

Controls.—The optimal supplement of CE (10 per cent) was used routinely. However, good growth could be obtained for a limited period at lower concentrations. In 1 per cent CE, a 4+ growth was obtained initially, but after a few weeks the cells began to decrease in growth rate and show cytoplasmic granularity. They could be completely restored at this stage by being returned to the standard medium. Even at a CE concentration of 0.1 per cent a 3+ growth might be obtained from newly transferred cells; this was maintained through only a few transfers.

Total deprivation of CE resulted in gradual deterioration, terminated by death of the cultures in the 4th or 5th week. In the first few days after transfer the cells spread out in sheets of a limited extent, which did not become completely confluent. Degenerative changes soon occurred: cells became vacuolized and rounded up, eventually aggregating in clusters, and departing entirely from sheet formation.2

When EE (BSS extract of 9-day chick embryos) is substituted for CE as 10 per cent of the medium, deterioration follows the same course but is more rapid, terminating in cell death in about 2 weeks (Fig. 2).

Experimental.—As shown in Table 1, hyaluronate in an equivalent concentration might be substituted for the whole cord extract. The ratio of hyaluronate in the CE supplement to whole medium was calculated as 6 mg/ml. At this concentration a 4+ growth was maintained (Fig. 3). At lower concentrations (3 mg., 0.3 mg., and 0.03 mg/ml), cell behavior paralleled that in reduced concentrations of whole cord extract. No difference in activity was detectable between hyaluronate.

1 Lately we have been able to omit the amniotic fluid from the medium, replacing it with an equal amount of Eagle’s medium.

2 In the last 2 months the controls deprived of CE have exhibited longer survivals, especially after repeated transfers. These cells grown in the absence of CE, however, present significant cytoplasmic granularity and a slower growth rate than those supplemented by cord extract.
nate extracted from human umbilical cord and that from human neoplasms.

Table 1 also summarizes results when CE was replaced by enzymatic digests of hyaluronate. Almost no growth was obtained when tetrasaccharides and monosaccharides were used singly, in weights equivalent to the optimal amount of hyaluronate. Only a minimal and transient growth was observed in cultures fed with both monosaccharides, N-acetylglucosamine and glucuronolactone, together.

The replacement of CE by chondroitinsulfate C was followed by a 3+ growth when this mucopolysaccharide was present at 0.03 mg/ml (Fig. 4). Growth was poorer at both higher and lower concentrations (Table 1). Administration of hyaluronate and chondroitinsulfate C simultaneously resulted in 4+ growth.

Effects of three polyvinylpyrrolidones, substituted for CE, varied somewhat with the molecular weights of these polymers (Table 2). When PVP K-90, of average molecular weight 360,000, was used at 6 mg/ml for replacement, its concentration in the whole medium represented a molarity of $1.66 \times 10^{-6}$, which was very close to that of hyaluronate in the same circumstances ($1.71 \times 10^{-6}$).

In the first few days after transfer, the best growth increment was seen in cultures supplemented with K-30. However, by the end of a week the cultures supplemented with K-90 showed a progressive improvement, whereas those fed with K-80 and K-30 remained stationary. From the 3d week on, cultures fed with K-90 resembled the controls in CE, but without ever exceeding a 3+ grade. With K-60, growth was maintained at a 2+ to 3+ level, whereas with K-90 it did not exceed 2+. Indeed, after the initial growth stimulation that followed transfer, the K-30 tubes regressed to a growth level comparable to that of controls from whose medium CE was omitted altogether. The growth of all tubes fed with PVP improved immediately after transfer, but the cells consistently showed a cytoplasmic granularity and, to a lesser extent, vacuolization, which was absent from optimally maintained controls. After repeated transfers the cultures supplemented with K-90 were almost indistinguishable from those fed with CE, but the growth rate of the former still appeared somewhat slower than that of the latter.

**DISCUSSION**

The BT 20 strain is purely epithelial, as determined by continuous observation. No fibroblasts or other cell types have ever been seen to contaminate these cultures. The close dependence of BT 20 cells on CE is all the more interesting if one considers that non-neoplastic epithelial cells iso-

### TABLE 1

**GROWTH OF BT 20 CELLS SUPPLEMENTED BY ACID MUCOPOLYSACCHARIDES**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration/ ml of medium</th>
<th>Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cord extract</td>
<td>10%</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>0.1%</td>
<td>++++</td>
</tr>
<tr>
<td>2. Hyaluronate</td>
<td>6 mg.</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>0.3 mg.</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>0.03 mg.</td>
<td>++++</td>
</tr>
<tr>
<td>3. Tetrasaccharides</td>
<td>6 mg.</td>
<td>0</td>
</tr>
<tr>
<td>4. Glucuronolactone</td>
<td>6 mg.</td>
<td>0</td>
</tr>
<tr>
<td>5. N-acetylglucosamine</td>
<td>6 mg.</td>
<td>0</td>
</tr>
<tr>
<td>6. Glucuronolactone</td>
<td>3 mg.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>[N-acetylglucosamine]</td>
<td>0</td>
</tr>
<tr>
<td>7. Chondroitinsulfate C</td>
<td>0.3 mg.</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.03 mg.</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.003 mg.</td>
<td>++</td>
</tr>
</tbody>
</table>

* Optimal growth is graded ++ , whereas complete failure of growth is graded 0. Intermediate + grades are given to intermediate degrees in rate of multiplication and morphological achievement of the cultures. These results are persistent over a period of months except for the growth in 6, which subsides shortly after 6 days of administration.

### TABLE 2

**GROWTH OF BT 20 CELLS SUPPLEMENTED BY POLYVINYLPYRROLIDONES (PVP)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration/ ml of medium</th>
<th>After 5 days in vitro</th>
<th>After 10 days in vitro</th>
<th>After 45 days in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP K-50</td>
<td>6 mg.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PVP K-60</td>
<td>6 mg.</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>PVP K-90</td>
<td>6 mg.</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Cord extract</td>
<td>10%</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Control</td>
<td>No suppl.</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
lated from human female mammary gland can spread and grow without the supplement of umbilical cord extract (11).

It is apparent that the active components of CE are the acid mucopolysaccharides, chiefly hyaluronate. Cord extract and hyaluronate produce exactly the same stimulating effects on our cultures. Chondroitin sulfate C can also maintain the growth of BT 20 cells, but not as well as hyaluronate. It is nevertheless to be considered as an active component of CE, although a secondary one.

It is unlikely that CE acts as a nutritional substance. Chick embryo extract, which has been much used in tissue culture as a growth stimulant (15), cannot replace CE in the maintenance of BT 20 cells. Furthermore, the enzymatic digests of hyaluronate—tetrasaccharides and monosaccharides—are not effective. It appears, therefore, that the action of hyaluronate is accomplished only when its entire molecule is made available to the cells.

The effect of CE in producing an even spreading of the newly explanted cells (11) suggests that it is acting on the cell membrane. The necessity for highly polymerized mucopolysaccharides to be present suggests further that this action is of a physical or physico-chemical nature, occurring at the surface, rather than that these materials are taken into the cell. This supposition is strengthened by the observations that chondroitin sulfate C of low molecular weight can only partially replace CE, that polyvinylpyrrolidones (which are nutritionally inert) can largely replace hyaluronate, and that the most effective of the three polymers assayed is K-90, which most closely approximates the molecular weight of hyaluronate. If the viscosity of the medium is a governing or limiting factor in the cells' response, this parallelism in molecular weight might be explained.

Recently Katsuta and co-workers succeeded in substituting polyvinylpyrrolidones, as well as other high molecular weight substances unrelated to cell nutrition, for varying amounts of serum in cultures of rat ascites hepatoma cells (8), L strain mouse fibroblasts (9) and Yoshida sarcoma cell (7). Analogously, Evans et al. (6) point out the favorable action on proliferation of strain L fibroblasts and Yoshida sarcoma cells by Methocel (mol. wt., 15,000–50,000) and by Dextran (mol. wt., 25,000–200,000). In addition, Bryant et al. (4), working with strains of fibroblasts and of monkey kidney cells, report good growth in a protein-free chemically defined medium (NCTC 109) supplemented by Methocel.

In our BT 20 cultures PVP K-90 can replace the acid mucopolysaccharides of CE only after a protracted period of cultivation and after repeated transfers. Furthermore, judging by the rate of growth of the various groups of cultures, it appears that BT 20 cells are more active metabolically when supplemented by CE or hyaluronate than when supplemented by PVPs. Consequently, it seems probable that other properties, more specific than high molecular weight and its related viscosity, are also involved in the growth-promoting action of the mucopolysaccharides. It has long been suggested by K. Meyer that in vivo these anionic mucopolysaccharides exert, among other functions, a control on the exchanges of metabolites and water (18). Dorfman and Schiller (5) also point out that these polyelectrolytes possess a high negative charge which interacts with cations and is in turn influenced by the concentration and nature of the cations. They suggest that these properties of acid mucopolysaccharides should be considered in problems concerned with exchange of electrolytes between circulating and extracellular fluid and between extracellular fluid and cells. A similar phenomenon may be expected to occur in the BT 20 cultures in relation to the cell-medium exchange.

It is of interest to note that, in the case of tumors of hamster kidney and rat liver, the cancer cells carry at their surface a considerably higher negative charge than do the homologous normal cells (8). It has also been demonstrated that Walker tumor cells agglutinate in the presence of positively charged polyelectrolytes, while they remain in suspension in the presence of heparin, which is a negatively charged mucopolysaccharide (2). It is therefore possible that acid mucopolysaccharides act on the cell membrane of BT 20 cells, favoring their spreading by virtue of their negative electrical charge. This is further supported by the observations that in absence of acid mucopolysaccharides BT 20 cells tend to gather in globular aggregates, and that normal breast epithelial cells can grow and spread in the absence of CE.

Recent experiments of Allfrey and Mirsky (1) have shown that it is possible to remove up to 76 per cent of the DNA of isolated nuclei of thymocytes and to substitute other polyanions for the missing nucleic acid to restore the biochemical activity of the nucleus, as tested by its capacity for ATP synthesis, amino acid incorporation into protein, and adenosine uptake into nuclear RNA. They suggest that a pattern of negative charge is correlated with the biochemical activity of the chromosome. Further investigations may indicate that these findings are also applicable to the behavior of the BT 20 strain.
The observations and hypotheses relating to the action of acid mucopolysaccharides on this particular strain of cancer cells may be of especial interest because of the large amounts of acid mucopolysaccharides that are found generally in the stroma of human mammary carcinoma.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. Karl Meyer for his valuable suggestions and for providing the acid mucopolysaccharides and their enzymatic digests.

REFERENCES

Growth-promoting Activity of Acid Mucopolysaccharides on a Strain of Human Mammary Carcinoma Cells

Luciano Ozzello, Etienne Y. Lasfargues and Margaret R. Murray


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/20/5_Part_1/600

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/20/5_Part_1/600. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.