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Configuration of Surfaces of Human Cancer Cells in Effusions

A Scanning Electron Microscopic Study of Microvilli

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Summary. The surfaces of viable cells of metastatic human carcinomas of various histologic types and primary origin, suspended in pleural and ascitic fluids, were shown by scanning electron microscopy to be covered by microvilli of variable configuration and distribution. Microvilli of some cancer cells appeared biologically active since they were capable of forming extensions and anastomoses when settling on glass. The possible specificity and significance of microvilli in the light of the experimental data were discussed.

Key words: Pleural effusion – Ascitic fluid – Human cancer cell surfaces – Microvilli.

Introduction

The configuration of surfaces of human carcinoma cells in vivo, or under conditions as closely reflecting the natural state as possible, is of a major current investigative interest. Such studies may contribute to our understanding of many phenomena presumably vested in the cancer cell surfaces, such as the interrelationship of cancer cells to each other and to their environment [4, 17, 19, 30, 32].

Effusions occurring in patients with metastatic carcinoma are a natural culture medium [16] wherein cancer cells may proliferate freely and are accessible to sampling by tapping. Effusions also contain a variety of benign cells such as mesothelial cells, macrophages, and leukocytes which can serve as natural controls. Surface features of the various cell types found in effusions were

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described in a prior communication [6] using light microscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) for identification and correlation. One of the preliminary conclusions of this study was that the surfaces of human cancer cells in effusions were endowed with microvilli (MV), a feature that was conspicuously lacking in the benign cells. However, this study was based on air dried material and thus subject to critique for technical reasons.

The purpose of the present study was to determine by the use of critical point drying technique and SEM whether the prior observation on the configuration of human cancer cell surfaces could be confirmed and expanded. We were interested in determining whether the organ of origin and the histologic type of cancer had any bearing on the configuration of cancer cell surfaces. It was also of interest whether the MV represented merely a feature of membrane configuration or whether evidence could be elicited under simple experimental conditions that MV may have an activity or function of their own in living human cancer cells.

Material and Methods

There were 19 pleural and ascitic fluids containing cells of metastatic carcinoma. The sites of origin, and histologic types of primary tumors are summarized in Table 1. Ten fluids from patients free of cancer, listed in Table 2, served as controls.

An aliquot of approximately 10 ml of the freshly drawn fluid was centrifuged for 10 min at 200 g. The supernatant was discarded and the sediment washed twice in Hank's Balanced Salt Solution (HBSS) by centrifugation for 5 min at 200 g. Following the second wash the samples were divided into 2 approximately equal aliquots, processed in a different manner.

Aliquot 1 was processed as follows: the samples were washed for the third time in HBSS. The supernatant was discarded except for a few drops to avoid drying, and the sample was fixed in 5 ml of 2% glutaraldehyde in cadodylate buffer pH 7.3 for 2 h at 4° C. After a wash in cacodylate buffer (5 min at 200 g), the supernatant was discarded, new buffer added, and the sample placed in a refrigerator at 4° C until further processing (usually for 48 h). Subsequently the sediment was spun down for 5 min at 200 g and transferred by Pasteur pipette into one well of Linbro plate covered by specially cut narrow cover slip sealed with Scotch tape, and centrifuged for 5 min at 200 g. Cover slips with cells deposited by centrifugation was then immediately transferred into a vial with 1% OsO_4 in cacodylate for postfixation at 4° C.

Table 1. Site of origin and histologic type of primary tumors

Site of origin	No. of cases	Pleural fluid	Ascites
Breast (Adenocarcinoma)	8	8	–
Lung (Adenocarcinoma)	2	2	–
Lung (Epidermoid carcinoma)	1	1	–
Colon (Adenocarcinoma)	3	2	1
Ovary (Adenocarcinoma)	2	–	2
Cervix uteri (Squamous carcinoma)	1	1	–
Stomach (Adenocarcinoma)	1	1	–
Pancreas (Adenocarcinoma)	1	1	–
Total	19	16	3

Table 2. Clinical diagnosis in cases negative for cancer

Diagnosis	No. of cases	Pleural fluid	Ascites
Congestive heart disease	3	3	–
Pulmonary emboli	1	1	–
Pneumonia	1	1	–
Liver cirrhosis	4	–	4
Unknown	1	–	1
Total	10	5	5

Aliquot 2 was processed as follows: the samples were washed for the third time in HBSS. The supernatant was discarded and 1 ml of fresh HBSS added to the sediment and mixed gently. An aliquot was transferred into a specially prepared short glass tube 1 cm in diameter with a cover slip attached by dental wax, and placed in inverted position. The cells were allowed to sediment onto the cover slip for 15 min at room temperature. 0.5 ml of HBSS was removed and 1 ml of glutaraldehyde added drop by drop in order to fix the cells in situ on the cover slip. After 15 min of fixation the cover slip was transferred into cacodylate buffer and placed at 4° C until further processing. The cover slip was postfixed with OsO_4 at 4° C.

Following postfixation both aliquots were dehydrated in ascending series of acetones and in amyl acetate, critical point dried in a Denton DCP-1 apparatus, coated with gold-palladium and examined with JSM-43 scanning electron microscope.

Viability of cells in the sample prior to fixation was tested by eosin exclusion. Very few cells accepted eosin and, conservatively, at least 90% of the cells were judged viable. All SEM findings were carefully controlled by light microscopic preparations stained according to Papanicolaou whereby identification of cell types could be accomplished with ease [16].

Selected aliquots of the same samples were studied by transmission electron microscopy (TEM). For this purpose, following the 3rd wash in HBSS, the samples were centrifuged for 15 min at 200 g and the sediment fixed in 2% glutaraldehyde in cacodylate buffer for 2 h at 4°. Postfixation in OsO_4 for 8 h followed. The samples were dehydrated as described and embedded in Epon. Ultrathin sections were examined with UEMW 100 microscope.

Results

Identification of Cell Types

Cells of metastatic carcinoma were readily identified in SEM because of their large size (20 to 40 μ in diameter) when compared with all other cell constituents of the fluid (Fig. 1) and because of their characteristic arrangement in 3-dimensional clusters that has not been observed in any of the controls. The number of component cells in the clusters varied from a few to several hundred. The general configuration of the clusters was usually spherical or irregular (Fig. 2). The cells were often demarcated from each other by invaginations of variable depth, giving the cluster a morula-like appearance (Fig. 3). Surface cell characteristics of clustered and single cancer cells were compared and found to be identical. Other cell types seen in effusions that could conceivably be mistaken for carcinoma cells were histiocytes and mesothelial cells. The cells of both types were considerably smaller than most malignant cells and had surface features, which allowed for their identification in nearly all instances (see below). Simulta-

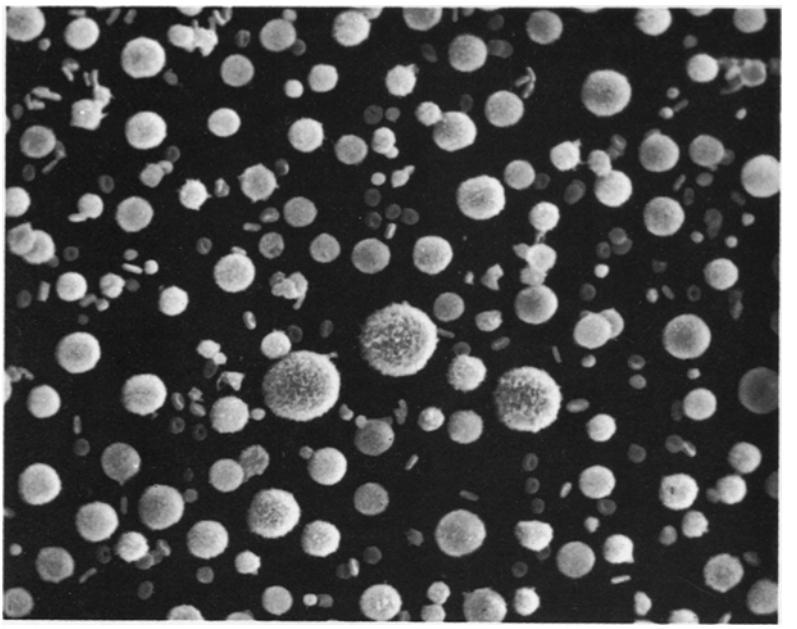


Fig. 1. Carcinoma of breast-pleural fluid. Note the large size of cancer cells when compared with erythrocytes and lymphocytes in the same field. × 300

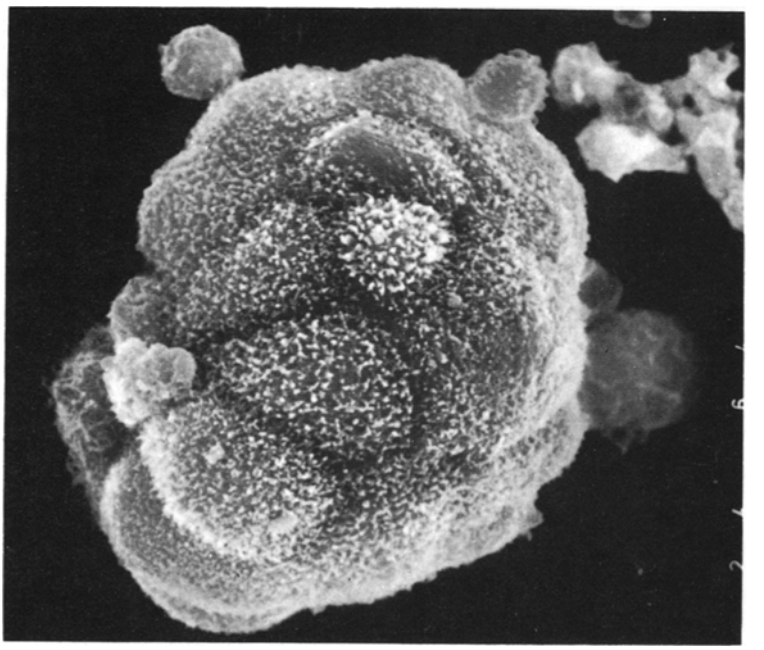


Fig. 2. Carcinoma of breast-pleural fluid. Cluster of cancer cells with ill-defined borders and the characteristic cell surfaces with microvilli of variable configuration. Three smaller cells attached to the periphery of the cluster have the surface characteristics of histiocytes (cf. Fig. 9). × 1700

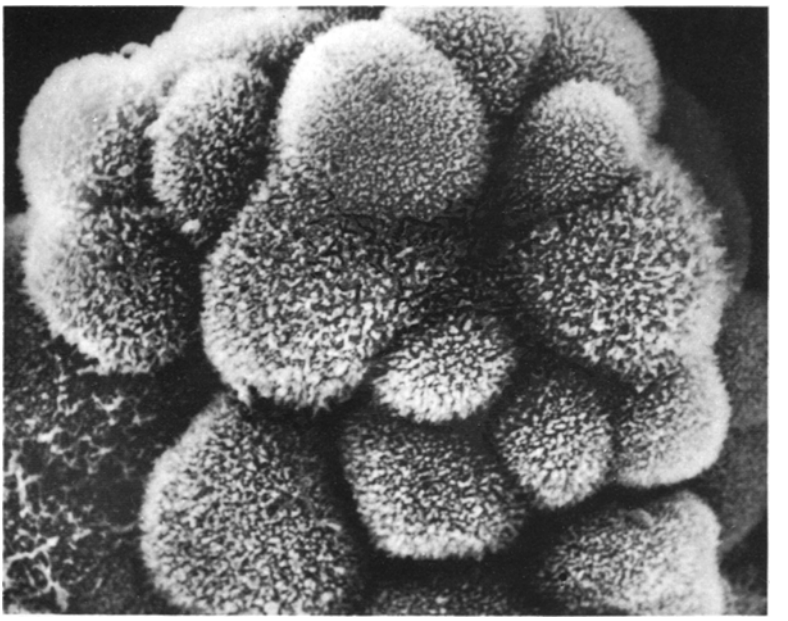


Fig. 3. Ovarian adenocarcinoma. Group of cancer cells with deep intercellular spaces. Note microvilli of variable configuration on cell surfaces. ×2300

neous TEM and light microscopic studies carried out on several samples from our current material fully confirmed the criteria of cell identification.

Single cancer cells predominated in fluids from patients with breast and gastric carcinomas. Cancer cell clusters were more commonly encountered in carcinomas of other primary origins.

Surface Features of Carcinoma Cells in Effusions

The presence of innumerable microvilli (MV) was the characteristic trait of the surfaces of carcinoma cells in effusions regardless of anatomic origin and histologic type of primary tumor. The MV were observed on surfaces of cancer cells in clusters (Figs. 2 and 3) but could be better studied on single cells. The latter were for the most spherical and resembled chestnut burs covered with innumerable tiny spines (Fig. 4). Occasionally indentations or invaginations of the surface were observed (Fig. 5). The MV generally covered the entire visualized surfaces of the cells except for an occasional small area which was MV-free. The MV varied in configuration, size, and density (Figs. 2–6). The length of MV varied from 0.1 μm to 0.5 μm but occasionally tufts of much longer MV could be observed (Fig. 6). The diameter of MV varied from 0.1 to 0.2 μm. The shape of the MV was also variable. Some MV had a relatively regular, rod-like configuration (Fig. 6). In other instances, the base was broader than the tip, and in yet other instances the free end of a microvillus was

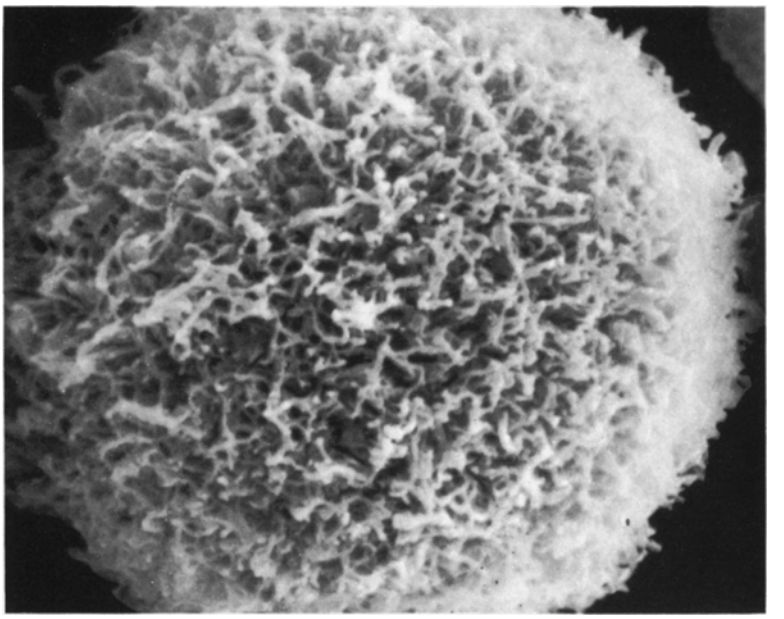


Fig. 4. Higher magnification of a breast cancer cell from the center of Figure 1. The cell is about 25 μm in diameter. Note numerous long, delicate MV covering entire cell surface. × 3000

Fig. 5. Epidermoid bronchogenic carcinoma. Cancer cell, about 20 μm in diameter, with surface invagination. The microvilli are sparser and shorter when compared with those shown in Figure 4. × 4600

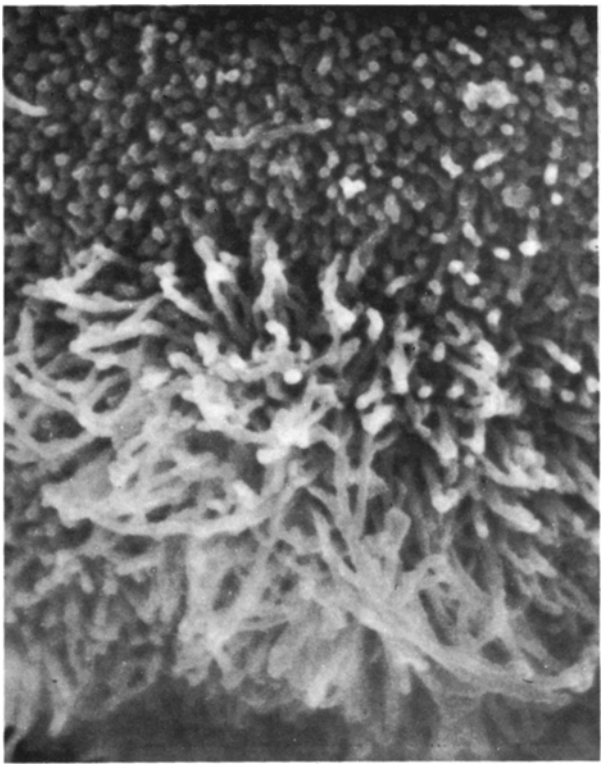


Fig. 6. Adenocarcinoma, lung. Cancer cell with a tuft of long MV at one pole. The remaining surface is covered with short, stubby microvilli. ×5500

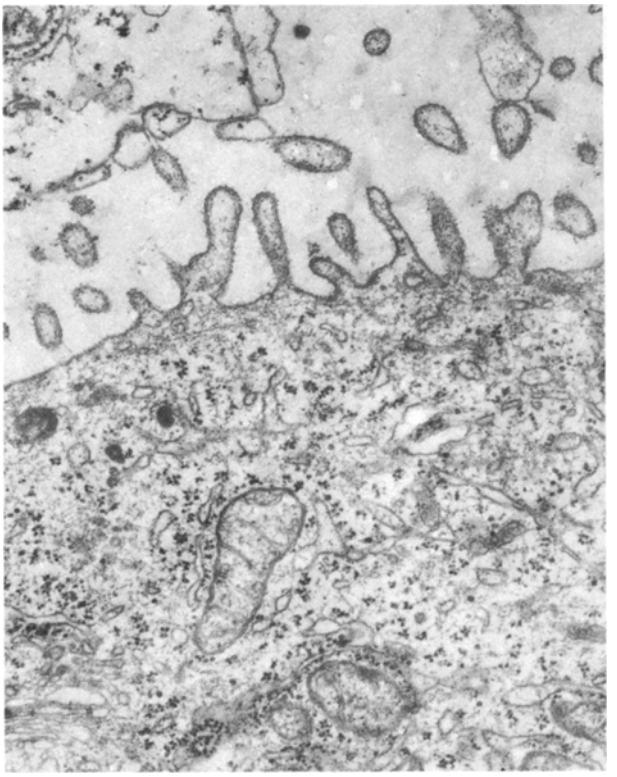


Fig. 7. Lung, adenocarcinoma, pleural fluid. Transmission electron microscopy. Note numerous microvilli on the surface of the cancer cell (cf. Fig. 6). ×27,900

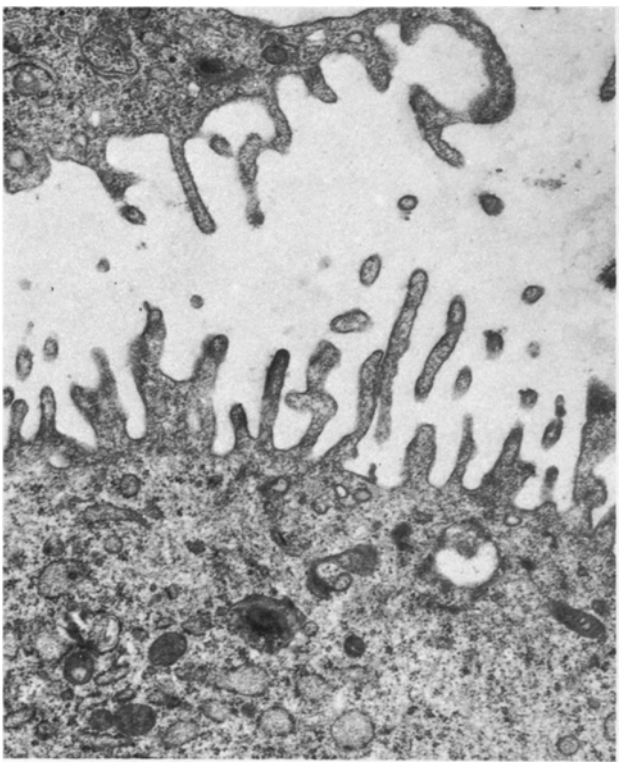


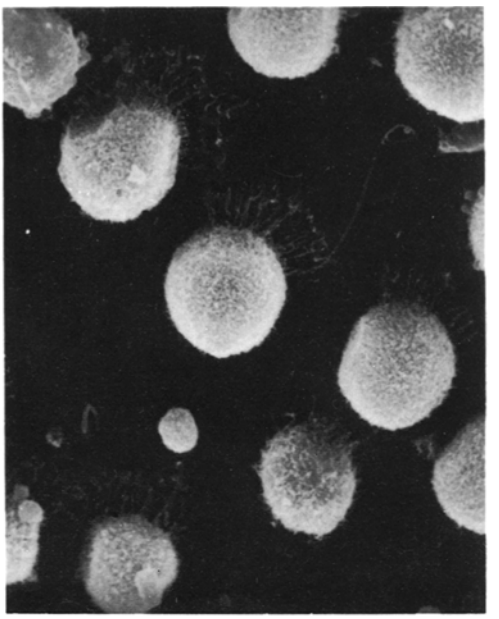
Fig. 8. Ovarian carcinoma, ascitic fluid. Transmission electron microscopy. Note numerous microvilli of unequal configuration on the surface of the cancer cell. Fusion of microvilli may be noted (cf. Fig. 3). ×25,000

bulbous and wider than the base. Branching and fusion of MV were repeatedly observed (Figs. 4, 7, 8). There were not only cell-to-cell differences in each sample but MV variability could be observed on the surface of a single cell (Fig. 6). No evidence could be obtained of a MV pattern that could be considered as specific for any tumor type or organ origin.

TEM studies of cancer cells in selected samples fully confirmed the presence of MV of uneven size and configuration on the surface of cancer cells of various primary origin (Figs. 7 and 8). This technique also confirmed excellent preservation of this material.

Behavior of Microvilli in Carcinoma Cells Settling on Glass Surface Prior to Fixation

Cancer cells that were allowed to settle on glass for 15 min prior to fixation had the surface texture as described. In all cases a spread of MV on the glass surface was observed. In most instances the MV in contact with the glass formed short spikes. In 2 cases of carcinoma of the breast the MV were extended



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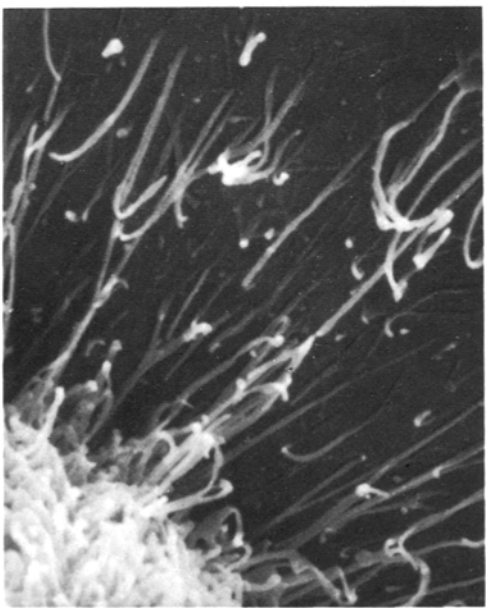

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Figs. 9 and 10. Cytoplasmic extensions in breast cancer cells which were allowed to settle down on the glass cover slip for 15 min prior to fixation. Same specimen as shown in Figures 1 and 4. **Fig. 9.** All cancer cells, covered by MV, are attached to glass by long cytoplasmic extensions. ×1000. **Fig. 10.** The details of the lower portion of the cell located in the center of Figure 7. Note the variable thickness of cytoplasmic extensions, some of which are broken, presumably as the result of contraction due to fixative. ×9000

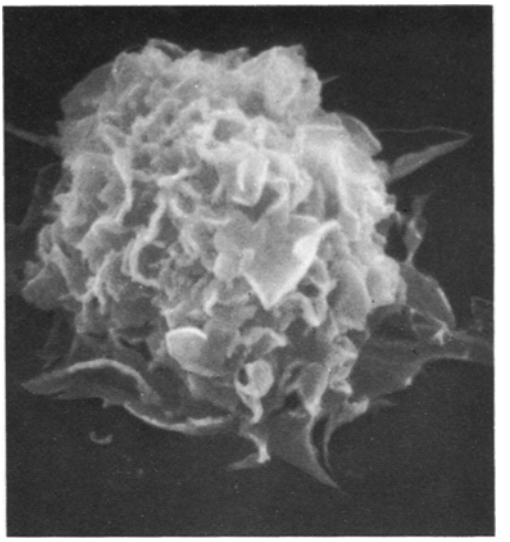

Fig. 11. A histiocyte, about 10 μ in diameter, with numerous surface ruffles. There are no MV. ×5500

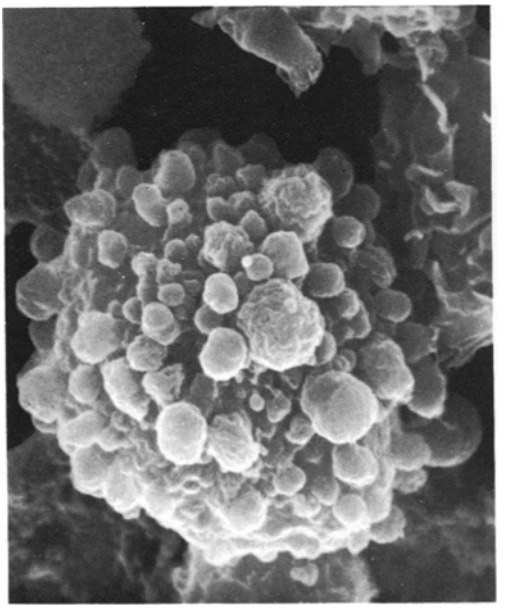

Fig. 12. Typical mesothelial cell, about 15 μ in diameter, covered with numerous blebs of different size. ×4600

for a considerable distance around the cells. Not only the MV in direct contact with the glass but also the MV from adjacent portions of the cell formed long, 8 to 10 μm extensions of variable diameter onto the glass surface, some of which, due to contraction of the cell body during fixation, were ruptured (Figs. 9 and 10).

Control Fluids – Macrophages and Mesothelial Cells

In the 10 control fluids no cells with surface features of cancer cells were observed. These fluids confirmed that the surfaces of macrophages and mesothelial cells were quite distinct. The surface of macrophages was made up of irregular ridges or ruffles of different size and width (Fig. 11). All macrophages which were allowed to settle on glass prior to fixation were attached to the glass by thin, veil-like, cytoplasmic processes.

Typical free-floating mesothelial cells were covered by blebs of different size and shape, some of them connected to the cell body by a narrow cytoplasmic neck (Fig. 12). This cell appearance may indicate dying or damaged mesothelial cells. There were a few occasional mesothelial cells covered by short, uniform MV only. Histiocytes and macrophages were also identified in fluids containing cancer cells.

Discussion

SEM is the ideal technique for the study of configuration of cell surfaces [4, 11, 12]. It offers the additional advantage of allowing the inspection of a very large number of cells. The present study was targeted on human cancer cells in their natural setting and with a minimum of experimental manipulation. This work confirms and extends prior findings [6] that the presence of MV of variable sizes and configuration characterizes all free-floating human carcinoma cells in fluids, regardless of histologic type or primary tumor and of organ of origin. SEM evidence that irregular MV may occur on surfaces of human cancer in other settings as well has been recorded in carcinomas of the uterine cervix and of the breast [15, 36, 42, 48].

There were prior light microscopic observations of cytoplasmic processes on the surface of some cancer cells in fluids [39, 40, 41]. The "brush borders" or "pseudocilia" presumably referred to the light microscopic appearance of the cancer cells with tufts of long MV such as shown in Figure 6.

The presence of MV has been previously recorded by TEM on the surfaces of free-floating human cancer cells from fluids [14, 22, 40, 41, 50, 51] and on free surfaces of cancer cells within various solid human tumors [1, 23, 31, 43], yet this feature has been rarely emphasized. The significance of this finding may have eluded the observers because TEM reveals only that part of the cell that is in the plane of the ultra-thin section, offering a limited view of the surface of the entire cell.

It must be stressed that MV are not exclusive feature of surfaces of cancer cells. They may be observed in a variety of anatomic situations on cell surfaces in normal adult mammals [3, 8, 47], on embryonal cells [26], and on some metaplastic cells [49]. However, the MV on normal cells exhibit polarity, i.e., they are located only on a well demarcated area of cell membrane, usually at the portion facing the lumen of a gland or a cavity. Such MV are uniform in length, shape and diameter and are evenly distributed throughout the MV-bearing area [9]. Only slight deviations from this rule have been recorded:

the number and morphology of MV on the cell surface may vary with different periods of the cell cycle [5]. Small differences in the numbers of MV observed on the surfaces of cells lining the bronchial and the tracheal mucosa were thought to be the result of continued regeneration of the bronchial epithelium [11]. Similar differences have been described on and among neighboring cells of duodenal epithelium in the early stages of embryonal development [26].

The MV on the surface of free-floating human cancer cells in fluids differ from those on benign cells in tissues in two respects:

1. They have no polarity and cover the entire surface of the cell. Moreover, with regard to groups of cancer cells, the phenomenon of "inverse polarity" has been previously observed in TEM [6]: the surfaces of cancer cells lining intercellular spaces inside the cluster were devoid of MV, whereas MV were abundant on the outer free surface, facing the fluid environment.

2. They are highly diversified in terms of length, width, shape and distribution on the cell surface.

Crucial evidence that MV are not artifacts of fixation or passive excrescences on the surfaces of human cancer cells was provided by the simple experiments described above: MV of some cancer cells settling on glass showed ability to spread for considerable distances while retaining their thickness. This suggests that the observed MV are active, dynamic structures, perhaps capable of proliferation and attachments to tissue surfaces in vivo. The mechanisms that triggers this proliferation is unknown but has many similarities to the spread of cell processes on hard surfaces in tissue culture [2].

A comparison of surface features of mesothelial cells and cancer cells sheds some light on the possible specificity of MV in cancer. Under physiologic conditions, the surfaces of mesothelial cells facing the lumen of the pleural or peritoneal cavity are covered by numerous MV. They have been described in the light microscope as a brush border [16] and have been observed with SEM [3, 47].

The surfaces of free-floating mesothelial cells in fluids are usually covered by surface blebs (Fig. 14), probably as a sign of cell injury or commencing cell death. It is known that mesothelial cells change their configuration in response to various environmental conditions [2, 21]. Therefore, it appears likely that mesothelial cells detached from their natural setting lose their MV and acquire surface blebs, in contrast to cancer cells which either retain or acquire MV.

It must be noted that occasional surface blebs have been observed also on the surfaces of Chinese hamster ovary cells during the G-phase of the cell cycle [28]. It is not clear whether or not this observation is pertinent to the human mesothelial cells in fluids. We have no information on the status of the cell cycle in cancer cells in fluids but so far have not observed blebs on carcinoma cells believed to be viable.

It has been observed that certain variables may have some bearing on the occurrence of MV on the surfaces of cells in tissue culture: thus, changes in metabolic activity [7], cell density in culture [24], and the type of growth medium [37] may increase or decrease the number of MV per cell. Perhaps

most pertinent to our observations is the increase in the number of MV in cultured cells transformed by oncogenic viruses [27, 45].

SEM studies epithelial surfaces in experimental bladder and cervix carcinogenesis have also emphasized the appearance of microvilli of diversified configuration once the malignant process has been established [13, 35]. Rubio [35] suggested the term anisovillosis to describe the phenomenon. In a more recent TEM study of experimental bladder carcinogenesis is the rat it has been emphasized that microvilli occur on the surfaces of all transformed epithelial cells and reach the epithelial surface relatively late [18]. This evidence indirectly supports the observations recorded here.

Formation of MV has been linked to intracellular levels of dibutyryl (3′5′) cyclic adenosine monophosphate (c-AMP) [7, 19, 29, 38]. For example, cultured tumor cells have much lower levels of c-AMP than normal cells from which they were derived [25], and they also exhibit more surface MV than do their benign counterparts [27]. Furthermore, it has been noted that experimental tumor cells treated with c-AMP can no longer be induced to cluster by exogenous lectins [4]. In support of this relationship, we have preliminary evidence that treatment of human cancer cells in effusions with c-AMP analogue added prior to fixation induces dramatic morphologic changes in cell surfaces with diminution or complete loss of MV (Domagala and Koss, unpublished data).

Much of the more recent work on cancer cell surfaces, such as lack of contact inhibition in tissue culture [2], response to various lectins (review in [30]), and biochemical studies (review in [33]), has been based mainly on experimental cell systems in vitro. The signal advantage of such studies is their reproducibility and controllability. The use of free-floating cancer cells in human effusions may serve as a counterpart to these experimental studies as has been suggested before [16].

Significance of MV on Surfaces of Cancer Cells

The presence of MV on the surfaces of human cancer cells and in experimental systems of epithelial carcinogenesis [13, 18, 35] suggests that this cell configuration is not purely incidental. However, neither the mechanism of formation of MV nor their biologic significance is known at this time. Several speculations appear worthy of a brief comment suggesting possible future directions of investigation.

MV may serve to increase the surface of cancer cells for purposes of absorption, as is the case in normal intestinal epithelium [44] or serve to maintain a constant cell surface area [10]. MV may increase the degree of exchange of fluids and metabolites with the environment and thus are engaged in active trans-membrane uptake and exchange [7, 34] or serve to produce a microenvironment of its own as a buffer zone between the cell and its environment [44]. MV may play a role in cell-to-cell and cell-to-substrate contacts [9, 12]. Some of our observations on behavior of MV recorded above support this hypothesis. Finally, the MV which increase the surface of the cell may play a role in

dissipation of heat, reflecting altered pathways of cell metabolism, as originally suggested by Otto Warburg [46].

After completion of this paper, a communication by Krivinkova et al. [20] reported the results of scanning electron microscopy of human cells in effusions carried out in short-term tissue cultures. These authors confirmed the presence of prominent microvilli as an exclusive feature of cancer cells. The identification of other cell types in culture corresponded closely to the observations recorded here.

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