MICROFILAMENTS IN EPIDERMAL CANCER CELLS

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ABSTRACT

The occurrence and structure of microfilaments in epidermal cancers induced in mice by treatment with 3,4-benzpyrene were investigated with the electron microscope. With malignant change, pleomorphic, undifferentiated cells with a cortical zone of microfilaments became increasingly abundant. The microfilaments were 40 Å in diameter and occupied the cortex of the cells beneath the plasma membrane, extended into cell processes, and were situated in the cores of microvilli. At high magnification, the filamentous areas were formed by an interconnected meshwork of filaments which in favorable planes had a polygonal arrangement. When exposed to high concentrations of cytochalasin B, the microfilaments became clumped and moderately disrupted. At the same time, the processes and microvilli of the cells were blunted. The structure of these filaments and their sensitivity to cytochalasin B place them in a class of microfilaments believed to be related to cell motility. Their presence in malignant cells may be correlated with the motile, invasive properties of these cells.

INTRODUCTION

Cytoplasmic microfilaments variously reported as 40-90 Å in diameter have been found in a wide variety of cell types. In many cases, their presence has been found to accompany such diverse cellular movements as cytokinesis (1, 2), cytoplasmic streaming (3), phagocytosis (4-6), extension of microvilli (7), axon elongation (8), morphogenetic movements (9, 10), and active migration of cells (3, 4, 11-18). Cytochalasin B, a mold metabolite, has been shown to disrupt microfilaments while at the same time altering cellular motile activities (2, 4, 5, 8, 10, 17, 19). Biochemical studies have demonstrated the presence of proteins resembling muscle actin in physical, chemical, and structural properties within cells showing motility or contractility and containing microfilaments (3, 14, 20-26). These studies provide evidence that microfilaments composed of an actin-like protein comprise a contractile system responsible for cellular motility.

Invasion and replacement of surrounding tissue is one of the primary behavioral characteristics of malignant neoplasms that distinguishes them from benign neoplasms and normal adult animal tissues. Because of the high degree of correlation between the presence of microfilaments and active cellular surface activity and movement, it is conceivable that the increased motility of cancer cells might be accompanied by the appearance of cytoplasmic microfilaments. To investigate the occurrence of filamentous structures in invasive cancer cells, skin cancers were induced in mice by 3,4-benzpyrene and observed at the fine structural level. Newly appearing microfilaments were tested for sensitivity to cytochalasin B. This study reveals the appearance of cytochalasin B-sensitive microfilaments in cancer cells. A com-
plete description of other cytotological changes occurring in the epidermis during carcinogenesis is presented elsewhere (27).

MATERIALS AND METHODS

6-wk old female Balb/C mice were obtained from an inbred colony maintained at the Yale University School of Medicine by Dr. William U. Gardner. The polynuclear aromatic hydrocarbons 3,4-benzpyrene and 1,2:3,4-dibenzanthracene (Sigma Chemical Co., St. Louis, Mo.) were employed as carcinogen and noncarcinogen control, respectively (28). Both are soluble in acetone and both enter epidermal cells of exposed mouse skin as shown by binding to cellular proteins (29, 30).

Acetone solutions of 0.5% 3,4-benzpyrene or 0.5% 1,2:3,4-dibenzanthracene were prepared and stored in 1-ml samples in sealed glass vials. These were kept at 4°C in the dark for the duration of the experiment. Previous dose-response studies have shown that application of 100 µg of 3,4-benzpyrene (0.5% in acetone) three times a week to mouse skin produces tumors within 10–15 wk (31). The solutions were applied externally onto the inner side of the pinna of the mouse's ear, dropwise using a tuberculin syringe with a filed-down 26G needle (3 drops = 100 µg).

The pinna proved to be especially advantageous as a site of application because it is thin and hairless, and tumors are easily observable grossly. Furthermore, the two epidermal surfaces lie within 0.5 mm of each other, allowing comparison of treated and untreated surfaces in a single section. 3,4-Benzpyrene was applied to the right ear of 12 mice. The same volume of pure acetone was applied to the left ear of the same mice. With six mice, 1,2:3,4-dibenzanthracene was applied to the right ear and acetone to the left ear. Chemicals were applied three times a week for up to 24 wk.

Tissue samples were taken from untreated mice and from treated mice at 6, 12, and 24 wk. Biopsies were taken of the pinna containing the desired region of epidermis or tumor. Except in the case of deeply invasive carcinomas, this procedure did not require sacrifice of the mouse and allowed most mice to be followed for the full duration of the experiment. The observations and figures are representative of a survey of tissues from at least three mice at each stage of malignant transformation.

Cytochalasin B (Imperial Chemicals Ltd., Macclesfield, Cheshire, England) dissolved in dimethyl sulfoxide (25 mg/ml) was added to culture medium 199 (unmodified, Earle's base, Grand Island Biological Co., Grand Island, N. Y.) to produce final concentrations of 50–100 µg/ml. Solutions containing dimethyl sulfoxide (0.4%) but lacking cytochalasin were also prepared. Thin tissue slices of tumors were placed in the cytochalasin B medium or control medium for 1 h at room temperature.

All tissue samples were cut into small rectangular pieces to allow orientation and fixed for electron microscopy. Tissue samples were fixed for 2 h at 4°C in cold 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). They were then rinsed in cold buffer several times and fixed for 2 h in 2% osmium tetroxide buffered with 0.05 M sodium cacodylate (pH 7.2) at 4°C. After osmium tetroxide fixation, the tissue samples were washed in cacodylate buffer (pH 7.2) and then several times in Michaelis buffer (pH 5.0). The tissues were stained en bloc with aqueous uranyl acetate by placing them in 0.5% uranyl acetate in Michaelis buffer (pH 5.0) for 2 h at room temperature (32). The tissues were then washed in Michaelis buffer (pH 5.0) and dehydrated in a graded series of ethyl alcohol. After carefully orienting the rectangular tissue blocks to permit sectioning in a plane perpendicular to the surface of the epidermis, they were embedded in Maraglas (The Marlborite Co., Div. of Allied Products Corp., Long Island City, N. Y.).

Thick sections were cut on a Sorvall MT-2 microtome (Ivan Sorvall, Inc., Newtown, Conn.) with glass knives, stained on glass slides with 1% toluidine blue, and surveyed for areas to be examined at the fine structural level. Thin sections cut with glass or diamond knives were stained with lead citrate (33) and examined with an Hitachi 8B electron microscope. Measurements were performed with a 7X ocular micrometer on micrographs with a magnification of 100,000 or above. The magnification of the microscope was calibrated with a carbon grating replica grid.

RESULTS

Light Microscope Structure of Tumors

Exposure of the pinna to benzpyrene results in the appearance of focal areas of hyperplasia at 6 wk, papillomas as 12 wk, and invasive carcinomas by 24 wk. The carcinomas underlie hyperplastic epidermis and invade deep layers of connective tissue down to the central layer of cartilage. The boundary between the tumor and underlying connective tissue is not readily evident in many areas. From the main tumor mass, tongues of carcinoma cells project into the subcutaneous tissues and surround connective tissue elements, blood vessels, nerves, and muscle. Cells in contact with the dermis are often elongated with processes extending into the connective tissue (Fig. 1). Intercellular spaces are present between individual cells and in the central regions of the tumor.
Figure 1 Periphery of an invasive carcinoma after 24 wk of exposure to 3,4-benzpyrene. Note the variation in size, shape, and staining characteristics of individual cells. Many cells at the edge of the tumor project long cytoplasmic processes (arrows) into the adjacent connective tissue (C), while cells deeper within the tumor tend to be round or polygonal in shape. Fibroblasts and macrophages occur in the connective tissue. A mitotic cell is present in the lower left portion of the micrograph. × 800.

they are bridged by thin cytoplasmic extensions. The cells vary in their staining characteristics, some being intensely stained with toluidine blue. Nuclei are often large with multiple nucleoli, and mitotic figures are common.

Fine Structure of Cancer Cells

There is variation in the cytology of the cells comprising the tumors, although the majority of cells fit into one of two categories. Central regions of tumors are composed largely of polygonal, closely packed cells which appear to be more differentiated, containing short bundles of tonofilaments, moderate amounts of rough-surfaced endoplasmic reticulum, and large numbers of ribosomes organized into polysomal clusters. Many small microvilli and pseudopodial processes arise from the surface of central cells. The second type of cell is more common at the periphery of the tumors, especially in the leading edges of tumor infiltration. These cells are highly irregular in shape with large processes and occasional microvilli (Figs. 2, 3). Free cytoplasmic ribosomes are common and often occur singly. There is a relative paucity of rough-surfaced endoplasmic reticulum. Tonofibrils are very scarce, but bundles of microfilaments are abundant in these cells. The tonofibrils are reduced to a few short electron-dense, spike-like structures which are often the only means of determining the cell's epithelial origin (Fig. 2). The tonofilaments comprising the tonofibrils are approximately 100 Å in diameter (50 filaments, mean diameter 105.9 ± SD 31.6 Å). Intercellular spaces between the less differen-
FIGURE 2 Part of a carcinoma cell at the edge of an invasive squamous cell carcinoma. 24-wk exposure to 3,4-benzpyrene. Exposed en bloc to 0.4% dimethyl sulfoxide in vitro for 1 h. The cell extension is situated in the dermis and is irregular in outline with several long, slender microvilli. There is no basement lamina although flocculent, amorphous material adheres to the cell surface. Microfilaments (Mf) occur in the peripheral cytoplasm. Some of the microfilament bundles are oriented in the long axis of the process (arrows). Also within the cell are microtubules (Mt), free ribosomes, and some cisternae of rough-surfaced endoplasmic reticulum. Tonofilibrils (T) are scarce, occurring as small short bundles of tonofilaments. X 37,000.

Tiated, peripheral cells are large and irregular. Masses of moderately dense, amorphous material occupy the intercellular spaces (Figs. 2–4). Occasional desmosomes connect the processes of adjacent cells (Fig. 3) but in general these cells show a reduced number of desmosomes and other specialized junctions in comparison to normal epidermal cells. A few small hemidesmosomes may occur along the surface of the cell facing the connective tissue (Fig. 3). A distinct basement
lamina does not delineate the tumor from surrounding connective tissue. However, fine flocculent material may occur in patches along the cell surface or form an irregular thin layer on the surface (Fig. 3).

**Microfilaments**

Microfilaments fill most of the cortex of the pleomorphic undifferentiated cells at the periphery of the tumor (Figs. 2–4). A small number of similar cells containing microfilaments were first observed in papillomas, but have not been seen in normal or hyperplastic epidermis. Cells in the central regions of cancers contain few microfilaments. Commonly, the filaments run in a band about 0.1–0.2 μm thick along the periphery of the cells beneath the plasmalemma (Figs. 3, 4). Filaments extend into the cell processes and occupy the cores of microvilli (Fig. 3). In some places, the filaments appear to be very closely associated with the plasma membrane (Fig. 5). Regions con-
**Figure 4a** Cortex and portion of the nucleus (N) of a carcinoma cell. Exposed en bloc to 0.4% dimethyl sulfoxide in vitro for 1 h. 40 Å microfilaments (Mf) are oriented parallel to the plasma membrane in the cortex of the cell. There is no basement lamina separating this cell from the connective tissue (C). Flocculent material occurs extracellularly. X 47,000.

**Figure 4b** High magnification of the blocked portion of Fig. 4a. Transverse connections can be seen between individual longitudinal filaments. X 183,000.
FIGURE 5 Very high magnification of the edge of a carcinoma cell showing a small number of 40 Å microfilaments (Mf) adjacent to the plasma membrane (PM). The interconnections between microfilaments form a polygonal network. The centers of the polygons are marked by stars. The plasma membrane above the microfilaments is sectioned obliquely, and in this region the microfilaments appear closely associated with or attached to the membrane. Just below the microfilaments is a tonofibril (T). X 294,000.

Measurement of 100 distinct filaments yielded a mean diameter of 37.2 ± SD 12.7 Å. Beadlike densities along the filaments are of larger diameter. Low power micrographs give the impression that the bundles are composed of parallel arrays of straight filaments (Fig. 4 a). However, higher magnification of different planes of section shows that the organization of the filaments is more complex. In sections parallel to a main bundle, filaments appear to run longitudinally, but are connected to adjacent filaments by cross bridges (Fig. 4 b). In sections passing obliquely or transversely through the bundles, the interconnections between filaments are especially apparent. The filamentous areas thus appear to be comprised by a meshwork of interconnected filaments. In some places, a pattern of uniform and delicate polygons is formed (Fig. 5).

Effects of Cytochalasin B

In vitro treatment of thin slices of carcinoma tissue with 100 µg/ml of cytochalasin B results in morphological alteration of the microfilaments and some of the cell processes. Cytochalasin B causes clumping of the long cortical bundles of microfilaments, thickening of filaments, and the appearance of many small focal, irregularly shaped densities within the disrupted filamentous material (Fig. 6). In addition, the oriented appearance and polygonal structure of the microfilament bundles become less apparent. The cell surface is less irregular and the processes and microvilli seen on the untreated cells are blunted and reduced in number. No other cellular structures are markedly affected by cytochalasin. The effects of 50 µg/ml of cytochalasin are not so apparent or consistent as the effects of 100 µg/ml.

The in vitro controls exposed to 0.4% dimethyl sulfoxide are indistinguishable from the carcinoma cells fixed immediately after excision (Figs. 2, 4). Microfilaments and cell processes are unchanged.

1,2:3,4-Dibenzanthracene and Acetone Exposure

At 24 wk of exposure to dibenzanthracene the epidermis is hyperplastic, being two to three times normal thickness. However, no papillomas or carcinomas appear, even after 1 y. Cell contact relationships and basement lamina are unaltered. The cytological structure of the cells is almost indistinguishable from that of the untreated epidermis, and no microfilaments are seen. Epidermis exposed to acetone for 24 wk is indistinguishable from normal at the light and electron microscope levels.

DISCUSSION

Various theories have been proposed to explain the origin of the motive force responsible for the spread of tumor cells into adjacent normal tissue. One hypothesis is that increased pressure within the growing tumor provides the force for a passive intrusion of tumor cells into surrounding tissues (34). This, along with inflammation and perhaps release of proteolytic enzymes or cytotoxic substances by tumor cells (35), could provide...
the necessary conditions for penetration of adjacent tissues.

On the other hand, the most widely accepted theory of local tumor invasion suggests that active motile activity on the part of the tumor cells is responsible for tumor invasion (36, 37). It has been shown that placing a malignant tumor beside a normal tissue in culture results in invasion of the normal tissue by tumor cells (38, 39). Furthermore, Wood et al. (40), studying in vivo movement of cells using a rabbit ear chamber and time-lapse cinemicrography, found that under the same conditions, carcinoma cells and polymorphonuclear leukocytes moved at similar rates while normal epidermal cells did not move at all. In addition, the carcinoma cells continually shifted arrangements with respect to one another. These studies indicate that the actual force for local invasion is generated within the individual cells themselves.

It was found in the present study that during malignant transformation induced by benzpyrene, epidermal cells lose their characteristic 100 Å tonofilaments and acquire a new population of thinner microfilaments. Microfilaments were not seen in normal epidermal cells or hyperplastic cells, but were abundant in the cells at the leading edges of invasive cancers. Because of the high degree of correlation between the presence of microfilaments and cellular motility (see Introduction), the presence of microfilaments in the pleomorphic cells of invasive carcinomas may represent the mechanism providing the force for the motile activities of these cells.

The microfilaments within the cancer cells are similar to those described in a variety of other cells (see Introduction). Close association with the plasma membrane (6, 8, 11, 17) and polygonal configurations (8) have been described. The filaments of cancer cells seem slightly smaller than
those of most other cells. Because several distinct classes of microfilaments occur in cells (17, 18), functional comparisons on the basis of size alone should be viewed with caution until the precise functions of the different types of filaments in each cell type are determined.

The microfilaments in the pleomorphic, undifferentiated cells were disrupted by exposure to high doses of cytochalasin B. Cytochalasin B has been shown to disrupt cytoplasmic microfilaments while at the same time altering cell behavior (see Introduction). Cytochalasin B may act directly on microfilaments, causing their disassembly (10), or it may block cell motility by altering mucopolysaccharide synthesis (41). While the mechanism of action of cytochalasin B is unknown, the sensitivity of microfilaments in the cancer cells to cytochalasin suggests that these filaments are similar to those associated with motility in other cells. The effect of cytochalasin B on the motility of the cancer cells was not studied, although it was observed that cellular extensions and villi were reduced in number and blunted or retracted after treatment. Its effect on the microfilament system provides a basis for investigation of the effects of cytochalasin B on tumors characterized by invasion and migration of cells.

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