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Fibronectin in Foreign Body-Induced Sarcomas and Preneoplastic Cells[1] (41532)

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Abstract. Foreign body (FB)-induced murine sarcoma cells and advanced preneoplastic cells as well as normal fibroblasts produce fibronectin (FN) in primary culture; cells at early preneoplasia do not. Hence, the neoplastic properties of FB-induced sarcomas do not depend on absence of FN. Temporary FN repression during early preneoplasia is associated with certain phenotypic cell characteristics.

Fibronectin (FN) is a glycoprotein found intracellularly, on cell surfaces, and in extracellular filamentous matrix of mesenchymal cells, and as a soluble substance in plasma (1). Malignant transformation of fibroblasts *in vitro* by oncogenic viruses or chemical carcinogens decreases both intra- and extracellular FN. This is accompanied by morphological cell change, loss of cellular adhesiveness, enhanced proliferation and movement (2). The study of FN has, therefore, become an important area of cancer research.

We examined FN in foreign body (FB) tumorigenesis (3) for two reasons. First, the carcinogenic agent, a nonbiodegradable implant, has no cell-invasive properties. Second, the tumor progenitor cells can be isolated *in vitro* for direct studies. Thus it was possible to follow FN through sequential stages of preneoplasia.

It takes on the average 12 to 14 months for sarcomas to develop in CBA/H mice upon subcutaneous implantation of a rigid, nonbiodegradable film of polyvinylchloride/acetate (PVCA) copolymer, 22 × 15 × 0.2 mm in size. Monoclonal tumors originate from microvascular stem cells which at the time of carcinogenic initiation are located in FB-reactive tissue distant from the implant. Progression depends first on the development of chronic FB reaction characterized by macrophage phagocytic inactivity and fibrotic encapsulation of the implant. Later, the preneoplastic cells attach to the FB surface before attaining neoplastic autonomy. Clonal preneoplastic cells can be cultured (a) from the FB-reactive capsule tissue at 3 months postimplantation and thereafter, (b) from the FB surface at 6 to 7 months postimplantation, but rarely earlier. The cells, especially when cultured from early capsules, appear in two morphological phenotypes. "Type III cells" (4) resemble cells cultured from retinal capillaries. They are very slender, have single nuclei with heavily marginated heterochromatin, and grow in linear cords or networks often across or alongside fibroblasts. Their attachment to culture surfaces is loose. "Type IV cells" (4), similar to cultured endothelial cells, are polygonal or stellate with short cytoplasmic processes and large round nuclei. They form pavement-like monolayers without piling up and are firmly attached to culture surfaces. Electron microscopically, preneoplastic types III and IV cells, as well as later sarcoma cells show similar ultrastructural characteristics (5). If clone related, they possess identical aneuploidies and marker chromosomes. Single-cell cloning of type III cells revealed that they convert into type IV cells during prolonged culture and are, hence, considered different developmental phenotypes.

Materials and Methods. *Sarcoma induction by FB in vivo and cultivation of preneoplastic and tumor cells in vitro.* Sarcomatogenic FB reaction was induced in groups of CBA/H mice by subcutaneous implantation of a PVCA film, 22 × 15 × 0.2 mm in size. After 3, 4, 5, 7, 9, 10, or 11 months, segments

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TABLE I. FN DETERMINATIONS ON CULTURED NORMAL FIBROBLASTS, FB-INDUCED PRENEOPLASTIC CELLS (PHENOTYPES III AND IV), AND SARCOMA CELLS

Cell types tested	Age of FB reaction (months)	Number of FN determinations	FN (E-FN and/or S-FN)
Normal fibroblasts	—	7	Positive
Phenotype III	3–7	9	Negative
Phenotype IV	3–7	7	Positive
Phenotype III	9 and over	2	Negative
Phenotype IV	9 and over	21	Positive
Sarcoma	—	33	Positive

of films and surrounding fibrotic capsule tissues were excised and cultured separately according to routine protocol (4). Part of each film capsule complex remained in the animal until tumor development. Aneuploid cell lines with type III and/or type IV characteristics were established within 3 to 6 passages. Upon transplantation to CBA/H-T6 recipients they formed sarcomas after clone-specific latencies (4–6).

Variations of cell culture conditions prior to FN determinations. Cell lines were grown on glass coverslips under varied conditions. (a) Uniform cell numbers were seeded on the coverslips and incubated until desired cell densities were reached. Under these circumstances, culture times varied relative to line-specific cell doubling times. (b) Cell numbers of the inocula were adjusted so that each line reached the desired cell density after approximately the same time in culture. (c) Cell lines were grown in standard medium (McCoy's 5a) supplemented with 10, 20, and 30% fetal calf serum to check for possible uptake of soluble FN.

FN determinations. The coverslip cell cultures were carried through the following steps: fixation with 1% glutaraldehyde in Dulbecco's phosphate-buffered saline (PBS) at 37°C for 20 min; incubation on a shaker at room temperature with 0.05 *M* glycine in PBS for 3 to 5 min, with appropriate dilutions of rabbit antifibronectin in PBS containing 1% normal calf serum (CS-PBS) for 12 hr, with excess goat anti-rabbit immunoglobulin (in CS-PBS) for 20 min, with an appropriate dilution of rabbit antiperoxidase–peroxidase complex for 20 min. Between steps, the cells were rinsed five times in PBS or CS-PBS. The preparations were mounted with Permount, photo-

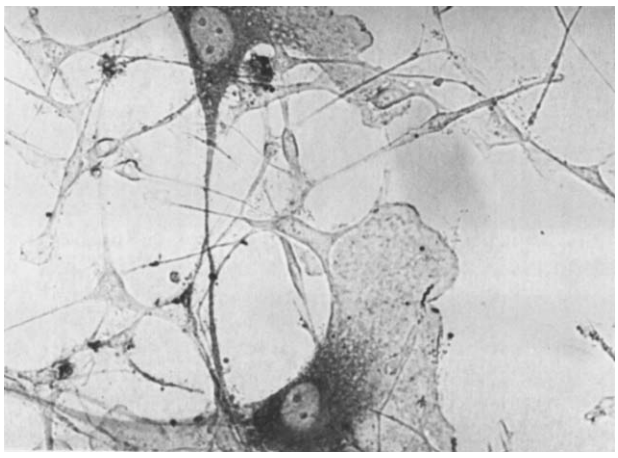

FIG. 1. Preneoplastic cells cultured from murine FB-reactive tissue, 3 months after implanting a standard PVCA film. The spindly FN-negative cells are type III cells. Cells in transition from type III to type IV are flattened and show perinuclear FN (400×).

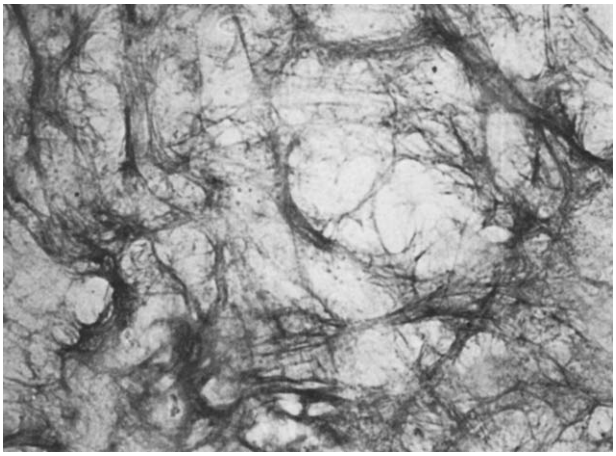

FIG. 2. Preneoplastic type IV cells cultured from murine FB-reactive tissue, 9 months after implanting a standard PVCA film. Maximum extracellular FN in confluent culture (400×).

graphed, and examined. The peroxidase–antiperoxidase precipitate is dark brown and easily identifiable by light microscopy or on color micrographs. Black and white illustrations (Figs. 1, 2, and 3) are obviously less distinct. The preparations were scored for the presence of cell surface FN (S-FN) and extracellular FN (E-FN) after Chen *et al.* (7). Presence of S-FN was evidenced by diffuse staining of cell plasma membranes. E-FN appeared as three-dimensional ropes spanning and overlapping the cultured cells with pronounced accumulations at contact sites.

Results. FN determinations were repeatedly done on 18 preneoplastic type III and type IV cell lines as well as on mixed populations, on 13 original and cell line-derived sarcomas, on 4 euploid fibroblast lines derived from normal CBA/H mouse embryos and adult connective tissue. As Table I and Figs. 1, 2, and 3 show, the normal fibroblasts, the preneoplastic type IV cells, and the sarcoma cells were found FN positive. In contrast, no FN was demonstrable in preneoplastic type III cells. These results were consistent no matter whether the preneoplastic

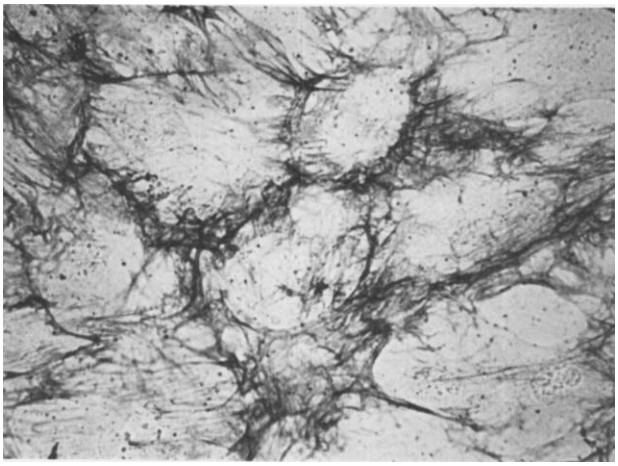

FIG. 3. PVCA film-induced, fully neoplastic murine sarcoma cells in confluent culture showing maximum extracellular FN (400×).

cells were cultured from early or from late FB-reactive tissues, whether cell densities or culture times were held constant, and whether the cells were grown with 10, 20, or 30% serum supplement. Sarcoma and preneoplastic type IV cells examined at low density usually exhibited surface FN (S-FN) only; at confluence or shortly before, maximum extracellular FN (E-FN) was seen; past confluence (when cells began to round up and loosen their attachment) both S-FN and E-FN decreased in intensity. Although the cell lines differed in several criteria such as growth rate, degree of contact inhibition, or tendency to pile up after reaching confluence, the results regarding FN were consistent throughout.

As an example, preneoplastic cell line 11496 was at the time of testing composed of both cell types III and IV. At low density, single type III cells were FN negative, single type IV cells showed S-FN. After more prolonged culture, islands of confluent type IV cells exhibited E-FN. Cells in transition between types III and IV formed intracellular FN in the perinuclear region (Fig. 1).

Discussion. This investigation has unequivocally shown that FB-induced sarcoma cells of mice produce FN. The presence of FN may be related to the general finding that these sarcomas fail to metastasize hematogenously (7). Otherwise, the tumors must be considered malignant: cell doubling time increases abruptly when autonomous growth commences; growth control seems irreversibly lost; histopathologically, many of the tumors are highly anaplastic; they are transplantable; local invasiveness is always apparent although initial growth is nodular; metastases per continuity into regional lymph nodes do occur. It follows that these properties of malignancy are not contingent on absence of FN.

Preneoplastic cells were temporarily found FN negative while in the type III phase. This phase seems to be characteristic of early preneoplasia because type III cell culture lines were more frequently obtained from early than from advanced FB-reactive tissues. The type III cells appear spindly and grow in cords or networks. Their attachment to the culture surface is loose. Conceivably, the absence of FN relates to these properties. In prolonged culture and prior to attaining neoplastic autonomy FN-negative type III cells convert to FN-positive type IV cells. In this state, the cells flatten and form a firmly adhering pavement-like monolayer.

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