Considering the absence of therapeutic effect of HS under experimental conditions on phosphoenolpyruvate carboxykinase activity demonstrated previously [5] under similar experimental conditions, the data described in this paper are evidence against Gold's hypothesis [12], at least so far as the two tumors which we studied are concerned. In our opinion the mechanism of the therapeutic action of HS on malignant tumors must be sought in its effect on other biochemical stages. The resultant effect in this case will be determined by its action on several such stages, one of which, but by no means the most important, may be gluconeogenesis, since its inhibition would be revealed in any event in intact rats during the first few hours after administration of a toxic dose of HS, several times greater than the therapeutic dose which we used [13]. In particular, the action of HS on vitamin B_6 metabolism [8] and its effectiveness as an inhibitor of biotransformation [7] have been established by the present writers. This last effect causes changes in the level of endogenous metabolites, which could affect both the status of the tumor-bearing animal and also growth of tumor cells. The antimonoamine-oxidase effect of HS has also been demonstrated [8]. Considering the clinical picture during treatment with HS [10], we are inclined to regard this particular effect as the most important in the action of HS on tumor-bearing animals.

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DISTRIBUTION OF SURFACE FIBRONECTIN IN LOW- AND HIGH-DENSITY CULTURES OF NORMAL AND TRANSFORMED MOUSE FIBROBLASTS

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UDC 616-008.93:577.112.853]-092.4+612.015.2:577.112.853]-085.23

KEY WORDS: fibronectin; fibroblasts; transformed cells.

Fibronectin is a high-molecular-weight adhesive glycoprotein synthesized by cells of different tissues. It is present on the cell surface and in biological fluids [2, 5, 6]. Surface fibronectin is often deposited in the form of fibrillary polymers, and together with collagen and glycosaminoglycans it forms the external supporting matrix of the cells. It has been shown by lactoperoxidase iodination that malignant transformation (especially virus-induced) of connective-tissue cells is usually accompanied by partial or complete loss of

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(Presented by Academician of the Academy of Medical Sciences of the USSR N. A. Kraevskii.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 1, pp. 74-76, January, 1984. Original article submitted February 17, 1983.

0007-4888/84/9701-0090$08.50

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surface fibronectin. Similar results have been obtained for several types of cells by the immunofluorescence method [2, 8]. Loss of fibronectin is considered to be one of the factors responsible for weakening of adhesion of tumor cells to the substrate compared with normal cells. Nevertheless, some transformed cells have considerable quantities of surface fibronectin [2, 3]. It is interesting to study the organization of fibronectin on the surface of those transformed cells which still retain it.

In the investigation described below the distribution of surface fibronectin was studied in cultures of normal and transformed fibroblasts by the indirect immunofluorescence method.

EXPERIMENTAL METHOD

Secondary cultures of normal embryonic fibroblast-like cells of C3HA mice (MEF) and transplantable cultures of 4 transformed lines of mouse fibroblasts (L, LSF, M-22, and MTs-1) were used. The origin of the mouse lines and conditions of culture were described previ-

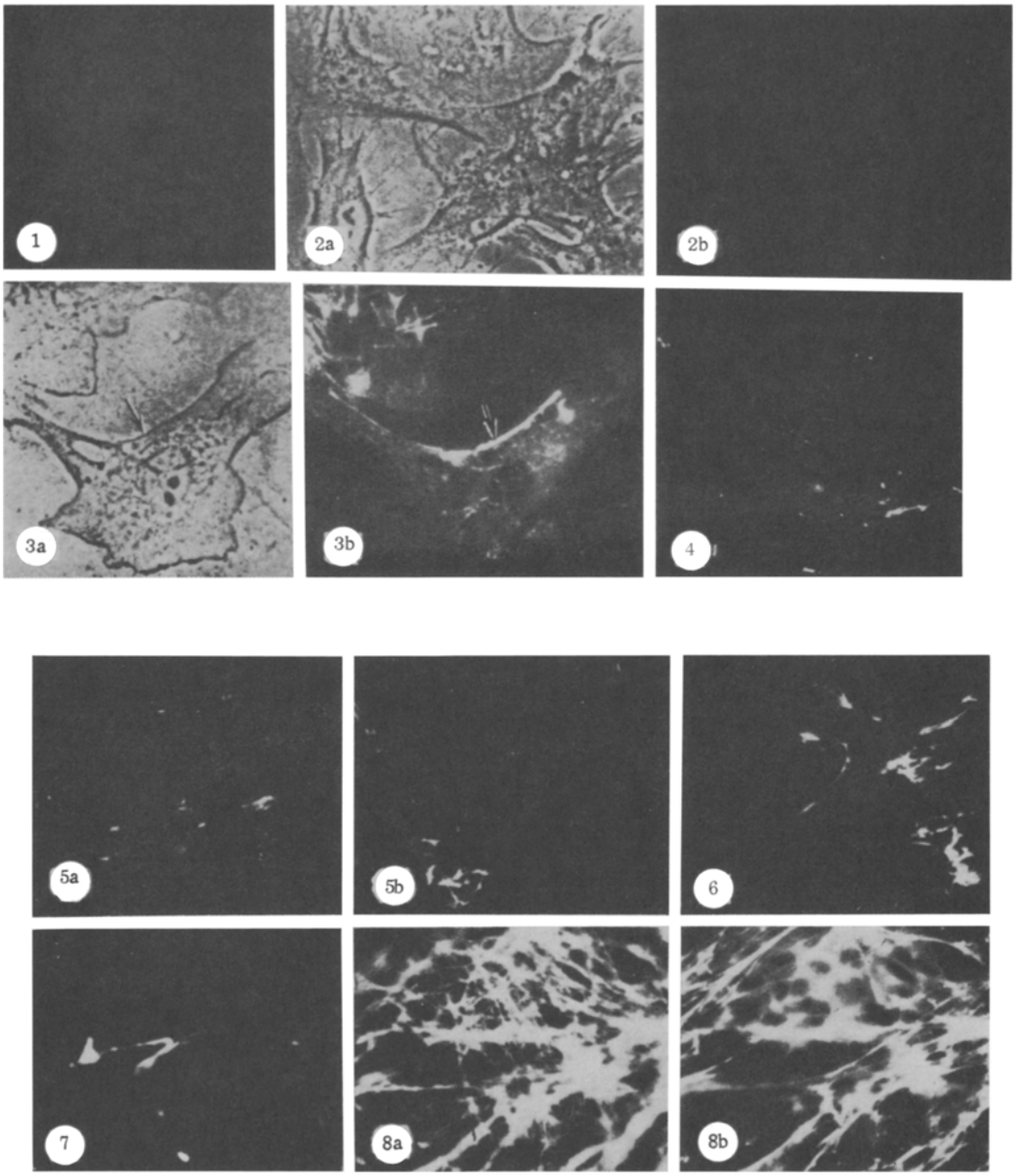

Fig. 1 (1-8)

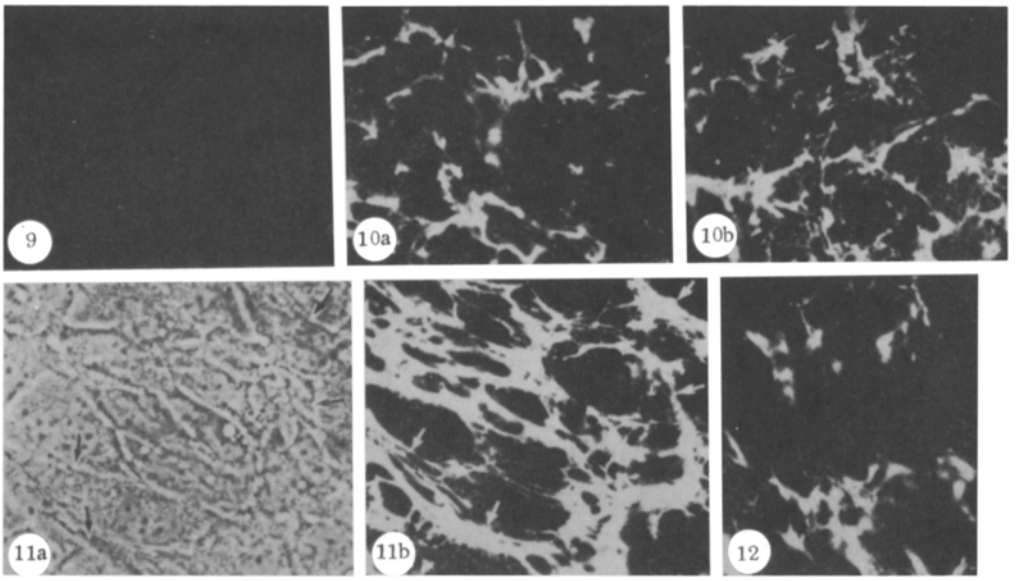

Fig. 1. Distribution of fibronectin in low- and high-density cultures of different mouse fibroblasts. 1) High-density culture (HDC) of MEF. Stained with antiserum preincubated with equal volume of fibronectin (1 mg/ml); 2 (a, b) — low-density culture (LDC) of MEF after treatment with trypsin; 3 (a, b) — LDC of MEF. Arrow indicates stable border of cell, stained for fibronectin; 4) LDC of L cells; 5) LDC of LSF in medium with (a) and without (b) serum; 6) LDC of M-22; 7) LDC of MTs-1; 8) HDC of MEF. Focus on lower (a) and upper (b) surface of monolayer; 9) HDC of L cells; 10) HDC of LSF in medium with (a) and without (b) serum; 11) HDC of M-22. Arrows indicate cell granules, stained for fibronectin; 12) HDC of MTs-1. 2a, 3a, 11a) Phase contrast; in other cases, fluorescence. 570 ×.

ously [3]. The cells were grown in plastic flasks (Falcon, USA). Low-density 2-3-day cultures and high-density 4-6-day cultures were used in the experiments. For indirect immunofluorescence the cultures were washed 4-5 times with Hanks' solution and fixed for 30 min with 4% formaldehyde solution in Dulbecco's physiological saline without Ca^{++} and Mg^{++} (DPS), pH 7.3-7.4 (all procedures were carried out at room temperature). Fixed preparations were kept at 4°C. They were then treated at room temperature by the method in [8]. The cells were washed carefully to remove fixative in DPS and incubated for 1 h with monospecific rabbit antiserum (dilution 1:100) against human fibronectin. The antiserum was generously provided by Professor A. Vaheri (Helsinki University, Finland). The reason why antifibronectin antisera could be used in a heterologous system is that they give a considerable cross reaction with each other irrespective of the source of the fibronectin [4]. Before the work the antiserum was exhausted with culture serum under the control of double immunodiffusion in agar, so that the exhausted antiserum reacted only with cellular fibronectin. The cells were then washed a further three times, for 10 min each time, in DPS and incubated for 1 h with fluorescein isothiocyanate-labeled pig antiserum (dilution 1:20) against rabbit immunoglobulins (from Sebia, France). After vigorous washing in DPS (3 times, 10 min each time) the cells were mounted in nonfluorescent glycerin in DPS and examined in the III photomicroscope (Opton, West Germany) with an oil immersion objective (40) and 12.5 ocular. Each field of vision was photographed under fluorescence and phase contrast conditions. Controls for autofluorescence of the cells, background fluorescence after treatment with the first antiserum, and for nonspecific fluorescence due to adsorption of the second antiserum, were all completely negative. Fibronectin was isolated from C3HA mouse blood plasma by consecutive affinity chromatography on columns with gelatin-sepharose (type I gelatin, from Sigma, USA; sepharose 4B, activated by cyanogen bromide, from Pharmacia, Sweden) and arginine-sepharose [7]. The preparation was homogeneous, as shown by electrophoresis in a 7.5-15% gradient of polyacrylamide gel and sodium dodecylsulfate and it effectively neutralized antifibronectin antiserum (Fig. 1), whose monospecificity had been confirmed. To prove the surface localization of the fibronectin, cultures were treated with trypsin (180 units/mg, from Serva, West

Germany) in low doses (2-10 μg-ml, 10 min, 37°C), and then prepared for immunofluorescence as described above. In every case trypsin caused disappearance of specific fluorescence (Fig. 1: 2a, b), demonstrating the surface location of the fibronectin.

EXPERIMENTAL RESULTS

In low-density MEF cultures thin fibronectin fibrils were found on the surface of the cells. They were located mainly in the perinuclear region (but not beneath or above the nucleus) or along the stable borders of the cells (Fig. 1: 3a, b). Fibrils were found less frequently in the region of the cell junctions. Some were located on the upper surface of the cells. By altering the focusing of the microscope fibrils could also be found on the lower surface, in the region of contact of the cells with the substrate. In the zone of the lamelloplasm specific fluorescence was observed only on the lower surface of the cells, when it appeared as dots or very short fibrils.

In low-density cultures of transformed fibroblasts (especially in lines L and LSF) the quantity of surface fibronectin was usually less than in MEF. Immunofluorescence revealed fibronectin fibrils or punctate fluorescence mainly on the lower surface of the cells. Antigen was found mainly at the sites of cell junctions and also along the stable borders of the cells and their long processes (Fig 1: 4-7).

In high-density cultures of MEF, fibronectin formed a dense network of interwoven fibrils, straight or curved in shape (Fig. 1: 8a, b). They were located on the lower surface of the cells and, probably, on the substrate. Rather fewer fibrils were present on the upper surface. Just as with low-density cultures, fibrils usually were not discovered directly beneath or above the nuclei.

An increase in the content of surface fibronectin with an increase in density of the culture also was observed in most of the transformed lines. Just as in MEF, in dense cultures of lines LSF, M-22, and MTs-1 a network of fibronectin fibrils was found mainly on the lower surface of the cells (Fig. 1: 10-12). Practically no fibrils were seen below or above the cell nuclei. Outlining of the intercellular boundaries by fibronectin fibrils was observed much more often than in MEF. Organization of the fibronectin network in line LSF was similar whether the cells were grown with or without serum. The content of surface fibronectin was indistinguishable from normal in line M-22, it was smaller in lines LSF and MTs-1 than in MEF, and in line L (Fig. 1: 9) it was virtually absent.

In low-density cell cultures (except of line L, which contained hardly any fibronectin) there was much less surface fibrin than in high-density cultures. Dependence of the fibronectin content on culture density also was found in the case of tumor cells. The results are evidence that changes in the fibronectin content during transformation must be studied in high-density cell cultures, where its maximal expression on the surface is observed. This applies in particular to lines such as LSF, the cells of which had little fibronectin in low-density cultures and contained much more of it in high-density cultures (Fig. 1: 5, 10). Changes in the fibronectin content in the tumor lines compared with those in MEF, revealed by the immunofluorescence method, are in full agreement with the results obtained previously by lactoperoxidase iodination [3].

The below-normal content of fibronectin (lines MTs-1 and LSF) or its weaker binding with the surface (line M-22 [3]) did not lead, as this investigation showed, to any appreciable change in its ability to be implanted into the intracellular matrix, with the formation of a network of fibrils. It can be tentatively suggested that in such cases weakening of the adhesiveness of the transformed cells [1] was attributable not (or not only) to fibronectin deficiency, but to a change in the other components of the supporting matrix, such as collagen, which also is frequently lost during transformation of fibroblasts [2]. In this connection it would be interesting to study the content and organization of collagen in the types of cells studied in the present experiments.

The author is grateful to Professor Yu. M. Vasil'ev for useful advice and discussion and to Professor A. Vaheri for providing the antifibronectic antiserum.

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