CARBONIC ANHYDRASE: A MARKER OF CELL TYPES IN GUINEA PIG VAS DEFERENS CELL CULTURE

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During culture of guinea pig vas deferens cells the problem of identification of cell types arises. Besides smooth-muscle cells (SMC), fibroblastlike cells (FLC) and epithelial cells (EC) also are present here. To identify these three types of cells, three types of immunohistochemical markers are usually used. In practice, however, it is preferable to use one marker, which is expressed in these cells with different immunomorphologic features.

In this study, a comparative immunomorphologic investigation was made of the principles of expression of the enzyme carbonic anhydrase III, together with a study of the distribution of cytokeratins and of smooth-muscle myosin.

EXPERIMENTAL METHOD

Male guinea pigs weighing 300-400 g were killed by ether anesthesia, the vasa deferentia were isolated under sterile conditions and freed from connective tissue and blood vessels, and prepared for organotypic and cell culture. Longitudinal strips 4 mm long and with a diameter of cross section of 0.75 mm, and with an undamaged layer of epithelial cells lining the lumen of the duct, were used for organotypic culture. Explants were arranged on a clean coverslip in a plastic Petri dish 40 mm in diameter. Nutrient medium was poured in to wet the sides of the dish, while a concave meniscus was formed at its center. The explants must be kept above the level of the medium by a distance equal to half of their own height. This satisfies the conditions of organotypic tissue culture on the boundary between liquid and gaseous phases [1]. For cell culture the epithelial layer was removed completely from the vas deferens. The tissue was cut into pieces, each measuring 1 mm^3, and covered with dissociating medium, consisting of collagenase 2 mg/ml, bovine serum albumin 2 mg/ml, soy trypsin inhibitor 0.5 mg/ml, and DNase 2 μg/ml, in Dulbecco's buffer without Ca^{2+} and Mg^{2+}. Incubation was carried out for 1 h at 37°C with gentle mixing. The sample was then pipeted 20 times, and the first portion of cell suspension thus formed was centrifuged 3 times in Dulbecco's buffer for 5 min at 1000 rpm each time. The cells which settled were resuspended in nutrient medium and seeded on coverslips in Petri dishes with a density of 10^5 cells/ml. The remaining tissue fragments were covered with fresh dissociating mixture and incubated for a further 2 h. The sample was pipeted 100 times and the second portion of cell suspension thus obtained also was centrifuged 3 times in buffer for 5 min at 1000 rpm each time. The cells were resuspended in medium and seeded on coverslips in a Petri dish with a density of 10^5 cells/ml. Medium of the following composition was used for culture: DMEM medium ("Sigma") 90%, fetal calf serum 10%, insulin 0.8 U/ml, HEPES 10 mM, sodium bicarbonate 1 M, penicillin 10 U/ml, and streptomycin 50 μg/ml. The Petri dishes with the cultures were transferred to airtight miniexsiccators of our own design [2], aerated with a gas mixture consisting of

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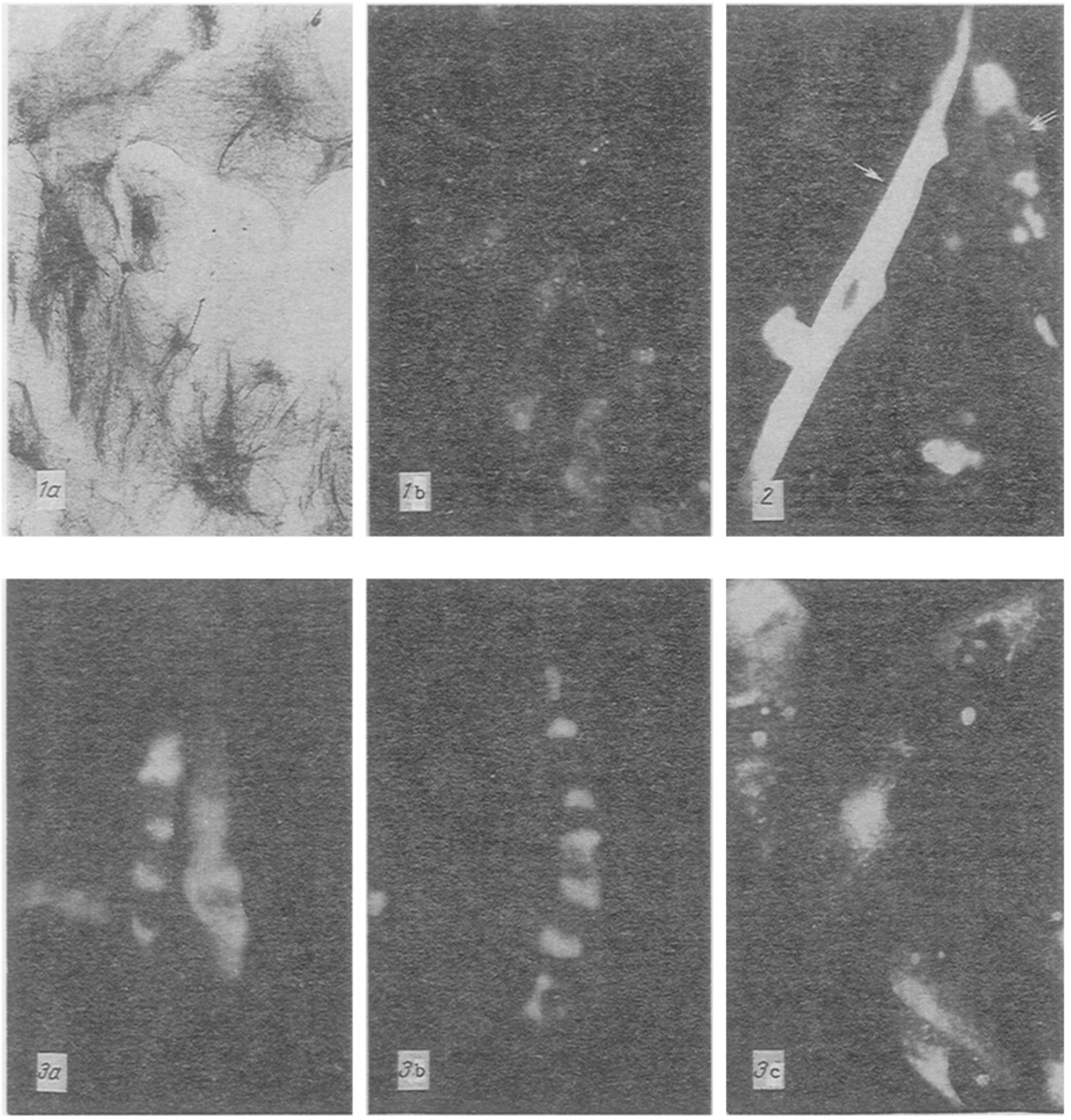

Fig. 1. Epithelial cells having migrated from explants: a) staining with MCAB for cytokeratins by PAP method, b) staining by PCAB for carbonic anhydrase III. Immunofluorescence (objective 40, ocular 5).

Fig. 2. Smooth muscle cell (arrow) and fibroblastlike cell (double arrow) stained by PCAB to myosin from smooth muscles. Immunofluorescence (objective 40, ocular 5).

Fig. 3. Expression of carbonic ahnydrase III in smooth muscle cells (a, b) and fibroblastlike cells (c). PCAB. Immunofluorescence (objective 40, ocular 5).

60% O_2, 35% N_2, and 5% CO_2, and incubated at 37°C. The medium and gas mixture were changed every 3 days. For immunohistochemical investigations frozen sections were cut from explants on the 9th day of culture, as well as sections from freshly isolated vasa deferentia. Cells migrating from the explants on to coverslips and cultures from

the first and second portions of the cell suspension on the 9th day of culture were washed with Hanks' medium and fixed 3 times with methanol for 5 min each time at 4°C, and then dried in air. The immunohistochemical investigation was conducted with the aid of polyclonal antibodies (PCAB) to carbonic anhydrase III, which were generously provided by Professor H.K. Väänänen (Finland). Monoclonal antibodies (MCAB) to cytokeratins were obtained from the "Diagnostikum" Combine (All-Union Oncologic Research Center, Academy of Medical Sciences of the USSR, Moscow). PCAB to myosins from smooth muscles were obtained by N. Sh. Shamsutdinov and co-workers at the V. M. Danilova Kiev University and of the Department of Histology, Kazan' Medical Institute, by M. E. Valiullina. PCAB to carbonic anhydrase III and to myosins were determined by Coons' immunofluorescence method; donkey antibodies against rabbit globulins, labeled with FITC (N. F. Gamaleya Institute of Epidemiology and Microbiology) were used as secondary antibodies. MCAB to cytokeratin No. 8 (clone H1, IgG1) and also MCAB H4 (IgM), reacting with a broad spectrum of cytokeratins of simple and complex epithelia [4], were used in the peroxidase-antiperoxidase (PAP) method. The PAP kit produced by the "Preparat" Combine (Nizhnii Novgorod) was used.

EXPERIMENTAL RESULTS

In frozen sections of cultured and control explants MCAB to cytokeratins stained a thin layer of epithelial cells on the tissue surface but did not stain the SMC layer. PCAB to myosins stained the SMC layer intensely, but were not detected in EC. PCAB to carbonic anydrase III were distributed uniformly throughout the tissue. In many cases, however, precise identification of the cells in frozen sections was made difficult by the overlapping and masking of the fluorescence by neighboring cells.

Cells which migrated from the explant and formed a dense monolayer expressed cytokeratins intensively (Fig. 1). Cytokeratins, as a very thin network, filled the whole of the cytoplasm with condensation in the perinuclear zone. These cells did not stain with PCAB to myosins and had morphological features of EC. Carbonic anhydrase III was clearly revealed in EC in the nuclei only, and was present very thinly along the nuclear membrane.

The culture of the first portion of the cell suspension contained a mixture of SMC and FLC (Fig. 2). Fusiform SMC with elongated nucleus were easily distinguished under the light microscope from spreading FLC with wide processes of cytoplasm and a large nucleus. However, some SMC were small, with a broadened body, and were not easy to distinguish from FLC.

PCAB to myosins stained SMC intensively but diffusely throughout the cell. If the SMC were elongated, the translucent nucleus was easily distinguished. In a shortened SMC the nucleus was masked by perinuclear fluorescence. An FLC alongside the nucleus of a fluorescent SMC appeared very pale (Fig. 2). Under high power, a very thin network of filaments could be seen in the cytoplasm of FLC, mainly in the cell processes. PCAB to carbonic anhydrase III stained SMC in two different ways: diffusely throughout the cytoplasm, not involving the nucleus, and discretely, in the form of transverse luminous lines alternating with dark areas. These transverse lines, numbering 6-8 or, less frequently, 12-16 were uniformly distributed along the length of the cell (Fig. 3). The lines were slightly concave toward the nucleus and decreased in size closer to the end of the cell, where a luminous ellipsoidal body could be seen. Two concave transverse lines around the poles of the nucleus sent filaments from one to the other. In this case the nucleus appeared surrounded by a luminous membrane. Other transverse lines were connected by almost invisible filaments, running beneath the cell membrane itself. The presence of two forms of expression of carbonic anhydrase III can evidently be associated with differences in metabolism and structure of the cytoskeleton of SMC. In FLC, MCAB to carbonic anhydrase III stained the nucleoli most brightly. The cytoplasm exhibited focal granulation, mainly in the perinuclear zone, and mainly toward the direction of movement of the cell. The cytokeratins tested could not be seen in SMC and FLC.

Testing cultures from the second portion of the cell suspension showed that in this case only SMC were present. Evidently the FLC, located in connective tissue on the surface of the vas deferens, were removed by the first incubation with dissociating mixture. Further evidence in support of the absence of FLC in the smooth-muscle layers is given by the fact that during thorough removal of connective tissue from the explants, no migration of fibroblasts into the zone of growth took place. Similarly, we also observed the complete absence of FLC in the tunica media of the aorta [3].

Thus types of cells of the vas deferens can be identified immunohistochemically with the aid of a set of different antibodies. SMC are indicated by their intense staining with PCAB to myosins from smooth muscles.

Antibodies to nonmuscular forms of myosin may perhaps be present in these PCAB [3], and in FLC they stain only the network of filaments. EC are revealed by MCAB to cytokeratins. Carbonic anhydrase III was found in all types of cells studied, diffusely and discretely in the form of transverse bands in SMC, but only in the nuclei and along the nuclear membrane in EC, and in the nucleoli and in the form of focal granulation in the cytoplasm in FLC.

These differences in expression of carbonic anhydrase III described above are such that it can be used as a general marker for the identification of cell types in cultures of guinea pig vas deferens cells.

LITERATURE CITED

1. B. K. Gavrilyuk and V. P. Safronov, Organotypic Tissue Culture [in Russian], Moscow (1983).
2. R. S. Nizamov, Lab. Delo, No. 2, 75 (1990).
3. W. J. Cliff, Blood Vessels, London (1976).
4. S. M. Troyanovsky, V. I. Guelstein, T. A. Tshipysheva, et al., J. Cell Sci., **93**, 419 (1989).