AN APPLICATION OF ELECTRON MICROSCOPIC CYTOCHEMISTRY TO HUMAN TUMORS

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Prostatic carcinoma, renal cell carcinoma and melanoma were studied by electron microscopic enzyme cytochemistry. ACPase of prostatic cancer cells was found in lysosomes, secretory vacuoles and vesicles as well as some Golgi apparatuses. The enzyme localizations remained unchanged in tumor cells two weeks after castration when cells were viable. Renal cell carcinomas revealed to have positive ALPase activity at microvilli resembling brush border and lateral plasma membranes facing adjacent tumor cells. The enzyme was negative at basal plasma membrane of the tumor cells. Proximal tubule cells of human kidney showed ALPase activity only at apical membranes. This indicated functional dedifferentiation of plasma membrane in the tumor cells. These observations were verified and substantiated by TPPase, IDPase and G6Pase localizations in tumor cells. The tumor cells also showed positive G6Pase in endoplasmic reticulum. DOPA oxidase was successfully detected in smooth endoplasmic reticulum close to Golgi apparatus, innermost Golgi cisternae and premelanosomes both in cultured melanoma cells and melanoma tissues, which may have diagnostic value. Thus, EM enzyme cytochemistry was considered to be applicable and useful for human cancer study concerning histogenesis, diagnosis and to assess the treatment.

The contribution of electron microscopy to cancer research has been remarkable in recent fifteen years. Numbers of controversies at the time of light microscopy have been solved by EM studies. Yet, the new approach has brought up much more fundamental problems. One of them is the function of tumor cells in relation to cellular organelles. In this respect, enzyme EM cytochemistry has been applied to human tumors by the author. This has been prompted by the fact that human prostatic cancer cells are rich in acid phosphatase (ACPase) and are androgen dependent.

MATERIALS AND METHODS

Three prostatic carcinomas, ten renal carcinomas and three melanomas were studied. Prostatic tumor tissues were obtained by open perineal biopsy before and two weeks after bilateral orchiectomy. In addition, human melanoma cells

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grown *in vitro* (17) were used to standardize the DOPA reaction at EM level. Tissues were fixed in 2% glutaraldehyde for varying time periods from 15 min to 2 hr, and were incubated in several phosphatase detecting medium in the form of 40 μ section for 5–60 min. Alkaline phosphatase (ALPase) at pH 9.2–9.4 and ACPase at pH 5.0 were detected in both prostatic carcinoma tissues and renal carcinoma tissues according to the method by Gomori (5) and Mayahara *et al.* (12), respectively. Thiamine pyrophosphatase (TPPase) at pH 7.0, inosine diphosphatase (IDPase) at pH 7.0 (13) and glucose-6-phosphatase (G6Pase) at pH 6.7 (21) were also examined in renal carcinoma tissues. DOPA oxidase activity is detected in the medium (14) at pH 6.9 in cultured melanoma cells and melanoma tissues. Cells were sometimes immersed in DOPA medium up to overnight at 4°C and incubated again at 37°C for up to 6 hr. Tissue sections were osmificated, dehydrated in alcohol and propylene oxide and embedded in Epon. Monolayer cells were processed by the technique reported by Chang (4). As control study for enzyme detection, omission as well as change of substrate, chemical inhibition and heat inactivation of the enzyme were employed.

RESULTS AND DISCUSSION

Prostatic cancer cells before and after castration

Human prostatic carcinomas regress by anti-androgenic treatment. Fine structural study revealed tumor cell degeneration after castration (20). EM cytochemistry showed ACPase localization in lysosomes, secretory vacuoles and multivesicular bodies in the prostatic cancer cells. ACPase activity in Golgi apparatuses varied among the organelles. The enzyme localizations in cancer cells were similar to those in epithelial cells of benign prostatic hypertrophy. ALPase was found only at capillary endothelial cells and young fibroblasts.

Biochemically, ACPase activities in cancer tissues decreased markedly two weeks after castration. EM cytochemically, ACPase localizations remained unchanged when cancer cells survived the treatment and appeared viable (1). Degenerative cells failed to show any ACPase activities. Diffuse lead precipitates were sometimes found in the damaged cells. This may indicate limited value of EM cytochemistry. Specimen must be obtained earlier than two weeks in this study. Multiple biopsies in sequence might help to overcome this limitation. Repeated biopsies, however, are difficult to perform considering the surgical risk and benefits of the patient.

Phosphatases in renal cell carcinoma

There has been a big controversy on the histogenesis of the human renal carcinomas, as was represented by the name of hypernephroma (6). Recent ultrastructural studies (15, 16, 18) and histochemical studies showing ALPase activity (2, 10) favored renal tubular origin of the tumor. Precise localization of the enzyme is not clear because of limited resolving power. Ultrastructurally, renal carcinoma cells have pleomorphic nuclei and large lipid droplets, abundant glycogen particles, lysosomes and numetous microvilli. Several tumor cells are seen attached to each other by junctional complexes (Fig. 1). 7 of 9 tumors

(Scales indicate 1 μ .)

exhibited positive ALPase activities. The enzyme was localized to microvilli resembling brush border (Fig. 3) as well as lateral plasma membrane. Basal plasma membranes apposing basement membranes were enzyme negative (Fig. 4). Proximal tubule cells of human kidney showed ALPase activity at brush border, apical invagination and multivesicular body (Fig. 2). The difference of ALPase localizations were interpreted as indicating the functional dedifferentiation of plasma membrane of the tumor cells (23). This concept has been verified and substantiated by the finding of TPPase, IDPase and G6Pase.

Renal carcinoma cells revealed TPPase activity at apical and lateral plasma membrane as well as Golgi apparatuses. IDPase was found positive at apical and lateral membrane as well as Golgi apparatuses. Endoplasmic reticulum was IDPase negative. G6Pase was also positive at apical and lateral membrane in addition to endoplasmic reticulum (Fig. 5). ACPase activities were confined to lysosomes and some Golgi apparatuses. Enzyme localizations in human proximal tubule cells are: TPPase in Golgi apparatuses, IDPase in Golgi apparatuses and endoplasmic reticulum, G6Pase in endoplasmic reticulum (Fig. 6) and ACPase in lysosomes and Golgi apparatuses. No part of plasma membranes showed the four enzyme activities.

When both tumor tissue and kidney tissue were incubated in media without substrates, no reaction product was seen for all enzymes. The addition of 0.01 M NaF to the incubating medium for ACPase resulted in complete suppression of

FIG. 1. Ultrastructure of a renal cell carcinoma. Microvilli resembling brush border and cytoplasmic glycogen particles are observed. Two tumor cells are attached by a junctional complex. $\times 29,000$.

FIG. 2. ALPase activity in proximal tubule cells of human kidney. Tissues were fixed in 2% glutaraldehyde for 60 min and were incubated for 15 min. Reaction products are shown at brush borders, apical invaginations and a multivesicular body. Note the absence of activity at the lateral plasma membrane. $\times 33,000$.

FIG. 3. ALPase activity in a renal carcinoma cell is positive on the microvillous projections of apical plasma membrane. Fixation, same as Fig. 2; incubation, 60 min. $\times 27,000$.

FIG. 4. ALPase activity in renal carcinoma. Lateral plasma membranes of tumor cells facing adjacent tumor cells show activity, while basal membranes do not. A mitotic tumor cell is observed at right lower corner. Fixation, same as Fig. 2; incubation, 30 min. $\times 13,000$.

FIG. 5. G6Pase activity in a renal carcinoma cell is confined to endoplasmic reticulum. Fine lead precipitates are found at the brush border-like structure of the tumor cells. Fixation, same as Fig. 2; incubation 60 min. $\times 42,600$.

FIG. 6. G6Pase activity in a proximal tubule cell of human kidney. Enzyme is localized in endoplasmic reticulum and nuclear envelope (left lower corner). Brush borders are G6Pase negative. Fixation and incubation, same as Fig. 5. $\times 56,000$.

FIG. 7. DOPA oxidase activity in a cultured melanoma cell. Reaction products are found in Golgi cisternae and vesicles as well as melanosomes. Cells were immersed in DOPA medium 4 hr at 4°C, fixed in 2% glutaraldehyde for 1 hr and incubated again for 4 hr at 37°C. $\times 56,000$.

FIG. 8. DOPA oxidase activity in a human amelanotic melanoma. Reaction products are confined to melanosomes and golgi vesicles. Fixation, 1 hr in 2% glutaraldehyde; incubation, 45 hr. $\times 56,000$.

the enzymatic reaction. ALPase medium containing 0.005 M EDTA yielded a negative enzymatic reaction. Suppression of TPPase activity was achieved by addition of 0.05 M NaF to incubating media, though a lower concentration (0.01 M) of NaF failed to inhibit the reaction.

Immunohistochemical study in renal tumor showing brush border antigen in the tumor tissue also supported the proximal tubule origin of the tumor (23). Functional alteration of the plasma membranes has been suggested in proliferating hepatocytes (9).

Based on the findings stated above, following conclusions were obtained. 1) Renal carcinomas arise from proximal tubule cells. 2) Functional dedifferentiation of the plasma membrane actually takes place in this tumor. 3) Metabolic difference is implicated between tumor cells and proximal tubule cells.

DOPA oxidase in cultured melanoma cells and melanoma tissues

Electron dense materials indicating DOPA oxidase activity were localized in smooth ER system close to typical Golgi lamellae, Golgi cisternae and Golgi vesicles, as well as premelanosomes exhibiting various stages of melanization both in cultured melanoma cells (Fig. 7) and melanoma tissues (Fig. 8). Some premelanosomes remained negative. Cells and tissues incubated in medium from which substrate was deleted were completely devoid of reaction products. Immersion of the cells in 95°C buffer solution for 20 min completely abolished the reaction. HeLa cells incubated in DOPA medium also failed to show the reaction products.

The enzyme localizations in human melanoma tissue and cultured melanoma cells were similar to those of B-16 and Harding-Passey melanoma (14), hamster melanoma cells *in vitro* (19), melanocyte of the fowl (3) and regenerating fowl feather (11), and human melanocytes (7, 8).

The DOPA oxidase localizations demonstrated an important role of Golgi apparatuses and associated smooth ER concerning melanogenesis. Furthermore, presence of reaction products in smooth ER and Golgi apparatus may serve as a clue to distinguish a melanoma cell from a migrating macrophage in tumor tissues. This certainly had diagnostic value when a tumor tissue showed weak or focal DOPA reaction by light microscopy, as was the case shown in Fig. 8.

Some problems encountered in human tumor cytochemistry

Several limitations experienced in applying EM cytochemistry to human materials are as follows: 1) ischemia during surgery can not be avoided and estimated, 2) warm ischemic time after tissue removal due to clinical and some other service is often too long and damages the tissue, 3) perfusion fixation cannot be employed, 4) human materials in question can not be obtained constantly and consistently, making it difficult to establish research project and program. The last factor is closely related to capacity and man-power of the EM cytochemical laboratory. The factors hindering the participation of basic scientists in this kind of work were discussed in the hope to promote more intimate co-operation among surgeons, pathologists and biologists, which is mandatory to accelerate EM cytochemical studies of human materials.

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REFERENCES

1. Aso, Y., Yokoyama, M., Okada, K., Tokue, A., Koiso, K., Murahashi, I. and Takayasu, H.: Ultracytochemical and biochemical study of acid and alkaline phosphatases in human prostatic cancer cell. *Invest. Urol.* 9; 329, 1972.
2. Braunstein, H. and Adelman, J. U.: Histochemical study of the enzymatic activity of human neoplasm. II. Histogenesis of renal cell carcinoma. *Cancer* 19; 935, 1966.
3. Brumbaugh, J. A.: The ultrastructural effects of the *I* and *S* loci upon black-red melanin differentiation in the fowl. *Develop. Biol.* 24; 392, 1971.
4. Chang, J. P.: A new technique for separation of coverglass substrate from epoxy-embedded specimens for electron microscopy. *J. Ultrastruct. Res.* 37; 370, 1971.
5. Gomori, G.: "Microscopic Histochemistry. Principles and Practice." Univ. Chicago Press, Chicago, Illinois, 1952.
6. Grawitz, P.: Die sogenannten Lipome der Niere. *Virchows Arch. Path. Anat.* 93; 39, 1883.
7. Hunter, J. A. A., Mottaz, J. H. and Zelikson, A. S.: Melanogenesis: Ultrastructural histochemical observations on ultraviolet irradiated human melanocytes. *J. Invest. Dermatol.* 54; 213, 1970.
8. Jimbow, K., Takahashi, M., Sato, S. and Kukita, A.: Ultrastructural and cytochemical studies of melanogenesis in melanocytes of normal human hair matrix. *J. Electron Microsc.* 20; 87, 1971.
9. Karasaki, S.: Subcellular localization of surface adenosine triphosphatase activity in pre-neoplastic liver parenchyma. *Cancer Res.* 32; 1703, 1972.
10. Manheimer, L. H. and Seligman, A. M.: Improvement in methods in histochemical demonstration of alkaline phosphatase and use in study of normal and neoplastic tissues. *J. Nat. Cancer Inst.* 9; 181, 1948.
11. Maul, G. G. and Brumbaugh, J. A.: On the possible function of coated vesicles in melanogenesis of the regenerating fowl feather. *J. Cell Biol.* 48; 41, 1971.
12. Mayahara, H., Hirano, H., Saito, T. and Ogawa, K.: The new lead citrate method for the ultracytochemical demonstration of activity of non-specific alkaline phosphatase (orthophosphoric monoester phosphohydrolase). *Histochemie* 11; 88, 1967.
13. Novikoff, A. B. and Goldfischer, S.: Nucleoside diphosphatase activity in the Golgi apparatus and its usefulness for cytological studies. *Proc. Nat. Acad. Sci.* 47; 802, 1961.
14. Novikoff, A. B., Albala, A. and Biempica, L.: Ultrastructural and cytochemical observations on B-16 and Harding-Passey mouse melanomas. The origin of premelanosomes and compound melanosomes. *J. Histochem. Cytochem.* 16; 299, 1968.
15. Oberling, C., Rivière, M. and Haguénau, Fr.: Ultrastructure des épithéliomas à cellules claires du rein (hypernéphromes ou tumeurs de Grawitz) et son implication pour l'histogénèse de ces tumeurs. *Bull. Cancer* 46; 356, 1959.
16. Okada, K., Yokoyama, M., Tokue, A. and Takayasu, H.: Ultrastructure of renal cell carcinoma. *Urologia* 36; 11, 1969.
17. Romsdahl, M. M. and Hsu, T. C.: Establishment and biologic properties of human malignant melanoma cell lines grown *in vitro*. *Surg. Forum.* 18; 78, 1967.
18. Seljelid, R. and Ericsson, J. L. E.: Electron microscopic observations on specializations of the cell surface in renal clear cell carcinoma, *Lab. Invest.* 14; 435, 1965.

19. Stanka, P., Kinzel, V. and Mohr, U.: Elektronenmikroskopische Untersuchung ueber die Praemelanosomenentstehung an Melanomzellen *in vitro*. *Virchows Arch. Abt. B. Zell-pathologie* 2; 91, 1969.
20. Takayasu, H. and Okada, K.: An electron microscopic study of prostatic cancer cells after castration. *Invest. Urol.* 5; 126, 1967.
21. Wachstein, M. and Meisel, E.: On the histochemical demonstration of glucose-6-phosphatase. *J. Histochem. Cytochem.* 4; 592, 1956.
22. Wallace, A. C. and Nairn, R. C.: Renal tubular antigens in kidney tumors. *Cancer* 29; 977, 1972.
23. Yokoyama, M.: Ultracytochemical and morphological studies of human renal cell carcinoma. *J. Urol.* 108; 62, 1972.