EXPRESSION OF ONCODEVELOPMENTAL MARKERS (REGAN ISOZYME, β -HCG, CEA) IN TUMOR TISSUES AND UNINVOLVED BRONCHIAL MUCOSA. AN IMMUNOHISTOCHEMICAL STUDY¹

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Eighty-nine human tumor tissues were examined for the presence of Regan isozyme of alkaline phosphatase, beta-subunit of human chorionic gonadotropin, and of carcinoembryonic antigen by immunoperoxidase staining.

CEA was the most commonly detectable antigen in the cases examined here, whereas Regan isozyme was rarely found in these tumor specimens.

Multiple expression of the marker antigen, such as concordance of CEA and β -HCG was shown in some cases.

Tumor marker and its incidence seemed to be different depending on the origin of the tumors, histological type, and the degree of differentiation.

Well differentiated tumors were likely to show a higher incidence of certain markers than poorly differentiated carcinomas.

Information is needed to answer fundamental questions regarding the significance of expression in tumors of antigenic proteins (1, 6, 7) which have an identity in prenatal life but are absent thereafter, "oncodevelopmental markers."

One such question is whether or not tumors express one or more oncodevelopmental markers; more specifically, will the same tumor cell express several such developmental proteins?

The answer is not forthcoming from quantitative measurements of these proteins in homogenates of tumor tissue because the homogenates can give only an average value of its constituents. Such results, however, have indicated multiple expression of these oncodevelopmental gene products (3, 5, 14, 16) and have motivated the present study.

More attractive is the possibility of demonstrating these oncodevelopmental

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proteins *in situ* with enzymorphologic and immunohistochemical techniques (5, 8, 9). In this respect, one wonders whether oncodevelopmental gene expression bears any correlation to tumor histology and to organ site. We are reporting our results on human chorionic gonadotropin; β -subunit, carcinoembryonic antigen and Regan isoenzymes on 89 tumor specimens, primarily of lung and breast which reveal information which is relevant to the questions defined.

MATERIALS AND METHODS

Tissues: Tumor tissues from patients with various malignancies were obtained either surgically, by aspiration, or at autopsy in Pondville State Hospital, Walpole, Massachusetts. Histological diagnosis of the tumors were made by Dr. L. Gandbhir, the Chief Pathologist of Pondville State Hospital. No tissue samples were available from normal or non-cancerous individuals. The tissues were fixed mostly in 10% formalin, or occasionally in 10% neutral formalin (phosphate buffered). Fixed tissues were processed in Technicon, and conventional paraffin sections were prepared. Fresh frozen sections or smeared cells were also used in some cases. They were stored at -20°C , until processed for immunohistochemistry. Paraffin sections were dewaxed in warm xylene for one hr and were dehydrated in graded series of ethanol. All the specimens examined by peroxidase-antibody technique were treated with 0.3% hydrogen peroxide in absolute methanol for 30 min to block the activity of endogenous peroxidase.

Antibodies: Anti-Regan isozyme (placental alkaline phosphatase) serum made in rabbit was provided by Dr. G. J. Doellgast, Tufts Cancer Research Center, Boston. Anti-beta-HCG (human chorionic gonadotropin) serum made in rabbit was kindly donated by Dr. A. Martinez-Fernandez, Colorado Medical Center, Denver. Anti-CEA (carcinoembryonic antigen) serum made in rabbit, and extensively absorbed with normal colon tissues, was given by Dr. A. Rule, Tufts Cancer Research Center, Boston. The specificity of each antiserum is described elsewhere (2, 10, 12). Fluorescein, or peroxidase-labelled anti-rabbit IgG goat IgG was commercially obtained from Cappel Laboratory, Downingtown, Pennsylvania.

Histochemical procedures: The tissue sections or smeared cells were incubated with each specific antibody diluted 1:25 to 1:100 for two hr at room temperature. After rinsing the samples, they were incubated with labelled anti-IgG diluted 1:50 to 1:100 for 45 min at room temperature. They were jet-washed between each step with phosphate buffered saline (0.05 M, pH 7.4), which was also used to make serum dilutions. Samples stained with fluorescein-antibody were observed without further treatment on a fluorescence microscope. Others stained with peroxidase-antibody were treated for three min with Graham-Karnovsky's solution (15) made in ammonium acetate buffer (0.05 M, pH 5.5), were counter-stained with methyl green, and were mounted in Diatex. Control experiments were performed by 1) substituting normal rabbit serum for each specific antiserum, 2) by staining without secondary (labelled) anti-bodies, and 3) by staining the sections only with Graham-Karnovsky's solutions. In the majority of these cases, pleural or peritoneal effusions as well as serum had been collected periodically, while the patients were hospitalized. Those fluids and tissue homogenates of the tumors were quantitatively analyzed for each marker by biochemical or immunochemical methods prior to the

TABLE 1. *Incidence of onco-developmental marker antigens in human cancer.* Immunohistochemical studies were performed on paraffin sections, fresh-frozen sections, and/or smeared cells of various tumors for Regan isozyme of alkaline phosphatase (R), beta-subunit of human chorionic gonadotropin (β -HCG or H), and carcinoembryonic antigen (CEA or C) simultaneously. Peroxidase-labelled antibody or fluorescein antibody methods were employed. Only those stained significantly were judged as positive, but the intensity of the reaction was not shown here.

	Case examined	Case with at least 1 antigen	Regan isozyme	β -HCG	CEA	Case with multiple antigens	R+H	R+C	H+C	R+H+C
Lung										
Adeno. ca.	15	12	1	3	10	3	0	0	3	1
Squam. Cell ca.	7	6	0	2	3	2	0	0	2	0
Undiff. ca.	13	5	0	2	3	2	0	0	1	0
Total	35	23	1	7	16	7	0	0	6	1
Breast										
Well-moderate diff. adenoca.	12	7	0	1	4	1	0	0	1	0
Poorly diff. adenoca.	4	0	0	0	0	0	0	0	0	0
Total	16	7	0	1	4	1	0	0	1	0
Oral cavity										
Well-moderate. diff. squam.	5	1	0	0	1	0	0	0	0	0
Poorly diff. squam. cell ca.	3	0	0	0	0	0	0	0	0	0
Total	8	1	0	0	1	0	0	0	0	0
Miscellaneous										
G-I tract	8	4	2	2	2	2	0	1	1	0
Ovary	4	2	1	1	1	1	0	0	1	0
Uterus	3	2	1	2	1	2	1	0	1	0
Urinary blad.	2	1	1	0	1	1	0	1	0	0
Parotid gld.	2	1	0	1	0	1	0	0	1	0
Prostatic gld.	2	0	0	0	0	0	0	0	0	0
Others	9	2	2	1	3	1	0	1	0	0
Total	30	12	7	7	8	8	1	3	4	0

experiments. Those results have been presented elsewhere (11).

RESULTS

Results are shown in Figs. 1-7.

Lung cases: Thirty five cases of lung cancer (15 moderately to well differentiated adenocarcinomas, seven moderately to well differentiated squamous cell carcinomas, and 13 undifferentiated carcinomas) were examined. Of the adenocarcinomas, 12 out of 15 cases showed at least one marker antigen; ten cases with CEA, and four cases with beta-HCG. Three out of latter four cases had double antigen; CEA and beta-HCG. In squamous cell carcinomas, six out of seven cases showed either CEA or beta-HCG, and two of them had both. In undifferentiated carcinomas, five out of 13 cases showed either CEA or beta-HCG, and one of them had both.

Breast cases: Sixteen cases of breast cancer; 12 moderately to well differentiated adenocarcinomas, and four poorly differentiated adenocarcinomas were examined. In the former group, seven out of 12 cases had either CEA or beta-HCG, and one of them had both markers. No marker antigens were detected immunohistochemically in all four cases of poorly differentiated adenocarcinomas in our study.

Oral cavity cases: (including esophagus, larynx, and tongue): Eight cases of squamous cell carcinoma; five moderately to well differentiated, and three poorly differentiated ones were examined. All of these cases except one showed none of these markers. Only one case of tongue cancer revealed CEA in the tumor cells.

Miscellaneous: Four stomach cancer, three colon cancer, and one rectum cancer were examined. Regan isozyme was detected in a case of stomach cancer, and in that of colon cancer. Two other stomach cancers gave no staining for such markers. In two cases of colon cancer, either CEA or beta-HCG was detected. CEA was detected in one case each of adenocarcinoma of ovary, uterus, parotid gland, gall bladder and pancreas, transitional cell carcinoma of urinary bladder, and mesenchymoma. Beta-HCG was detected in two cases of adenocarcinoma of uterus, one case each of ovarian adenocarcinoma, and of parotid gland. Regan isozyme was stained in single cases of ovarian adenocarcinoma, uterus, gall bladder, and transitional cell carcinoma of urinary bladder.

Tissues of various segments of the tracheobronchial tree: These were also examined in 13 cases. This preliminary study was performed in parallel with biochemical studies using cell homogenates from bronchial mucosa of cancer patients, in order to find any markers in morphologically normal tissues from cancer patients. In some cases, either CEA or β -HCG was detected biochemically in significantly elevated levels in

FIG. 1. Regan isozyme on paraffin section of endometrial tumor, stained by peroxidase antibody method. $\times 200$

FIG. 2. Regan isozyme on smeared cells of transitional cell carcinoma of the urinary bladder, stained by peroxidase-antibody method. Note the reaction exclusively on the cell surface. $\times 400$

FIG. 3. β -HCG on paraffin section of endometrial tumor, stained by peroxidase antibody method. The sample is identical with that of Fig. 1. $\times 100$

FIG. 4. β -HCG on paraffin section of squamous cell carcinoma of the lung, stained by peroxidase antibody method. Note the diffuse staining in the cytoplasm. $\times 400$

FIG. 5. The same with Fig. 4, another portion of the section. $\times 100$

FIG. 6. β -HCG on paraffin section of adenocarcinoma of the breast, stained by peroxidase antibody method. $\times 100$

FIG. 7. β -HCG on paraffin section of ovarian adenocarcinoma, stained by peroxidase antibody method. $\times 100$

cell homogenates of bronchial mucosa, though the bronchi were diagnosed as not invaded by tumor cells. In the present studies, conventional paraffin sections were used for histochemical staining of markers. Positive staining for either CEA or β -HCG was obtained in eight cases. Nevertheless, three of them showed no markers in the tumor tissue itself by biochemical methods. In five other cases, such markers were found both in tumor tissues and bronchial mucosa, even though the tumors

FIG. 8. CEA on the same sample of Fig. 7. $\times 100$

FIG. 9. CEA on paraffin section of tubular adenocarcinoma of the colon, stained by peroxidase antibody method. Note the reaction mainly on the luminal surface of the gland-like structure. $\times 200$

FIG. 10. CEA on paraffin section of undifferentiated lung tumor, probably oat cell cancer, stained by peroxidase antibody method. Diffuse cytoplasmic reaction is shown on this specimen. $\times 100$

FIG. 11. CEA on colon tumor of another case. Metastatic lesion in the liver, stained by peroxidase antibody method. $\times 100$

FIG. 12. CEA on the bronchial mucosa of the patient with lung cancer (CEA-positive). Some of the basal cells are intensely stained for CEA. $\times 100$

were not adjacent to the segment of bronchi examined here. Figure 7 shows presence of CEA in basal cell layer of bronchus of the patients with lung cancer. These experiments should be followed by extensive studies using non-neoplastic lung tissues from normal subjects.

	Cases	Markers (+) in Tumor	Markers (–) in Tumor
Markers (+) in Bronchus	(8)	5 cases	3 cases
Markers (–) in Bronchus	(5)	4 cases	1 case

DISCUSSION

Immunohistochemical detection of three onco-developmental markers (Regan isozyme, β -HCG, and CEA) was performed on 89 cases of human malignancies. Although the cases examined were rather few, and those were exclusively of cancer patients, some interesting findings seemed to be drawn from these results.

In lung cancer cases, a high incidence of marker antigens were detected both in adeno- and squamous cell carcinoma. Undifferentiated carcinoma showed such markers much less frequently as compared to another type of lung cancer. CEA was the most commonly detectable marker in the lung cancer in this study, and its incidence was almost similar both in adeno- and squamous cell carcinoma. Some cases of lung cancer had β -HCG or CEA. Regan isozyme was found in solitary case of lung cancer, and in this case, CEA and β -HCG was also found at the same time. The protocol of this particular case was presented in a previous report (11).

In the breast cases, the incidence of such markers in these tumor cells were a little lower than in the lung cases. In this group, also, CEA was found most commonly, but β -HCG or Regan isozyme was almost negative. Even CEA was not stained in poorly differentiated adenocarcinoma of the breast, which did not evidence any of the three markers studied.

In the tumors of the oral cavity (tongue, pharynx, esophagus), such markers were utterly negative, except one case with CEA.

The above findings seemed to show that the marker and its incidence was considerably different depending on the origin of the tumors, histological type, and the degree of differentiation. In the present study, well differentiated tumors were likely to show a higher incidence of certain markers such as CEA than poorly differentiated carcinomas. Mucin-producing tumors sometimes showed Regan isozyme or CEA, although the tumors were classified as poorly differentiated tumors.

Reviewing miscellaneous malignancies, Regan isozyme was detected in a wide range of tumor organ origins. Regan isozyme, known to be identical with term placental type of alkaline phosphatase, was not well-represented in the series of cases of lung or ovarian tumors presented here.

Multiple expression of marker antigens was found, though in few cases, in tumors of diverse organ sites. Concordance of CEA and β -HCG was most frequently shown in these cases, especially in lung adenocarcinomas. Some other reports pointed out the discordance of these two markers (13), however. The previous studies of our group showed the concordance of β -HCG and Regan isozyme in the biological fluids

from the patients with ovarian carcinoma (4). In the present study, however, such types of tumors were very few, and no comments could be made on the concordance or discordance of marker antigens.

The incidence of multiple staining for antigens on the histologic sections was much lower than expected from the results of biochemical studies. This might be due to the lesser sensitivity of the procedures of histological preparations. Nor have we yet been able to demonstrate multiple expression of markers on each tumor cell. However, considering the ratios of stained cells on serial sections, it would be safe to assume that at least some tumor cells produced multiple antigens. Goldenberg *et al.* reported the discrepancy of his results between biochemical and immunohistochemical studies (8). In fact, the staining for one marker was not always homogenous on a large section. That could not be explained from considerations of cell cycle, fixation, or other factors.

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