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# AN ENZYMOCYTOCHEMICAL STUDY OF ALKALINE PHOSPHATASE ISOENZYMES IN GASTRIC CANCER — LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS

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**By using light and electron microscopic cytochemical technique, the activities and distributions of AP isoenzymes in gastric cancers and benign gastric diseases were examined. The results showed: Nagao, Regan and Kasahara isoenzymes, which were not expressed in normal gastric mucosae and non - malignant lesions, might be considered as tumor markers of gastric cancer; The epithelium of intestinal metaplasia exhibited intestinal - type AP only, which was one of the markers of well - differentiated intestinal metaplasia. In the view - point of the gene expression of AP isoenzymes, two mutation hypothesis and recessive - gene mutation hypothesis might be fit for gastric cancer.**

**Key word: Alkaline phosphatase isoenzymes, Gastric cancer, Cytochemistry, Light microscopy, Electron microscopy.**

Neoplastic alkaline phosphatase (AP) isoenzymes were first reported by Fishman (1968) and a general interesting has been focused on it.[1] The studies of AP isoenzymes in gastric cancer were carried out mainly by using biochemical methods and few enzymocytochemical studies were reported in the literature.[2] The source of each AP isoenzyme and whether AP isoenzymes exhibited in precancerous lesions were not very clear. In the current study, the activities and distributions of AP isoenzymes in gastric cancer tissues and precancerous lesions were examined by using light and electron microscopic enzymocytochemistry. The types of AP isoenzymes and their significances in gastric cancer cells, the relationship between intestinal metaplasia, dysplasia and gastric cancer, and the mechnism of malignant transformation were discussed especially.

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## MATERIALS AND METHODS

Surgically resected specimens of 60 cases of advanced gastric cancer and 11 cases of benign gastric diseases (gastric ulcer 7 cases, chronic gastritis 3 cases and gastric polyp 1 case) including 38 cases of intestinal metaplasia and 42 cases of dysplasia were obtained. Normal full - term placenta, adult duodenum of human and kindney of mice were selected as control specimens for placental

- type (PAP), intestinal - type (IAP) and liver - type (LAP) respectively. After removal, the tissue pieces ($1.5 \times 1.0 \times 0.2 cm^3$) were immersed in the fixative immediately for following enzymocytochemical investigations.

## Light Microscopic Enzymocytochemistry

The serial cryosections with thickness of 8 $\mu$m were performed after fixation in precooled 4% formaldehyde (pH 9.3) for 3—5 hr at 4℃. For histological diagnosis and classification of intestinal metaplasia, HE and mucin histochemical stain—AB (pH 2.5)/PAS and AB (pH 1.0)/PAS were performed. The conventional AP activity stain was done by using modified Mayahara method.[3] According to the different sensitivities of AP isoenzymes to heat denaturation (65℃ 10 min and 20 min) and various inhibitors (12.5 mmol/L L - phenylalanine, 12.5 mmol/L L - leucine and 1 mol/L thiocyanate), a light microscopic cytochemical technique of AP isoenzymes was established.[4] The reaction mixture without substrate was used as negative control and three control specimens were used as positive controls. After incubation the sections were developed in 1% sulferamine and counterstained in 2% methyl green. AP activity was graded (Table 1) in order to analyze the types of AP isoenzymes correctly (Table 2).

*Table 1. The criteria of AP activity grades*

| Grade | Distribution | Colour |
|---|---|---|
| - | All cells | Negative |
| ± | Sparse local areas | Light to dark brown |
| + | More than 2/3 or less than 2/3 cells | Light brown or brown |
| + + | More than 2/3 or less than 2/3 cells | Brown or dark brown |
| + + + | All cells or more than 2/3 cells | Brown or dark brown |

## Electron Microscopic Enzymocytochemistry

After fixation in precooled 1.5% glutaral - dehyde (pH 7.4) for 1—2 hr at 4℃, the 40 $\mu$m serial cryosections were made and collected on the glass slides which coated with insulating plastic film which was previously immersed in 0.6% nitrocellulose in amyl acetate, airdried at room - temprature and stored at 4℃ before incubation. The incubation procedure, as same as the above, was performed simultaneously with light micros - copic enzymocytochemistry. After rinsed in cocodylate buffer, the sections were postfixed in freshly prepared 1% $OsO_4$ containing 1.5% $K_4Fe(CN)_6$ for 1 hr at 4℃. The processes of dehydration, immersion and embedding were demonstrated all over the plastic film. The appropriate sites selected under light microscope were digged out and adhered to the tip of waste Epon 812 embedding blocks. Ultrathin sections were observed and photographed under a transmission electron microscope.

*Table 2. The cellular origins and enzymologic features of AP isoenzymes*

| AP isoenzyme | Cellular origin | Activity | Heat | L-phe | L-Leu | thi |
|---|---|---|---|---|---|---|
| LAP | Interstitial & cancer cells | + + + | - | + + + | + + + | - |
| IAP | Intestinal metaplastic & cancer cells | + + + | - | - | + + + | + + |
| PAP (Regan isoenzyme) | Placental syncytiotrophoblast & cancer cells | +—+ + + | +—+ + + | - | +—+ + + | - |
| Nagao isoenzyme | Cancer cells | +—+ + + | +—+ + | - | - | + |
| Kasahara isoenzyme | Cancer cells | ± | - | - | - | - |

## RESULTS

Normal gastric epithelium showed no AP activity, but the interstitial cells (capillary endothelial cell, inflammatory cells and fibroblast) of lamina propria of normal gastric mucosa and benign gastric lesions exhibited LAP activity (+— + + +) invariably. Ultrastructically, LAP positive material was localized predominantly in the cytoplasmic matrix of capillary endothelial cell and on the cell membrane of lymphocyte and neutrophil looked "rail-like".

Intestinal metaplastic epithelium expressed IAP only. The positive rate was 81.6% (31/38) which was much higher than that in normal gastric mucosa, dysplasia and gastric cancer ($P < 0.05$). Generally, more IAP activity was found on the surface epithelium of intestinal metaplasia than in the crypt and deep intestinal metaplastic glands. According to the mucin histochemical stain intestinal metaplasia was classified into four types (Table 3).

*Table 3. The expression of IAP in intestinal metaplasia*

| Type of intestinal metaplasia | No. | Positive No. (%) | Activity |
|---|---|---|---|
| Complete colonic intestinal | 29 | 22(75.9) | ±—+ + + |
| Incomplete colonic intestinal | 17 | 6(29.4) | ± |
| Complete small intestinal | 16 | 15(93.8) | ±—+ + + |
| Incomplete small intestinal | 7 | 2(28.6) | ± |

In complete types more IAP activity was found and the positive rate was much higher than that in incomplete types ($P < 0.01$). but no significant difference was found between colonic and small intestinal types ($P > 0.05$). Under light microscope, the IAP activity was mainly localized at striated border and in the apical cytoplasm of absorptive cells. Under electron microscope, the microvilli and endoplasmic reticulum of absorptive cells existed IAP reaction products.

All of 42 cases of dysplasia including 24 cases of intestinal metaplastic dysplasia which originates from intestinal metaplastic epithelium showed no AP activity.

The AP positive rate was 21.3% (13/60) in gastric cancer, 24.6% (8/27) in differentiated type (papillary, tubular and mucoid adenocarcinoma) and 15.2% (5/33) in nondifferentiated type (poorly-differentiated adenocarcinoma and signet-ring cell carcinoma). There was no significant difference of AP positive rate between two types ($P > 0.05$). By analysing AP enzymologic characteristics five types of AP isoenzymes—LAP, IAP, Nagao, Regan and Kasahara isoenzymes were recognized in gastric cancer cells (Table 4). The cancer cells of different areas in the same case might exhibite different AP isoenzymes, and the cancer cells of the same area might also exhibite two types of AP isoenzymes. Under light microscope, the AP activity in well-differentiated adenocarcinoma was distributed at the lumen side of cancerous glands and in the apical cytoplasm with a certain polarity, and sometimes covered the whole cancer cell when the AP activity was intense (Figure 1). While in poorly differentiated adenocarcinoma the AP activity was relatively weeker and distributed diffusely in the cytoplasm, cell membrane and nucleus (Figure 2). Under electron microscope, 13 cases of gastric cancer were examined and four cases were AP positive. One case of poorly-differentiated adenocarcinoma showed LAP activity. Among the other three cases of tubular adenocarcinoma, one showed LAP and two showed Nagao isoenzyme activity. In tubular adenocarcinoma, no significant difference of activity and distribution between LAP and Nagao isoenzyme was found. The enzymoactivity was intense and distributed diffusely in the cytoplasmic matrix, cell membrane and nucleoplasm (Figure 3). While in poorly - differentiated adenocarcinoma, the LAP activity was weaker and mainly localized at cell membrane and nuclear membrane (Figure 4). In all of four AP positive cases, it was found that the AP positive and negative cancer cells often mingled with each other ultrastructically.

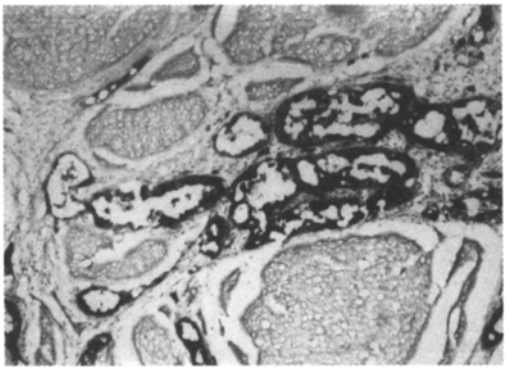

Fig.1. Tubular adenocarcinoma. The AP activity (+ + +) was insensitive to heat (65℃, 20 min). It was Nagao isoenzyme. Heating group 200×.

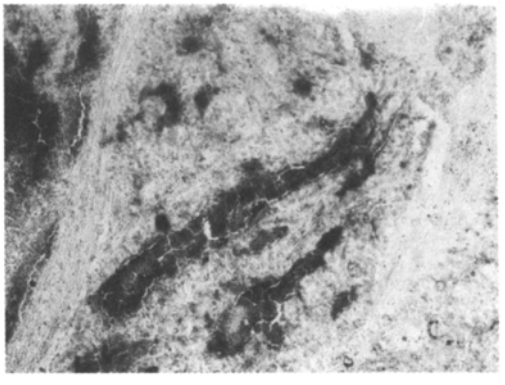

Fig. 2. Poorly - differentiated adenocarcinoma. The AP activity (±) distributed in sparse local areas with colour of dark brown and was sensitive to heat and three inhibitors. It was Kasahara isoenzyme. Conventional group 200×.

*Table 4. The expression of AP isoenzymes in gastric cancer*

| Histologic type | No. | Positive No. (%) | Activity | Type of isoenzyme (positive No.) |
|---|---|---|---|---|
| Papillary adeno-carcinoma | 3 | 1(33.3) | + + + | Nagao, a few of tubular adenocarcinoma IAP + LAP |
| Tubular adeno-carcinoma | 21 | 7(33.3) | ±—+ + + | Nagao(4), LAP(3), IAP(1) Kasahara(1) |
| Poorly-differen-tiated adenocar-cinoma | 32 | 5(15.6) | ±—+ + | LAP(3), IAP(1), Nagao(1) Regan(1), Kasahara(1) |
| Mucoid adenocar-cinoma | 3 | 0 | - | |
| Signet ring cell carcinoma | 1 | 0 | - | |

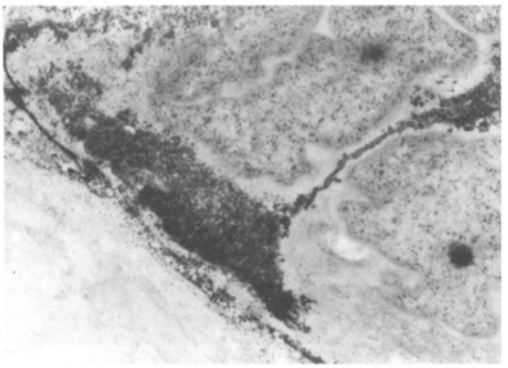

Fig. 3. Tubular adenocarcinoma. Besides some AP(+) fine granules on the cell membrane and in the nucleoplasm, a great number of high electron-dense granules existed in the cytoplasm. Con-ventional group 12,000×.

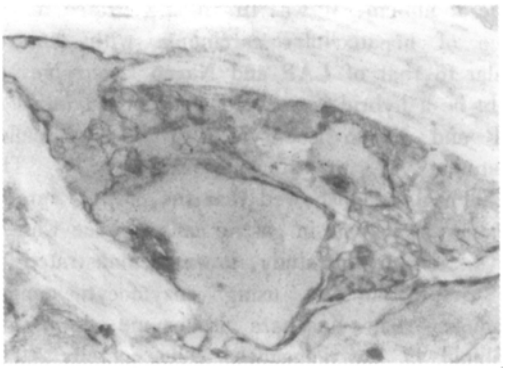

Fig. 4. Poorly-differentiated adenocarcinoma. The AP(+) reaction materials mainly localized on the cell membrane looked irregular "rail-like". Conventional group 12,000×.

## DISCUSSION

### The AP Isoenzyme Types and Their Significances in Gastric Cancer Cells

PAP exists in the syncytiotrophoblast of normal human placenta. The carcinoplacental PAP includes Regan isoenzyme (PAP) and Nagao isoenzyme (placental-like AP) which is a rare

variant of PAP whose features are similar to that of PAP except for more sensibility to L – leucine and EDTA and less sensibility to thiocytate. The carcinoplacental AP had been demonstrated in gastric cancer tissue by biochemical, enzymocytochemical and immunohistochemical techniques.[2,5,6] It might also be present in the sera of the patients with gastric cancer. In this paper the positive rate of carcinoplacental AP was 11.7% (7/60), and the most (6/7) showed Nagao isoenzyme with intense activity (+—+ + +). Since no carcinoplacental AP activity was exhibited in normal gastric mucosa and nonmalignant lesions, it might be considered as one of tumor markers of gastric cancer. If Nagao and Regan isoenzymes were present in sera, gastric juices and gastric mucosae of the patients suffering from chronic gastric diseases (except for pregnant woman), the posibility of gastric cancer should be considered seriously.

Kasahara isoenzyme exists in the cultural FL cells of amnion and not be found in any normal tissue of human. It was first demonstrated in the tissue of hepatocllular carcinoma with features similar to that of LAP and Nagao isoenzyme. It might be a hybrid isoenzyme made of subunites of LAP and Nagao isoenzyme.[1] It is also called hepatoma AP or Regan variant AP. The biochemical study showed that the positive rate of Kasahara isoenzyme in gastric cancer tissue was 7.3%(4/55). In this study, it was demonstrated for the first time by using enzymocytochemical technique that Kasahara isoenzyme might be exhibited in gastric cancer cells actually. The enzymoactivity in both positive cases was weak with distribution in sparse local areas and colour of dark brown (Figure 2). Since Kasahara isoenzyme was negative in normal gastric mucosa and nonmalignant lesions, it might also be regarded as one of tumor markers of gastric cancer and not be specific for hepatocellular carcinoma.

LAP is widely expressed in human tissues such as liver, kedney, bone, lung etc. LAP activity in gastric cancer tissue had been considered to originate from interstitial components, but in this paper it was first approved that the gastric cancer cells also expressed LAP with intense activity and more frequency (7/60) than any other AP isoenzyme. Since LAP also existed in normal gastric mucosa and benign gastric lesions with intense activity, so it could not be regarded as tumor marker of gastric cancer. It had been considered that the LAP expressed in tumor cells was early – placental AP.[7] Whether LAP in gastric cancer cells contained early – placental AP could not be testified in the present paper, and the further studies are needed.

**The Relationship between Intestinal Metaplasia, Dysplasia and Gastric Cancer and the Inquiry about Mechanism of Malignant Transformation**

The intestinal metaplastic epithelium expressed IAP only. The positive rate was obviously higher in complete types than in incomplete types ($P < 0.01$). The enzymoactivity was generally greater in surface epithelium of intestinal metaplasia which was differentiated better. Under electron microscope, the enzymoactivity was localized mainly at microvilli of absorptive cells which might be related to the function of material absorption. The findings above suggested that the expression of IAP in intestinal metaplasia was one of markers of well – differentiation from gastric to intestinal epithelium. It might be the result of activation of intestinal gene in stem cells in the process of benign directional differentiation. While in gastric cancer the AP expression was not related to the differentiation ($P > 0.05$). The phenominon that non-differentiated type of gastric cancer showed weaker enzymoactivity and lower positive rate of AP might be related to its lower functions and metabolism. The expression of three carcino – fetal AP (Regan, Nagao and Kasahara isoenzymes) indicated a fetalism tendency of gene expression in gastric cancer cells. The phenominon that the different AP isoenzymes were expressed simultaneously in the cancer cells of the same area indicated a "nondirective" disdifferentiation of ancestral AP gene in the process of malignant transformation. The phenomina that different AP isoenzymes existed in the cancer cells of different areas in a

same case and AP positive and negative cancer cells mingled ultrastructically with each other indicated lability of gene expression or heterogeneity. Therefore, the gene expression of AP isoenzymes in intestinal metaplasia and gastric cancer was different essentially, and no evidence of direct relationship between them was found in the view – point of gene expression of AP isoenzymes.

The biochemical studies[8,9] indicated that the PAP activity existed in dysplasia grade Ⅱ and grade Ⅲ, and PAP expression in dysplasia suggested malignant transformation. But in this study a contradictory result was obtained. It might be due to inaccurate localization of biochemical method, insensitivity of enzymocytochemical method and different grading critaria of dysplasia. The results indicated that even if the AP was expressed in displastic epithelium the enzymoactivity might be much weak, and it is necessary to use the method with both more sensitivity and precise localization for further investigations.

At present it is generally acknowledged that dysplasia is a precancerous change of gastric cancer. We think that the phenominon of "intestinal metaplasia (IAP +) → intestinal metaplastic dysplasia (AP –) → gastric cancer (AP isoenzymes +)" can be explaned by two mutation hypothesis and recessive – gene mutation hypothesis.[10] In intestinal metaplasia genic mutations may occure under the effects of various carcinogens. After first genic mutation the morphologic and metabolic changes of intestinal metaplastic epithelium are dysplasia with various degrees and decline or disappearance of IAP activity, etc. which may be the manifestations of "dedifferentiation" of intestinalized cells. But the first mutation can not inactivate both alletic genes of tumor repressor gene to cause malignant transformation, and also can not derepress AP genes to express tumor AP isoenzymes. So the intestinal metaplastic dysplasia shows AP negative. The second mutation may make tumor repressor gene inactivated absolutely to cause malignant transformation of the cell. At the same time some fetal genes closed in the fetal period may also be activated again. If AP genes which may be located at chromosome 21 are derepressed the various AP isoenzymes can be expressed. Besides some biological significances, the AP isoenzymes expressed in gastric cancer cells may become useful tumor markers in diagnosis of gastric cancer.

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