HISTOCHEMICAL STUDY OF MUCOPOLYSACCHARIDE IN GASTRIC CANCER

SHUNTA HIROSE, MASAYUKI YASUTOMI, NOBUHIRO MURAI,
ZENJI IWASA, MASAOKI HIROSE, KATSUHISA SHINDO,
REIZO ASO, NOBUTERU KIKKAWA,
and A.K.A. RAZZAQ

*The Second Department of Surgery, Osaka University Medical School
Osaka*

Received for publication September 12, and in revised form October 19, 1972

Mucopolysaccharide components at the infiltrating periphery of gastric cancer were examined by means of histochemistry. PAS reaction and alcian blue staining were only weakly positive and no changes were observed after digestion tests. Mowry's colloidal iron reaction was positive and it almost disappeared after incubation with hyaluronidase. These results suggested that Mowry's colloidal iron reaction was most excellent of the three methods of staining for digestion test of mucopolysaccharides. The main component of the substance under discussion was indicated to be hyaluronic acid.

Beyond the infiltrating front of the gastric cancer, Mowry's colloidal iron reaction was markedly intensified while the substance was negative after testicular hyaluronidase digestion, resisted to streptomycin hyaluronidase digestion. This substance was believed to be chondroitin sulfate.

There are many reports of the results of biochemical as well as histochemical studies on mucopolysaccharides in malignant tumor. Recently an increasing attention has been paid not only on tumor cell itself but also on the change of mucopolysaccharides which are the main components of the substances constructing connective tissue. Differences in certain properties of mucopolysaccharides in the normal and malignant tissues have been indicated.¹³⁾ Some scholars place importance upon the change of properties of mucopolysaccharides, which is claimed to be one of the biochemical peculiarities of cancer tissue.⁷⁾

Various methods have been reported for histochemical demonstration of mucopolysaccharides. At present, however, it is believed that none of them are specific enough to identify exact chemical properties of mucopolysaccharides.¹⁰⁾ In this study, we employed a combination of periodic acid Schiff (PAS) reaction, alcian blue staining and colloidal iron reaction method improved by us.⁴⁾

MATERIALS AND METHOD

Resected stomach of gastric cancer and gastric ulcer was fixed in 10% formaldehyde solution and after embedded in paraffin as usual, paraffin sections of 5–7 μ were prepared. The following staining was conducted to determine mucopolysaccharides.

(1) Periodic acid Schiff (PAS) reaction

- (2) Alcian blue staining
- (3) Colloidal iron reaction (as reported before, we employed the improved Mowry's method.⁹⁾)

Digestive examination by the following enzymes was also conducted as to these staining.

- (1) Diastase⁵⁾
Sections were incubated for 1 hour at room temperature (25°C) in 0.02 M phosphate buffer, (pH 6.0) containing 1% malt diastase and 0.8% NaCl.
- (2) Pepsin¹⁾
Sections were incubated for 1 min at 37°C in a solution of 1/200 N HCl containing 0.005% crystalline pepsin.
- (3) Hyaluronidase⁶⁾
 - (a) Sections were incubated for 4 hours at 37°C in 0.1 M acetate buffer, (pH 5.0) containing 5 T.R.U. (turbidity reducing unit)/ml of testicular hyaluronidase (Shionogi Co. Ltd.).
 - (b) Sections were incubated for 4 hours at 60°C in 0.1 M phosphate buffer, (pH 5.0) containing 200 T.R.U./ml of streptomyces hyaluronidase (Amano Co. Ltd.).
- (4) Sialidase (neuraminidase)¹¹⁾
Sections were incubated for 24 hours at 37°C in 0.05 M sodium acetate buffer, (pH 5.5) containing purified *Vibrio cholerae* neuraminidase at the ratio of 1 : 4, 0.9% NaCl and 0.1% CaCl₂. The enzyme solution containing 100 units of *Vibrio cholerae* neuraminidase per ml was made by Sigma Chemical Company.

RESULT

In the case of normal gastric epithelium, PAS reaction was particularly strong in the covering epithelium and in the part of goblet cells. It was also fairly strong in the pyloric gland cells and accessory cells of the fundic gland. In the case of intestinal metaplasia, an extremely strong reaction was noticed at the brush border and in the goblet cell. A medium degree of reaction was observed in the muscularis propria and serosa. Stroma of all layers of the gastric wall also showed in general positive reaction. In the case of ulcer, a medium degree of reaction was seen in the scar. As for epithelium of regenerative gland, practically no reaction was observed in immature gland. Out of various types of cancer, mucoid cancer and signet cell cancer indicated strong positive reaction (Photo 1) but generally speaking, the reaction of cancer cells was extremely weak. However in the case of cancer cell having goblet cells in well differentiated adenocarcinoma, such goblet part reacted strongly (Photo 2).

In the alcian blue staining, no reaction was observed in the normal gastric epithelium, but a medium degree of reaction was noticed at the part of goblet cells in the intestinal metaplastic epithelium. The reaction was extremely weak in the muscularis propria, serosa, stroma etc., but where there is an ulcer, a weak reaction was noticed in the scar. Among various types of cancers, muconodular cancer showed positive reaction, which was restricted to the region where mucus was deposited (Photo 3). Most of cancer cells showed no reaction. However, goblet cell components in carcinoma resembling intestinal metaplasia sometimes

showed positive reaction, and though very rarely, vacuoles of signet cell cancer were positive (Photo 4).

As for colloidal iron reaction, a medium degree of reaction was observed in an accessory cell of the fundic gland and a slight reaction was seen in the covering epithelium. Fairly strong reaction was observed in the brush border and in the goblet cell of intestinal metaplastic epithelium. In stroma of the gastric wall, an inner coat and an intermediate coat of blood vessel, fairly strong reaction was noticed, which differs from the findings of alcian blue staining. In case of the presence of an ulcer, the scarred part was stained intensively (Photo 5). In cancer, mucoid cancer and signet cell carcinoma showed strong reaction and the goblet cells of well differentiated carcinoma showed colloidal iron reaction (Photo 6). A fairly strong reaction was observed in the stroma at the front of carcinoma (Photos 7,8). In the presence of metastasis to lymph node reaction was observed in the stroma around the cancer cells (Photo 9). In such parts hematoxylin-eosin staining and Mallory azan staining revealed sometimes formation of connective tissue, but more often a bright space almost completely lacking cellular components was observed. This finding was particularly remarkable at the front of cancer infiltration. Such part, however, showed only weakest reaction to PAS reaction and to alcian blue staining.

Digestion tests: In PAS reaction and alcian blue staining, no remarkable change was noticed after digestion by diastase, pepsin, hyaluronidase and sialidase. In the case of Mowry's colloidal iron reaction, the situation was quite different, in the stroma at the periphery of infiltration by cancer. After hyaluronidase digestion, the stroma turned negative to Mowry's colloidal iron reaction, irrespective of whether testicular hyaluronidase or streptomyces hyaluronidase was applied (Photos 10, 11, 12). There was no remarkable difference between the digestive actions of these two kinds of hyaluronidase, but the reaction to Mowry's colloidal iron in the inner coat of the blood vessel resisted to streptomyces hyaluronidase. Also at immature granulation tissues of an ulcer, testicular hyaluronidase was effective, but as for streptomyces hyaluronidase, some reaction still remained. In some cases hystiocytes-like cells of connective tissue showed strong resistance to streptomyces hyaluronidase.

In the colloidal iron reaction at the periphery of cancer infiltration, most of the part was lost after digestion by these two enzymes but there remained some part which was not digested by streptomyces hyaluronidase (Photo 13). In such a part, the cells with bright cytoplasm are densely populated and resembled closely to immature granulation of scar of an ulcer. Such part was not noticed at the foremost end of cancer infiltration, but it was found at around the conglomerate of cancer cells. To summarize the colloidal iron reaction of cancer infiltration, the part showing positive reaction which does not accompany round cell infiltration was noticed and next to it was positive part accompanying round cell infiltration and at the region closer to cancer cell group, the positive parts having construction of immature granulation were observed. The latter was not digested by streptomyces hyaluronidase but other parts were all digested by hyaluronidase of either type.

Sialidase caused only slight decrease of reaction of accessory cell of fundic gland and when compared to the digestion by hyaluronidase, it had much weaker

PHOTO 1. Signet cell carcinoma. PAS reaction.

PHOTO 2. Well differentiated adenocarcinoma having goblet-like structure. PAS reaction.

PHOTO 3. Muconodular adenocarcinoma. Alcian blue staining.

PHOTO 4. Signet cell carcinoma. Alcian blue staining.

PHOTO 5. Scar in gastric ulcer. Mowry's colloidal iron reaction.

PHOTO 6. Well differentiated adenocarcinoma having goblet-like structure. Mowry's colloidal iron reaction.

PHOTO 7. Infiltration of cancer in muscularis propria. Mowry's colloidal iron reaction.

PHOTO 8. Another part of cancer infiltration in muscularis propria. Mowry's colloidal iron reaction.

PHOTO 9. Metastasis in lymph node. Mowry's colloidal iron reaction.

PHOTO 10. Infiltration of cancer. Same part as in Photo 7.

Testicular hyaluronidase digestion prior to Mowry's colloidal iron reaction. Stromal mucopolysaccharide has disappeared.

PHOTO 11. Infiltration of cancer. Same part as in Photo 8.

Testicular hyaluronidase digestion prior to Mowry's colloidal iron reaction. There is complete absence of mucopolysaccharide around the cancer cells.

PHOTO 12. Metastasis in lymph node. Same part as in Photo 9.

Testicular hyaluronidase digestion prior to Mowry's colloidal iron reaction. Mucopolysaccharide around the metastatic cancer cells has disappeared.

PHOTO 13. Infiltration of cancer. Same part as in Photo 7.

Streptomycetes hyaluronidase digestion prior to Mowry's colloidal iron reaction. There is the part which was not digested by streptomycetes hyaluronidase.

potency. Digestion of the reaction at the scar of ulcer was also very little in case of sialidase. In particular, as for colloidal iron reaction observed at around infiltration of cancer cells, practically no digestion was effected by sialidase.

In the digestion test by diastase and pepsin, practically no difference was observed between the time before digestion and after digestion.

DISCUSSION

In order to investigate the change of acid mucopolysaccharide at gastric cancer tissue, PAS reaction, alcian blue staining and colloidal iron reaction were conducted and digestive examination was made mainly with hyaluronidase and sialidase. Many reports have been made on these reactions of cancer tissue and the authors revealed their respective interpretations of the findings.

In this experiment, we noticed considerable differences between these three cases. In the case of PAS reaction, strong positive reaction was noticed at covering epithelium, goblet of foveolae epithelium and accessory cell etc. which contain mucus. Reaction was observed in wide range at stroma, muscularis propria, serosa, etc., though the degree of reaction was varied. Many researchers already pointed out that this reaction is noticed over a wide range and degree of reaction is quite strong.¹⁰⁾ Therefore although it contains much acid mucopolysaccharides, their specificity is rather low and their reaction to digestive examination is not clear. In the case of cancer cell, most of the cancer cells showed only weak reaction, except those which caused mucous degeneration or those having goblet part.

In alcian blue staining, specificity to mucopolysaccharide is fairly high. No reaction is observed with normal gastric epithelium while signet cell cancer or well differentiated cancer having goblet structure showed positive reaction.

In case of colloidal iron reaction, the results were closer to those obtained in alcian blue staining rather than PAS reaction but the reaction was extremely strong. Reaction of stroma was highly specific. In the light of these results we judged that for digestive examination of polysaccharides, colloidal iron reaction was most excellent of three methods of staining.

The principle of colloidal iron reaction is that the presence of mucopolysaccharides is determined by combining them with iron ion and reacting this iron by Berlin blue reaction. We reported previously two findings obtained in the past, that is, (1) in the detection of iron in vivo, an extremely favorable results were obtained when Berlin blue reaction was conducted in 70% acetone and (2) when this method was applied to colloidal iron reaction of acid mucopolysaccharides reaction appeared very strongly.⁴⁾ In the digestive test by modified method of Mowry, in which iron reaction was conducted in 70% acetone, sialidase showed only slight digestion, while only hyaluronidase indicated remarkable digestive effect. Most of the reaction at stroma is considered to have been caused by hyaluronic acid.

It is an extremely important subject to verify the specificity of enzyme digestion of polysaccharides. Hyaluronidase may be obtained from testis or streptomyces. It is generally believed that hyaluronic acid is digested by hyaluronidase but the enzyme which digest hyaluronic acid selectively is streptomyces hyaluronidase. Testicular hyaluronidase also digests chondroitin sulfate A, C. In the digestive test of hyaluronidase of this experiment, both testicular hyaluronidase and strepto-

myces hyaluronidase digested mostly the positive substance and it may be right to say that most of colloidal iron positive substance is hyaluronic acid. However the immature granulation of ulcer and tunica intima et media, etc. is digested only to a minor degree by streptomyces hyaluronidase and therefore it is supposed that chondroitin sulfate A, C are also mixed in them.

In the colloidal iron reaction at around the cancer infiltration, most of the positive substance was digested by the two kinds of hyaluronidase and it is thought to be the reaction caused by hyaluronic acid. Intensification of staining is remarkable at the front end of cancer infiltration and it does not agree with the formation of connective tissue fiber. A part of it shows the structure very similar to the granulation of ulcer and in the case of streptomyces hyaluronidase, we noticed the part not digested at all. Such undigested part is considered to be mainly composed of chondroitin sulfate. Not only its construction but also its reaction suggest that it is similar to immature granulation.

Vasiliev¹⁴⁾ stated that infiltration of cancer is the result of not only the strong growth of cancer cell itself but also of the added effect of destruction of surrounding connective tissue stroma and proliferation of immature connective tissue. According to our observation, those which appear to be the immature granulation are observed only locally at around the conglomeration of cancer cells and they do not exist at the foremost end of infiltration. Therefore we believed that proliferation of immature connective tissue is only a secondary phenomenon and it does not constitute the cause of cancer cell infiltration. We believe that increase of hyaluronic acid is not the phenomenon which is accompanied by formation of connective tissue fiber but it rather precedes the formation or connective tissue fiber. In the case of chondroitin sulfate we think that such deposition is resulted by granulation which is formed as the secondary phenomenon. Such increase of hyaluronic acid has the possibility to accelerate infiltration or growth of cancer as Meyer⁸⁾ reported, but it is extremely difficult to prove whether the cause of such phenomenon rests with cancer cell itself or with the surrounding stroma.

Other stroma which indicated strong reaction is scar of ulcer but it is well digested by hyaluronidase but hardly digested by sialidase. At this point, it is exactly same with the reaction of infiltration of cancer. In the case of scar, reaction is always accompanied by connective tissue and at this point it differs from infiltration of cancer. In this reaction, the fresher the scar, the stronger is its reaction while the ulcer which is in an advanced stage of healing, the reaction was weaker. From these view points, it is considered that fibroblasts in the immature stage is abundant in hyaluronic acid. Speaking about the infiltration of cancer, it may be right to say that the reaction of the part which does not accompany connective tissue fiber is the true reaction of cancer cell infiltration.

In the last place, we should like to discuss on the digestive examination by sialidase which was conducted to investigate the conditions of distribution of sialic acid. In 1957, Gottschalk³⁾ named the enzyme which acts upon oligosaccharide and decomposes the connection between ketone group of sialic acid and carbohydrate, neuraminidase. Spicer and Warren¹²⁾ applied this neuraminidase (sialidase) histochemically and stated that sialidase is an enzyme which selectively removes sialic acid from mucopolysaccharides and it reduces the reaction of metachromasia and alcian blue. Quintarelli¹¹⁾ examined the attitude of digestion by sialidase

in various polysaccharides staining including PAS reaction. Constantine and Mowry²⁾ applied sialidase for colloidal iron reaction. However in any of these reports, materials used were submandibular gland, sublingual gland, sweat gland etc. and none has used gastric tissue especially its cancerous tissue. Sialic acid indicates the reaction of acid mucopolysaccharides histochemically and they said that it is stained by various staining agents. Therefore it always causes problems in the staining of acid mucopolysaccharides. In the digestive test using sialidase, mainly accessory cell of fundic gland and positive substance in the surrounding stroma showed slight digestion.

REFERENCES

1. Arcadi, J. A.: Some polysaccharide components of the prostate gland of the dog. *Anat. Rec.* 112; 593, 1952.
2. Constantine, V. S. and Mowry, R. W.: Histochemical demonstration of sialomucin in human eccrine sweat glands. *J. Invest. Dermatol.* 46; 536, 1966.
3. Gottschalk, A.: Neuraminidase: the specific enzyme of influenza virus. *Biochim. Biophys. Acta*, 23; 645, 1957.
4. Hirose, S. et al: A modified method of the Prussian blue reaction for the histochemical demonstration of iron, and its application to the colloidal iron reaction of acid mucopolysaccharides. *Acta Histochem. Cytochem.*, 3; 18, 1970.
5. Lillie, R. D. and Greco, J.: Malt diastase and ptyalin in place of saliva in the identification of glycogen. *Stain Tech.*, 22; 67, 1947.
6. Mannozi, T. M.: *Boll. Soc. Ital. Biol.*, 17; 153, 1942.
7. Masamune, H. and Hakomori, S.: On the molecular alteration of mucoproteins and mucopolysaccharides in cancer. *Symposia Cell Chem.*, 10; 37, 1960.
8. Meyer, K. and Chaffee, E.: Hyaluronic acid in the pleural fluid associated with a malignant tumor involving the pleura and peritoneum. *J. Biol. Chem.* 133; 83, 1940.
9. Mowry, R. W.: Improved procedure for the staining of acid polysaccharides by Müller's colloidal (hydrous) ferric oxide and its combination with the Feulgen and the Periodic acid-Schiff reactions. *Lab. Invest.*, 7; 566, 1958.
10. Pearse, A. G. E.: *Histochemistry, Theoretical and Applied*, 2nd Ed., J. & A. Churchill, Ltd., London, p. 228, 1960.
11. Quintarelli, G., Tsuiki, S., Hashimoto, Y. and Pigman, W.: Studies of sialic acid containing mucins in bovine submaxillary and rat sublingual glands. *J. Histochem. Cytochem.*, 9; 176, 1961.
12. Spicer, S. S. and Warren, L.: The histochemistry of sialic acid containing mucoproteins. *J. Histochem. Cytochem.*, 8; 135, 1960.
13. Tsurumi, K.: *Saishin Igaku*, 13; 2498, 1958. (in Japanese)
14. Vasiliev, J. U. M.: The role of connective tissue proliferation in invasive growth of normal and malignant tissues: a review. *Brit. J. Cancer*, 12; 524, 1958.