A review for in situ zymography:
– method for localization of protease activities in a tissue –

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1. Introduction

Matrix metalloproteinase (MMP) and plasmin/plasminogen activator system express their functions in various topical parts in the body in formation of tissue (evolution), damage and repair of tissues, inflammation, tumor formation and so forth. Activities of these enzymes are regulated mainly by the following three ways, that is, regulation of biosynthesis of these enzyme precursors, regulation of process for activating their precursors and suppression of their activities by endogenous inhibitors. Thus, expressions of these enzymes occur only at the limited time periods and in the limited parts of the body. Accordingly, it is required for analyzing the functions of these proteases to assay their localized activities in addition to determinations of expressed amounts of protein or mRNA of these enzymes.

Many sorts of methods for determining enzyme activities localized in a tissue (in situ zymography) have been invented for this purpose. In the present paper, the authors review these methods and also introduce an assay method developed recently by the authors with a crosslinked gelatin membrane.

2. Examples of studies on localization of protease activities in a tissue with in situ zymography

In situ zymography visualizes localization of enzyme activities in a tissue using a cryosection adhered on a substrate thin membrane. Until now studies of in situ zymography have been performed on MMPs and plasminogen activators, and casein, collagen or gelatin have been used as substrates.

Studies of in situ zymography with plasmin/plasminogen system were performed on tissue of rheumatic arthritis1), colon adenoma2) and retina3) in humans, as well as on formation of corpus lutea in rats4), formation of placenta5), ovary in ovulation phase6) and normal kidneys7) in mice (animal models). On the other hand, studies with MMP in humans were the largest on circulation system such as atherosclerosis foci8), aortic aneurysm9), cerebral artery aneurysm10) and aortic occlusion10) and followed by studies on tumor tissue11) and skin (normal12) and psoriasis13)). In animals (animal models), studies were made on horse hoofs14), coronary artery occlusion in dogs15) and lung tissue in rats16). In addition, there are studies with cultured tissues.

As for the assay technique by in situ zymography, the study on measurement of activity in collagenase solution with a membrane made of collagen was already reported in the 1960’s17). Growing numbers of studies, however, have been reported in 1990’s on localization of enzyme activities in a tissue. The assay technique of activities of plasminogen activators was established by Sappino et al., in 19917). Since then, several reports on application of this technique were issued. Outline of the method is as follows. Mixed solution of agar, casein as an enzyme substrate and plasminogen, a precursor of enzyme, is prepared. A cryosection adhered on a slide-glass is immersed in the solution consisting of substrate and precursor and drawn up so that a thin membrane of the solution is formed on the surface of section. Enzyme activity is assayed by incubating the specimen at 37°C(C). When tissue-type or urokinase-type of plasminogen activator (tPA or uPA) exists in tissue, plasmin generated by activation of plasminogen in the thin membrane hydrolyses a substrate, casein, to give a signal. Changes in thickness of the membrane produced by hydrolysis of casein are determined with a
darkfield light microscope. Advantage of the method of Sappino et al. is that localization of respective enzyme activity of tPA or uPA can be detected inhibiting each enzyme selectively by addition of respective inhibitor, i.e., an uPA-specific inhibitor, amiloride, or neutralizing antibody of tPA into the substrate solution.

On the other hand, a representative example of in situ zymography with MMP among several techniques ever reported is the technique developed by P. Libby et al.8, 18), 1994. Two sorts of substrates, fluorescence-labeled casein or emulsion for photography (containing gelatin), were used for MMPs with different substrate specificity. In the former case, a cryosection adhered on a membrane made of casein-resorufin or casein-FITC and agarose was allowed to react. Localization of casein hydrolysing activity was identified by detection of the part with decreased fluorescence by means of a fluorescence microscope. In the second method to use emulsion for photography, a thin membrane of emulsion was formed on a tissue specimen by immersing and drawing up a cryostat section adhered on a slide glass in the emulsion for autoradiography. After incubation of the specimen, the thin membrane is treated by the same procedure to develop photographs. Localization of gelatinase activity is detected as a transparent spot produced due to hydrolysis of gelatin in the emulsion membrane by the enzyme. Since MMP activity is inhibited with chelating reagents such as EDTA or 1,10-phenanthroline, it can be confirmed if the substrate is hydrolyzed by MMP. Nevertheless, the pattern of hydrolysis of casein is almost the same as that of gelatin, and assay of MMP distinguishing its MMP-species has not been realized yet.

3. Film in situ zymography (FIZ) using a crosslinked gelatin membrane

All of the aforementioned methods have been used for qualitative detection of localized enzyme activities and could not be used for quantitative assay of enzymes. It is considered, however, that relative comparison of enzyme activity would be possible even in different experiments and an unified evaluation of experimental results can be made by organs or by tissues, if the same substrate and the same experimental condition are employed. On the other hand, there are several cases in which experimental results can not be reproduced even in cases of aforementioned representative methods (private communication), and it is known that the poor reproducibility arises from difficulty in controlling the thickness of membrane. In order to overcome the difficulty, the authors are developing a preparation method of substrate membranes of uniform thickness for in situ zymography with a homogeneous application technique used for preparation of photographic films. The substrate membrane is tentatively named film in situ zymography (FIZ) and its details are explained in the present paper.

3-1. Composition of film and method to use

A crosslinked gelatin thin membrane with about 7µm of thickness was prepared on a base film made of polyester. Upon use of the film, a tissue cryosection with about 4-8µm of thickness is adhered on the surface of gelatin membrane and incubated at 37°C for several to 24 hr in a moist chamber. Subsequently the film is immersed in dye solution to stain the gelatin membrane. Localization of gelatinase activity can be detected from the spots with lower intensity of staining produced by hydrolysis of gelatin with the enzyme. Optimal incubation period is several to 10Åıhr in tumor tissues with a high gelatinase activity and 16 to 27hr in those with a low gelatinase activity. Various tests revealed Amido Black 10B and Ponceau 3R are suitable dyes for staining. Gelatin is stained with dark blue by staining with the former and with red with the latter. Because of high intensity of staining with the former, spots are detectable with good contrast at the part where gelatin is hydrolyzed. On the other hand, localization of enzyme activity can be identified easily by the use of the latter dye, since its combined use is possible with nuclear staining dyes such as hematoxylin.

3-2. Examples of application of FIZ

The authors applied the prepared crosslinked gelatin membrane to squamous cell carcinoma of oral cavity. The results of study using HE-staining and FIZ exhibit the localization of gelatinase activities in the positions where tumor cells exist (Fig. 1). Okada et al.11) also showed the localization of gelatinase activity at the position where tumor cells existed applying prepared gelatin membranes on cryosections of thyroid carcinoma or brain tumor tissue.
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Fig. 1 Squamous cell carcinoma of oral cavity protease activity expression
a) HE stained
b) FIZ, Amido Black stained

Fig. 2 Breast cancer protease activity expression
a) HE stained
b) FIZ, Ponceau, hematoxylin double stained
c) FIZ, (1, 10-phenanthroline containing membrane), Ponceau, hematoxylin double stained
(Data courtesy of Dr. Hiroji Iwata, Department of Breast Surgery, Aichi Cancer Center Hospital)
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Enzyme inhibitors are generally used for examining if the gelatinase activity is derived from MMP. Gelatin membrane with enzyme inhibitors can be easily prepared by drying the gelatin film following its immersion in the solution that contains a chelating reagent such as 1, 10-phenanthroline. In Fig. 2 is shown an example of inhibition of gelatinase activity using a membrane prepared by drying after immersing a gelatin film in 100mM 1, 10-phenanthroline solution. In recent academic meeting, similar examples of inhibition were reported in uterine cancer and ovarian cancer of humans and papers describing these results would be published soon. Moreover, novel results were obtained in this study, that is, nuclei were identified by hematoxylin-staining in addition to detection of localization of gelatinase activity by Ponceau-staining.

Good reproducibility is obtained with FIZ because of usage of a homogeneous gelatin membrane and even routine form of assay would be possible if cryosections can be prepared. A relative comparison in a series of test specimens would be possible under the same experimental conditions, e.g., a specimen with high gelatinase activity can be distinguished from that with low gelatinase activity even in the same type of tumor samples. From the studies on correspondence of the data of FIZ to the data of clinical examinations, the technique might be applicable for not only medical research but for clinical practice.

Since FIZ is on a way of development, we are going to characterize the optimal properties of membranes from the results of tests with various types of test samples and also will develop the staining methods for a respective objective.

4. Conclusion

The largest advantage of in situ zymography is that the technique can visualize directly the localization of protease activity in tissue, intensity of which is regulated by a balance of amounts of activated-form of enzyme and amounts of endogenous enzyme inhibitors. Thus, the technique has a striking feature that differs from those of immunological staining or in situ hybridization. On the other hand, demerit of in situ zymography technique is difficulty in quantitation. Nevertheless, significant quantitation would be realized by means of the digital image analyzing system that has been developed recently.

The present status of methodology and application of in situ zymography as well as its possibility in the future were described briefly. In this review, explanation about the method was limited to in situ zymography in a narrow sense, i.e., a technique for examination of tissues. The authors would like to give a final comment on the fact that the method of assay of protease activity with a substrate membrane has been used for determining enzyme activity in solution and also for high-resolution in situ zymography to analyze an individual cell. The results of these studies are reported elsewhere.

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