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In situ zymography: topographical considerations

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Abstract

In situ gelatin zymography is a simple technique providing valuable information about the cellular and tissue localization of gelatinases. Until recently, the use of this technique has been confined to soft, relatively homogeneous tissue. In this report in situ zymography has been utilized to assess the sub-lamellar location of gelatinases in the hard, semi-keratinized epidermal layer and the adjacent soft connective tissue matrix of the dermis of the equine hoof. We show that alterations in the orientation at which the tissue is dipped and withdrawn from the emulsion cause profound alterations in emulsion thickness. Microscopic variations in the surface topography of frozen tissue sections also influence emulsion thickness making interpretation of the results difficult. Given these results, researchers must be aware of potential variations in zymographic analysis may be influenced by physical tissue parameters in addition to suspected gelatinase activity. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In situ zymography is a relatively simple technique enabling the localization of proteolytic enzymes at the cellular and tissue level [1]. Requiring only a gelatin-based emulsion overlay, available commercially, for autoradiography, in situ gelatin zymography enables localization of gelatinase activity, irrespective of whether the enzymes are in their active or inactive forms (proforms and molecules bound to inhibitors), as all

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molecular forms are deliberately activated prior to visualization. The principle is applicable to any proteolytic enzyme but to date, has only been successfully utilized for the visualization of gelatinases, a sub-group of the family of matrix metalloproteinases (MMPs) [1–5].

Gelatinases (Gelatinase-A (MMP-2), Gelatinase-B (MMP-9) and membrane bound metalloproteinase-1 (MT1-MMP)) are all capable of degrading denatured collagens (gelatins) [6,7], major components of extra-cellular matrix and basement membranes [8,9]. In vivo, these enzymes are involved in normal tissue remodeling processes [10–13] and are all tightly regulated by tissue inhibitors of metalloproteinases (TIMPs) [6]. In recent years MMPs have become increasingly implicated in pathological conditions, particularly cancer metastasis [14–16] and bullous skin diseases (for review see: [13]).

While in situ zymography has limitations regarding quantitation, it can be utilized effectively for localization of gelatinase activity at the cellular and tissue level [1–5]. During the course of previous experimentation, we noted some irregularities as a result of tissue orientation prior to dipping in emulsion. In the current study we investigated these variations in emulsion digestion, resulting from variations in emulsion thickness due to the topographical orientation of the tissue section.

2. Materials and methods

2.1. Tissues

Hoof tissue from normal horses was obtained from a commercial knackery (Meramist, Caboolture, Aust.), transported on ice to the dissection room within 60 min, where hooves were dissected to obtain pieces of tissue approximately 6 mm by 6 mm extending from the inner hoof wall through the lamellar junction to the dermal connective tissue. Tissues were rapidly frozen by immersion in liquid nitrogen and stored at -70°C until sectioned.

2.2. In situ zymography

In situ zymography was performed as described previously [4]. Briefly, 10 μm serial frozen sections were mounted on untreated glass slides, equilibrated to room temperature and dipped in autoradiography emulsion as per the manufacturer's instructions (EM-1, Amersham Int., Buckinghamshire, UK). Slides were dipped in emulsion containing 10 mM calcium chloride in addition to either: 0.7 mM *p*-aminophenyl-mercuric acetate [APMA, an organomercurial MMP activator; Sigma (St. Louis, USA)] dissolved in dimethyl sulphoxide (DMSO, Sigma), 0.7 mM APMA and 100 μM Batimastat (a specific MMP inhibitor British Biotech., Oxford, UK) dissolved in DMSO, 5% DMSO or 100 μM Batimastat. Coated slides were incubated in a humidity chamber at 37°C for up to 18 h then developed as per control slides. Control slides were developed immediately using D-19 developer (Eastman Kodak Co., Rochester, USA) and fixed with a 30% w/v solution of sodium thiosulphate (Sigma) in distilled water, as per the

manufacturers instructions. All tissues shown in Fig. 2. were incubated for 8 h. All sections were counterstained with Mayer's Haematoxylin (Sigma), dehydrated using ascending alcohol solutions, cleared with xylene and coverslipped using Depex mounting media (Gurr, BDH, Poole, UK). Slides were examined with an Olympus BX50 microscope and images captured and stored on a Pentium II PC (Scientific instruments and Optical Supplies, Normanby, Aust.) via a JVC 3-CCD digital camera (JVC, Yokohama, Japan) utilizing a Flashbus MV Pro frame grabber card (Integral Technologies Inc, Indianapolis, USA) and analyzed using ImagePro software (Media Cybernetics, Silver Spring, USA).

2.3. Histology

Ten μm serial sections were mounted on silanized glass slides and post-fixed in 1.6% formaldehyde for 10 min. Hoof lamellar basement membrane was demonstrated histochemically [17] using the periodic acid Schiff (PAS) method, while cell morphology was visualized by haematoxylin and eosin (Sigma) staining.

3. Results and discussion

Normal tissue morphology is shown in Fig. 1 for clarity. Haematoxylin and eosin staining (Fig. 1a) identifies the interdigitating lamellar structure characteristic of the hoof dermal–epidermal junction. The primary epidermal lamellae (Fig. 1a, PEL) is a hard keratinized backbone to which semi-keratinized secondary epidermal lamellae (Fig. 1a, SEL) are attached as fingerlike projections. PAS staining (Fig. 1b) demonstrates the basement membrane (BM) attached intimately to the epidermal basal cells (Fig. 1b, BC) surrounded by dermal extracellular matrix (ECM).

Tissue sections oriented such that the lamellae were perpendicular with the direction of emulsion run-off after dipping (Fig. 2a) showed an emulsion digestion pattern

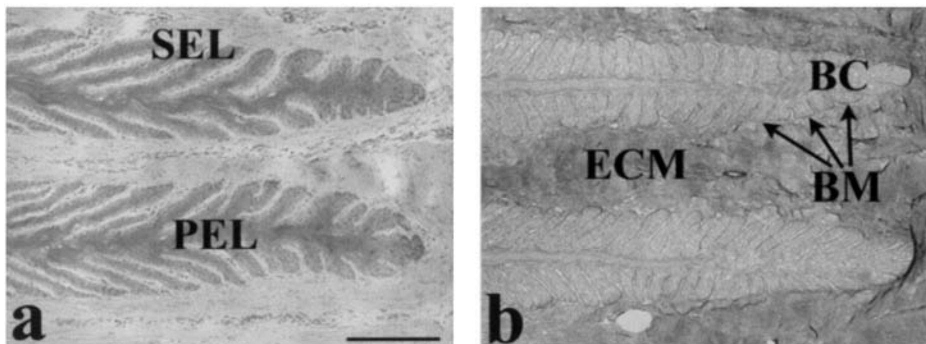

Fig. 1. Haematoxylin and eosin (a) and PAS (b) staining of normal hoof lamellae. SEL = secondary epidermal lamellae, PEL = primary epidermal lamellae, ECM = extracellular matrix, BC = basal cell, BM = basement membrane. Bar = 200 μm.

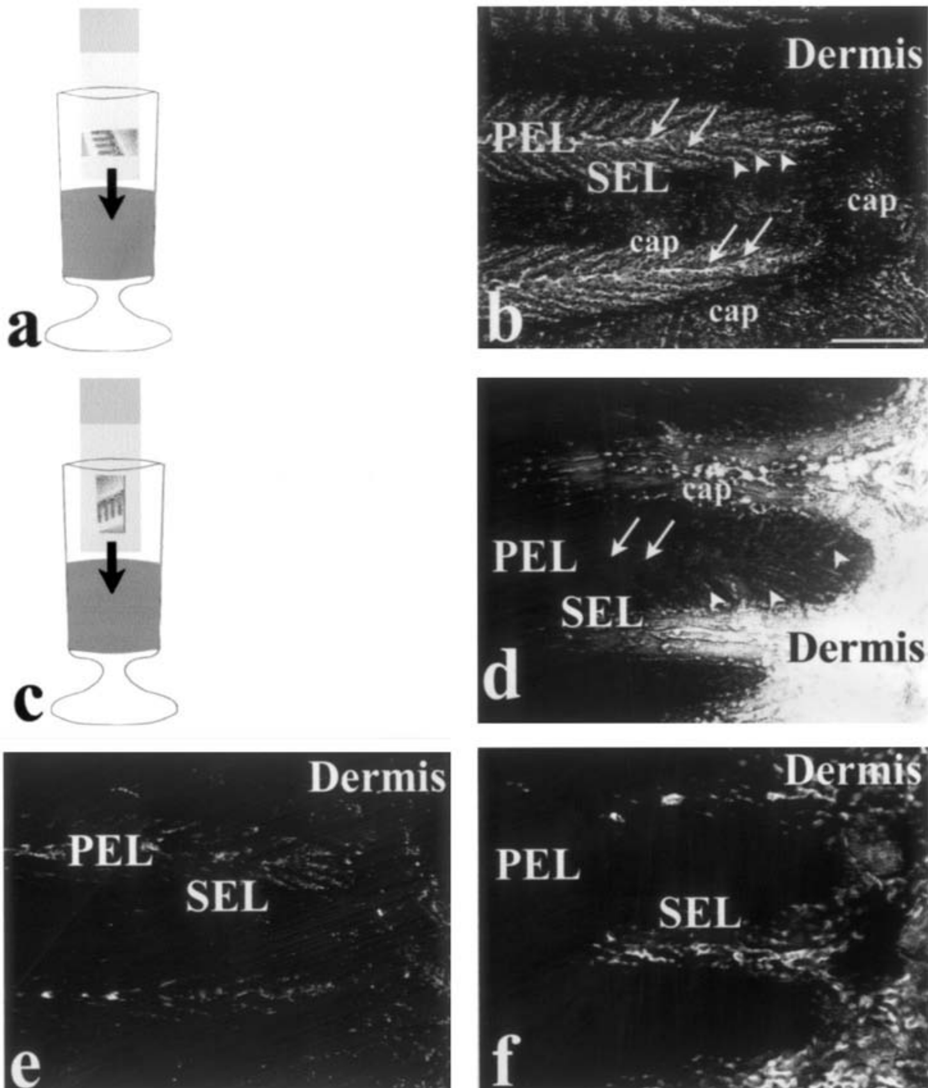

Fig. 2. Digestion pattern produced by tissue sections with the lamellae oriented perpendicularly (a) to the direction of emulsion run-off (arrow) (b) or parallel (c) to the direction of emulsion run-off (arrow) (d). Perpendicular (e) and parallel (f) lamellar sections treated with 100 μM batimastat. SEL = secondary epidermal lamellae, PEL = primary epidermal lamellae, cap = capillary. Bar = 200 μm.

predisposed to epidermal gelatinase activity. The PEL was almost devoid of emulsion overlay (Fig. 2b, arrows) while the SELs had started to degrade their overlying emulsion (Fig. 2b, arrowheads). The surrounding dermal tissue was interpreted to have relatively little gelatinase activity, with the exception of multiple ‘‘spots’’ attributable to capillaries cut in cross-section (Fig. 2b, ‘‘cap’’). In stark contrast, tissue sections oriented such that

the lamellae were parallel to the direction of emulsion run-off (Fig. 2c) showed an emulsion digestion pattern accentuating dermal gelatinase activity. Compared to the former orientation, there was essentially no emulsion digestion over the PELs (Fig. 2d, arrows) although the SELs still retained considerable gelatinase activity (Fig. 2d, arrowheads). The dermal tissue in general showed significant digestion of the emulsion overlay in a relatively non-specific pattern (Fig. 2d).

The emulsion thickness disparity appears to be attributable to variations in tissue composition and thus, elasticity. Dermal tissue, predominantly extracellular matrix and vascular tissue, is amenable to significant displacement, either as a result of load bearing, or as a result of desiccation. Hoof epidermis, in contrast, consists predominantly of keratinized tissue of varying age and density, providing a relatively inflexible ‘‘skeleton’’ supporting the dermal tissue. The juxtaposition of tissues of different compositions, as exists at the epidermal–dermal junction in the hoof, produces a predictable tissue surface topography when examined as frozen sections. As tissue sections are withdrawn from the emulsion, the liquid tends to run off under the force of gravity, according to the natural contours of the section, depending upon the orientation at which it was placed onto the slide.

Sections tested with the specific MMP inhibitor, batimastat were used to evaluate and differentiate the ‘‘real’’ gelatinase activity from potential artifact induced by topographical variations. Perpendicular lamellar sections (Fig. 2e) treated with batimastat showed minimal gelatinase activity confined to PELs and SELs. Reflecting the overall thinner emulsion coating, parallel lamellar sections (Fig. 2f) treated with batimastat showed more dermal digestion than their perpendicular counterparts but showed significantly less dermal emulsion digestion than untreated parallel sections indicating a significant portion of dermal ‘‘gelatinolytic’’ activity observed in untreated sections with this orientation as artifact. A similar level of epidermal gelatinase activity was observed with both orientations, probably reflecting the true level of enzymatic activity.

Non-incubated sections dipped in emulsion, developed but not counterstained or coverslipped, revealed a significantly different emulsion ‘‘settling’’ pattern when illuminated from above. When sections are dipped in emulsion such that the lamellae are perpendicular to the emulsion run-off they show a relatively even emulsion topography (Fig. 3a) with the PELs barely visible (Fig. 3a, arrows). Sections oriented parallel to the direction of emulsion run-off showed a stark topography under epi-illumination. The PELs (Fig. 3c, arrows) and the SELs (Fig. 3c, arrowheads) are easily visible above the emulsion level over the remainder of the section. Fig. 3b illustrates the anticipated run-off when the lamellae of the section are at right angles to the direction of run-off, while Fig. 3d presents the alternate orientation, parallel to the direction of emulsion run-off. Perpendicular lamellar sections (Fig. 3b) have PELs oriented to resemble corrugations, which impede the emulsion from running off the section evenly. This produces a slightly thicker coating over the dermal tissue, between the raised epidermal lamellae, and a thinner coat over the primary epidermal lamellae. This produces the pattern seen in Fig. 3a, perhaps accentuating the epidermal ‘‘gelatinase’’ activity observed in these sections. In contrast, there is significantly more run off from sections with lamellae parallel to the direction of emulsion run-off (Fig. 3d) producing an emulsion thickness more indicative of the natural tissue contours. Under these conditions

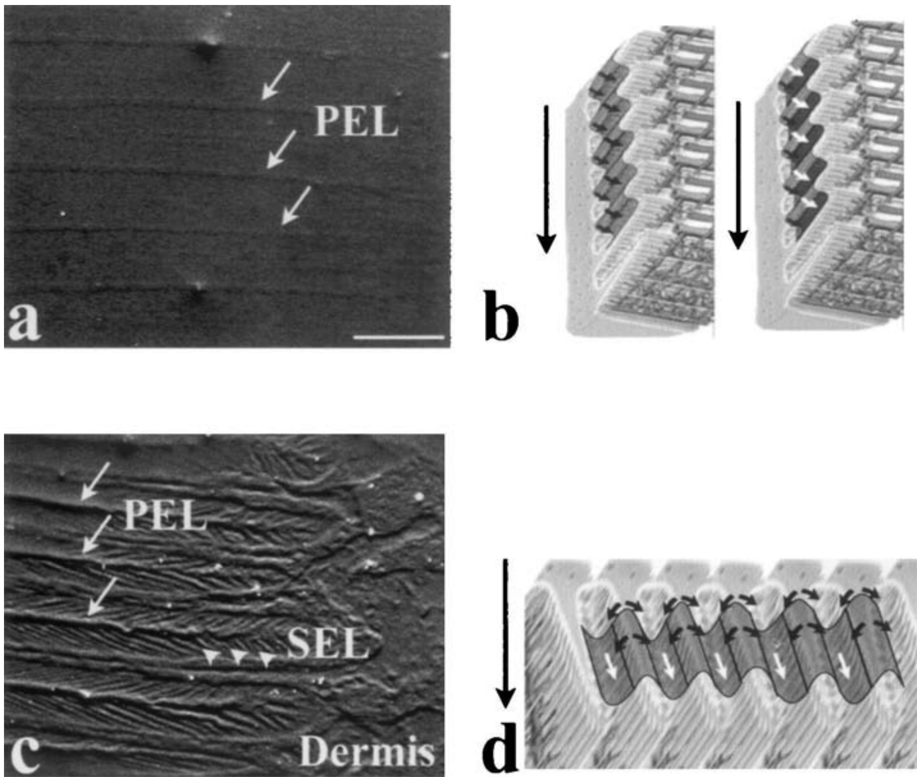

Fig. 3. Epi-illumination of the tissue section after dipping in emulsion. (a) Lamellae perpendicular to the direction of emulsion run-off showing the raised contours of the primary epidermal lamellae (PEL) evident against the smooth background. (b) Schematic representation of the emulsion run-off (arrow) from tissue sections dipped with perpendicular lamellae. (c) Lamellae parallel with the direction of emulsion run-off showing the natural contours of both the PEL (arrows) and secondary epidermal lamellae (SEL, arrowheads). (d) Schematic of the emulsion movements when the tissue section is dipped such that the lamellae are parallel with the direction of emulsion run-off (arrow). Bar = 500 μm.

the emulsion runs off the PELs (Fig. 3d, black arrows) as for perpendicular lamellar sections, but gravity then pushes the emulsion down the softer, indented dermal lamellae (Fig. 3d, white arrows) resulting in a much thinner emulsion overlay.

As can be seen, in situ zymography in different tissues must be individually planned for each tissue, in order to ensure that the orientation of the tissue section does not bias the results, otherwise quite dramatic variations in results can be produced. However, providing the same orientation is presented for each subsequent experiment, there is still scope for cross experiment comparison. We have previously reported the localization of gelatinase activity in normal hoof tissue [4] and the analysis of variations in gelatinase activity in laminitic hoof tissue when compared to normal hoof tissue is possible by maintaining the same tissue orientation throughout [5]. While it is clear from this study that lamellae oriented parallel to the direction of emulsion run-off does provide an

emulsion thickness more indicative of the natural contours of the tissue, it must be emphasized that the specific topography of each tissue investigated must be assessed to provide optimal resolution of the specific area under observation. Forearmed with this knowledge, researchers may be better equipped not only to initially troubleshoot this technique enabling them to highlight their area of interest, but most importantly, to accurately analyze and interpret their results in the context of tissue composition.

4. Simplified description of the method and its applications

In this study we have evaluated the influence of tissue topography on the technique of in situ gelatin zymography. This technique utilizes a gelatin overlay to localize at the cellular level, tissue gelatinases involved in remodeling of the ECM. Using equine hoof tissue, which displays a characteristic morphology of hard keratinized epidermal tissue interdigitating with soft, flexible dermis, we have demonstrated graphic variations in perceived results simply by altering the orientation of the tissues when treated with the emulsion overlay. While hoof tissue demonstrates a significant topographical influence on in situ zymography according to orientation, selection of orientation must be assessed individually for each tissue and may only be relevant when adjacent tissues vary greatly in their composition.

This technique is extremely useful for the detection and localization of gelatinases in situ as has been demonstrated in several unrelated tissues, but thus far, no attempt has been made to consider tissue composition in the interpretation process. To this end, the current study highlights the value of understanding the composition, and ultimately, the topology of the tissue under study when interpreting zymography results.

Acknowledgements

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