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Gelatin In Situ Zymography on Fixed, Paraffin-embedded Tissue: Zinc and Ethanol Fixation Preserve Enzyme Activity

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SUMMARY In situ zymography is a method for the detection and localization of enzymatic activity in tissue sections. This method is used with frozen sections because routine fixation of tissue in neutral-buffered formalin inhibits enzyme activity. However, frozen sections present with poor tissue morphology, making precise localization of enzymatic activity difficult to determine. Ethanol- and zinc-buffered fixative (ZBF) are known to preserve both morphological and functional properties of the tissue well, but it has not previously been shown that these fixatives preserve enzyme activity. In the present study, we show that in situ zymography can be performed on ethanol- and ZBF-fixed paraffin-embedded tissue. Compared with snap-frozen tissue, ethanol- and ZBF-fixed tissue showed stronger signals and superior morphology, allowing for a much more precise detection of gelatinolytic activity. Gelatinolytic enzymes could also be extracted from both ethanol- and ZBF-fixed tissue. The yield, as analyzed by SDS-PAGE gelatin zymography and Western blotting, was influenced by the composition of the extraction buffer, but was generally lower than that obtained from unfixed tissue. (*J Histochem Cytochem* 58:29–39, 2010)

KEY WORDS

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DURING THE LAST FEW YEARS, there have been attempts to get an overview of all proteases, protease homologs, and their inhibitors, as well as their association in a biological sample, namely, the “protease web.” The transcriptional level is currently studied by high-throughput DNA microarrays with special protease chips, and tandem mass spectrometry is used to determine presence/amount of proteases at the protein level (auf dem Keller et al. 2007; Overall and Blobel 2007; Doucet and Overall 2008). However, a successful system biological approach requires methods for precise localization and quantification of protease activity in a tissue, as well as reliable detection of physiological substrates.

Matrix metalloproteases (MMPs) constitute a family of proteolytic enzymes (Brinckerhoff and Matrisian 2002). Together these enzymes are able to digest all components of the extracellular matrix as well as many non-extracellular matrix proteins, and hence have complicated biological functions that play a role in physio-

logical as well as in pathological conditions (Cawston and Wilson 2006; Nagase et al. 2006). Despite MMPs having been extensively studied, their function is still not fully elucidated. In fact, many reports show conflicting results concerning the role of MMPs in diseases such as cancer (Chabottaux and Noel 2007; Duffy et al. 2008). Previous studies were focused mainly on the role of MMPs as enhancers of cancer cell invasion and metastasis. More recently, it has been revealed that their role in cancer is much more complicated, inasmuch as they may also regulate cancer cell growth and inhibit invasion and metastasis (Coussens et al. 2002; De and Mareel 2003; Cawston and Wilson 2006; Deryugina and Quigley 2006; Chabottaux and Noel 2007; Duffy et al. 2008).

Most MMPs are secreted as zymogens that require enzymatic cleavage to become active (Brinckerhoff and Matrisian 2002). The activity of the MMPs is further

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regulated by various inhibitors, including tissue inhibitors of MMPs (Clark et al. 2008). To explore the roles of MMPs in the pathogenesis of various diseases, it is essential to differentiate between active and inactive enzymes. However, the precise quantification and localization of proteolytic activity in tissues has proven difficult. It is therefore important to develop methods that enable high-resolution imaging of local proteolytic activity, as well as methods for reliable extraction and quantification.

Immunohistochemistry is a method frequently used to study the localization of specific MMPs and tissue inhibitors of MMPs in a tissue section. Although antibodies may discriminate between active and pro-MMPs, activity cannot be reliably predicted, owing to possible interactions with inhibitors. In contrast, *in situ* zymography is an appropriate method for detection and localization of enzymatic activity. *In situ* zymography is performed on frozen sections, because it is generally believed that fixation of the tissue inhibits enzyme activity (Galis et al. 1995; Mook et al. 2003; Yan and Blomme 2003; Frederiks and Mook 2004). Frozen sections present with poor tissue morphology compared with sections of fixed, paraffin-embedded tissue. Therefore, the precise localization of the enzyme activity is often difficult to determine by *in situ* zymography.

Among the fixatives commonly used are those based on aldehydes and alcohols. Aldehydes such as formaldehyde (formalin) and glutaraldehyde are electrophilic and form crosslinks with reactive functional groups in proteins, nucleic acids, and other macromolecules (Puchtler and Meloan 1985; Dapson 2007). Alcohol-based fixatives such as ethanol and methanol do not form crosslinks, but fix tissue by precipitation (Lykidis et al. 2007). Some years ago, a new fixative based on different Zn salts was introduced (Beckstead 1994). The exact mode of action for this zinc-based fixative (ZBF) is not fully understood, but it preserves the tissue very well. Judged by light microscopy, the morphology of ZBF-fixed tissue is as good as the morphology of tissue fixed in neutral-buffered formalin (NBF) (Beckstead 1994; Wester et al. 2003). In addition, ZBF-fixed tissue is better suited for many histochemical and biomolecular analyses than is NBF-fixed tissue (Beckstead 1994; Ismail et al. 2003; Wester et al. 2003).

A common way to analyze MMPs is to perform activity determination, zymography, or Western blotting on tissue extracts. Because MMPs are known to bind to various extracellular matrix components through diverse interactions (Yu and Woessner 2000), the yield of these molecules may vary depending on the composition of the extraction buffer.

In the present report, we show that ethanol- and ZBF-fixed, paraffin-embedded tissues are very well suited for *in situ* zymography analyses because such tissues show more-intense and sharper signals than does unfixed tis-

sue. The morphology of the fixed tissue was superior to that of the unfixed tissue, making possible a more precise localization of enzymatic activity. Further, our results show that functional gelatinolytic enzymes can be extracted from tissue fixed in both ZBF and ethanol, but the yield of the various enzymes is lower in fixed tissue compared with unfixed tissue. The composition of the extraction buffer affected the yield of gelatin-degrading enzymes from tissue homogenates.

Materials and Methods

Materials

Acetic acid, formaldehyde, Tris-HCl, calcium chloride dihydrate, zinc chloride, zinc acetate, calcium acetate, pefabloc, glycerol, PBS, ammonium persulfate, SDS, tetramethylethylenediamine, sodium chloride, xylene, methanol, acrylamide, Coomassie Brilliant Blue G-250, Tissue-Tek OCT, Triton X-100, and sodium acetate were purchased from VWR International (Oslo, Norway). DMSO, Hepes, Brij-35, EDTA, bromophenyl blue, and gelatin were purchased from Sigma-Aldrich (St. Louis, MO). Harris hematoxylin and Histokit were from Chemi-Teknikk (Oslo, Norway). Ethanol was from Arcus AS (Oslo, Norway). MMP-2 (ab37150) and MMP-9 (ab38898) polyclonal antibodies (for immunohistochemistry) were from Abcam (Cambridge, MA), and the EnVision+ system-HRP kit was from DAKO (Glostrup, Denmark). Dye-quenched gelatin, 4',6-diamidino-2-phenylidole, and SDS-PAGE NuPAGE Novex 4–12% gels were purchased from Invitrogen (Carlsbad, CA). Antibodies used for Western blotting included goat anti-mouse/rat MMP-2 (AF148) from R&D Systems, Inc. (Minneapolis, MN), and rabbit anti-MMP-9 (AB19016) and polyvinylidene difluoride (PVDF) membranes from Millipore (Billerica, MA). Horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody and Western blotting luminol reagent were from Santa Cruz Biotechnology (Santa Cruz, CA), and HRP-conjugated donkey anti-rabbit antibody was from SouthernBiotech (Birmingham, AL).

Tissue Fixation

Kidneys, liver, tongue, and heart from 10–12-week-old BALB/c nude mice were collected. For extraction experiments, each organ was cut into nine pieces. Each piece was accurately weighed before it was snap frozen in liquid nitrogen or fixed for 20–22 hr in either 4% NBF, 70% ethanol, or ZBF-containing 36.7 mM ZnCl₂, 27.3 mM ZnAc₂ × 2H₂O, and 0.63 mM CaAc₂ in 0.1 M Tris, pH 7.4. For immunohistochemistry and *in situ* zymography, each kidney was cut into two pieces and either snap frozen in liquid nitrogen and mounted with OCT, or fixed in NBF, 70% ethanol, or ZBF for 36–38 hr before dehydration and paraffin embedding. In addition, human tongue squamous cell carcinomas

(SCCs) from routine surgical pathology at University Hospital of North Norway, as well as tissue from mice with human xenograft SCC, were ZBF fixed, dehydrated, and paraffin embedded. The surgical pathology tissue was used after informed written consent given by the patient, and the use was approved by The Regional Committee for Research Ethics. Animal experiments were approved by the Norwegian Animal Research Authority.

Immunohistochemistry

Five- μ m-thick sections of fixed, paraffin-embedded or frozen OCT-mounted kidney tissue were cut on a paraffin or cryostat microtome, respectively, and mounted on Superfrost Plus slides. Primary polyclonal antibodies against human and mouse MMP-2 (1:350) and MMP-9 (1:1000) were used. Horseradish peroxidase-labeled secondary antibody and diaminobenzidine substrate were used for visualization. Paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol baths. Frozen sections were air dried for 10 min, fixed in cold acetone for 10 min, and then rinsed in three PBS baths. Thereafter, all sections went through the following procedure: First, they were incubated with peroxidase blocking solution for 5 min to block endogenous peroxidase activity. After three rinses for 5 min each in PBS, they were incubated with 1.5% normal goat serum in PBS for 20 min to reduce unspecific staining. Primary antibodies were incubated for 30 min, rinsed in PBS baths (three $\times$ 5 min), followed by incubation with a peroxidase-labeled secondary antibody for 30 min. After rinsing in PBS baths again (three $\times$ 5 min), sections were incubated with diaminobenzidine substrate for 10 min, rinsed as before, and counterstained with Harris hematoxylin, dehydrated in graded alcohol and xylene baths, and mounted with Histokit. As negative controls, the primary antibodies were replaced by 1.5% normal goat serum.

In Situ Zymography

For localization of gelatinolytic activity, in situ zymography was performed essentially as described by Sbai et al. (2008) and Miller et al. (2005). Eight- μ m-thick sections were cut from the same tissue that was used for immunohistochemistry and from ZBF-fixed human or murine tongue tissue. After the cryosections were air dried for 10 min, they were washed in PBS baths (three $\times$ 5 min) to remove traces of OCT. The fixed, paraffin-embedded tissue sections were heated at 59°C overnight, deparaffinized in xylene, and rehydrated in graded alcohol baths. Substrate was prepared by dissolving 1 mg DQ gelatin in 1.0 ml Milli-Q water, and this was further diluted 1:50 in a reaction buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl_2 , and 0.2 mM sodium azide (pH 7.6). Of this mixture, 250 μ l was put on top of tissue sections, covered with Parafilm, and incubated in a dark humidity chamber at

37°C. After 2 hr, the Parafilm was gently removed, and the sections were rinsed with Milli-Q water and fixed in 4% NBF for 10 min in the dark. Sections were then rinsed in PBS baths (two $\times$ 5 min) and mounted with glycerol containing DAPI to counterstain the nuclei. To verify the contribution of metalloproteases, control slides were preincubated with 20 mM EDTA for 1 hr. Twenty mM EDTA was also added to the substrate. The level of autofluorescence in the tissue was evaluated by incubating control sections at -20°C for 2 hr immediately after the substrate was added. To determine whether the fixatives or technical procedures could affect the activity of the gelatin-degrading enzymes, different pretreatments of frozen, unfixed tissue sections were performed before in situ zymography. Some sections were pretreated with either ZBF or ethanol for 20 min, others with xylene for 10 min followed by 10 min of ethanol. All of these incubations were performed at room temperature. In other experiments, unfixed frozen sections were incubated overnight at 59°C. Fluorescence was studied using a Leica TSC SP5 confocal laser microscope with Leica Application Suite Advanced Fluorescence software (Wetzlar, Germany).

Extraction of Proteases From Tissue

Kidney, liver, tongue, and heart tissue was cut into nine pieces and fixed for 22–26 hr in the different fixatives, and then rinsed in PBS baths (three $\times$ 5 min) to remove excess fixative. Four different lysis solutions were tested to homogenize the tissue and extract proteases: 0.25% Triton X-100 in 10 mM CaCl_2 (v/v); 10% DMSO in 10 mM CaCl_2 (v/v); loading buffer for zymography containing 1.0 M Tris, pH 6.8, 87% glycerol, 10% SDS, bromophenyl blue, Milli-Q water; and 1 M NaCl in 10 mM CaCl_2 . The tissue was homogenized in 2 ml safe lock tubes containing a 5-mm-diameter steel bead and 20 μ l of lysis solution per mg tissue (wet weight) using a tissue lyser (Qiagen; Hilden, Germany) at 25 Hz for 2.5 min $\times$ 2 (4°C). In some experiments, the tubes were maintained at room temperature for 0–3 hr after homogenization, to investigate whether this influenced the amount of enzymes extracted. The tubes were then centrifuged at $9500 \times g$ for 5 min (4°C). Before storage at -20°C , 0.1 M CaCl_2 in 1 M HEPES (pH 7) was added to the supernatants not containing CaCl_2 to give a final concentration of 0.01 M CaCl_2 .

Gelatin Zymography

SDS-PAGE substrate zymography was carried out as described previously (Malla et al. 2008a) with gels (7.5 $\times$ 8.5 cm $\times$ 0.75 mm) containing 0.1% (w/v) gelatin and 4% and 7.5% (w/v) polyacrylamide in the stacking and separating gels, respectively. Eight μ l of extract was mixed with 2 μ l of loading buffer (333 mM Tris-HCl, pH 6.8, 10% SDS, 0.03% bromophenol blue, and

50% glycerol). Seven μ l of this non-heated mixture was applied to each well of the gel, which was then run at 20 mA at 4°C. Thereafter, the gel was washed twice in 100 ml of washing buffer [2.5% (v/v) Triton X-100 in Milli-Q water] and incubated in 100 ml of assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM CaCl_2 , 0.2 M NaCl, and 0.02% Brij-35) for $\sim$ 20 hr at 37°C. Gels were stained with 0.2% Coomassie Brilliant Blue R-250 (30% methanol) and destained in a solution containing 30% methanol and 10% acetic acid. Gelatinolytic activity was evident as cleared regions.

To evaluate the nature of the enzymes responsible for the observed gelatinolytic activity, some gels were divided into three parallel parts after electrophoresis. One part of the gel was washed and incubated in buffers containing 10 mM EDTA (a metalloprotease inhibitor), the second part in buffers containing 1 mM pepabloc (a serine protease inhibitor), and the third part in buffers without inhibitors.

To determine whether the extracted enzymes represented active forms, extracts were incubated with 800 μ g/ml α 2-macroglobulin for 30 min at 37°C, prior to gelatin zymography. As a control, various amounts of trypsin-activated pro-MMP-9 were incubated with this inhibitor.

Evaluation of the Effect of the Zn Fixative Components

To investigate how each of the components of the ZBF affected the gelatin-degrading activity in the tissue extracts, they were dissolved in Tris buffer and added to extracts of unfixed material in the same final concentration as in the fixation buffer. Also, the complete ZBF was added to extracts of unfixed material. Zymography gels were run following the same procedure as above.

Western Blotting

Twenty μ l of the same undiluted extracts used for SDS-PAGE gelatin zymography were loaded on NuPAGE Novex 4–12% gels and subjected to non-reducing SDS-PAGE before transfer to PVDF membranes. The membranes were blocked in 5% skim milk in Tris-buffered saline-Tween 20 (TBS-T) (150 mM NaCl, 0.1% Tween-20, 20 mM Tris, pH 7.4) and incubated overnight at 4°C with goat anti-MMP-2 (1:1000) or rabbit anti-MMP-9 (1:1500) antibodies, washed in TBS-T, and incubated with HRP-conjugated donkey anti-goat (1:2000) or donkey anti-rabbit (1:2000) antibodies for 1 hr before visualization using luminol reagent.

Results

Tissue Selection

Tissue was obtained from mouse tongue, liver, kidney, and heart. Equal amounts (wet weight) were either fixed or snap frozen prior to extraction. The amount of

gelatinolytic enzymes was determined by gelatin zymography. The results revealed that the kidney contained much more gelatin-degrading enzymes than did the other organs (not shown). In addition, the mouse kidney was large enough to provide parallels for all preservation methods from the same animal. Kidney was therefore selected for further analysis.

Detection of Gelatinases by Immunohistochemistry

To investigate the presence and localization of gelatinases in kidneys, immunohistochemistry using antibodies against MMP-2 and MMP-9 was performed.

In ethanol-fixed tissue, MMP-2 staining was strong in tubuli and focally positive in glomeruli (Figures 1A and 1B). Staining for MMP-9 was negative, except for a few single cells (Figures 1C and 1D). Tissue fixed in NBF and ZBF showed the same staining pattern as the ethanol-fixed tissue. In contrast to the fixed tissue, snap-frozen tissue stained more weakly for both gelatinases. There was no significant difference in morphology between tissues treated with the different fixatives. As expected, the morphology of the fixed tissue was superior to that of the snap-frozen tissue.

Detection of Gelatin-degrading Enzymes by In Situ Zymography

To determine to what extent the gelatin-degrading enzymes were active in the tissue and where these enzymes were localized, in situ zymography was performed on serial sections of the same tissue used for immunohistochemical analyses. When snap-frozen tissue was used, weak activity was detected in both the tubuli and the glomeruli (Figure 2A). This activity was partly inhibited by the metalloproteinase inhibitor EDTA (Figure 2B). As expected, no activity was detected in the NBF-fixed tissue (Figure 2J). Surprisingly, both the ZBF-fixed (Figure 2D) and the ethanol-fixed (Figure 2G) tissue expressed much more intense and sharper signals compared with the snap-frozen tissue (Figure 2A). Activity was detected both in glomeruli and tubuli, but in contrast to the snap-frozen tissue, the strongest activity was detected in the tubuli. The activity was stronger in the ZBF-fixed tissue compared with the ethanol-fixed tissue. As for the frozen tissue, the activity in the fixed tissue was partly inhibited by EDTA (Figures 2E and 2H). As a negative control and a control of possible auto-fluorescence, one section of each sample was incubated for 2 hr at -20°C after the substrate was added. As seen in Figures 2C, 2F, 2I, and 2L, very little green fluorescence was detectable in these sections.

To determine whether the fixatives or the technical procedures could affect the activity of the gelatin-degrading enzymes, sections of unfixed frozen tissue were pretreated with either ZBF, ethanol, or xylene, and preheated before in situ zymography was performed.

Figure 1 Immunohistochemical staining of ethanol-fixed kidney. (A,B) Matrix metalloprotease-2 (MMP-2), (C,D) MMP-9.

None of these treatments affected the intensity of the in situ zymography signals (data not shown).

ZBF-fixed human tongue SCC from the routine surgical pathology (Figure 3A) and murine tongue with human xenograft SCC (Figures 3B and 3C) were subjected to in situ zymography. As can be seen in Figure 3A, there is a clear difference in gelatinolytic activity between the tumor tissue and the adjacent normal tongue tissue. Activity is mainly observed within tumor islands and in the dysplastic surface epithelium, but there is also a general increase in gelatinolytic activity in the stromal compartment close to the tumor, compared with the normal connective tissue. To illustrate the high level of detail obtained by performing in situ zymography on fixed tissue, Figure 3B shows a tumor island with intracellular gelatinolytic activity in the cytoplasm, and Figure 3C shows skeletal muscle fibers from tongue with gelatinolytic activity in a regular band-shaped pattern in 630 $\times$ magnification.

Detection of Gelatin-degrading Enzymes in Tissue Extracts

Tissue exposed to the different fixatives was treated with an extraction buffer containing Triton X-100 to investigate the extent to which it is possible to extract gelatin-degrading enzymes from fixed tissue. Gelatin zymography revealed that extract from snap-frozen tissue contained more gelatin-degrading enzymes than did the extract from fixed tissue (Figure 4A). Further, significantly fewer enzyme bands could be detected in extracts from the ZBF-fixed tissue compared with the

ethanol-fixed tissue. In all extracts, the majority of the enzymes had a high M_r (150–300 kDa). Zymography gels from extracts of frozen and ethanol-fixed tissue revealed a gelatinolytic band with an M_r of $\sim$ 200 kDa that became stronger with increasing dilution of the samples (Figure 4B). This may be due to the presence of both an enzyme and an inhibitor of the same molecular size, where dilution results in a shift in equilibrium toward the free form of the enzyme, making the band at this position stronger. In extracts from ZBF-fixed material, this gelatinolytic band became weaker with dilution. In addition, distinct bands at 92 kDa and 72 kDa were observed, where the former band was the more intense. These two bands had positions identical to pro-MMP-9 and pro-MMP-2, respectively.

Different Extraction Protocols

Proteases are known to bind to various extracellular matrix components through diverse interactions such as hydrogen, ionic, or hydrophobic bonds (Woessner 1995; Yu and Woessner 2000). The yield of different proteases may vary depending on the composition of the extraction buffer. Therefore, different extraction protocols were used to determine to what extent this influenced the yield of the gelatin-degrading enzymes.

DMSO is known to detach gelatinases from collagen and gelatin. We recently showed that DMSO and Triton X-100 have different abilities to detach gelatinase complexes from gelatin-Sepharose columns (Malla et al. 2008a). Therefore, DMSO was also used to extract gelatin-degrading enzymes in the present study. There

Figure 2 In situ zymography of normal mouse kidney tissue using DQ-gelatin substrate. Green fluorescence (FITC) shows gelatinolytic activity, whereas nuclei are shown in blue (DAPI). **A–C**, unfixed tissue; **D–F**, zinc-buffered fixative (ZBF)-fixed tissue; **G–I**, ethanol-fixed tissue; and **J–L**, neutral-buffered formalin-fixed tissue. **A,D,G,J**, control. To demonstrate the contribution from MMPs, 20 mM EDTA was added to the substrate (**B,E,H,K**). As a control of autofluorescence in the tissue, sections were incubated at -20°C (**C,F,I,L**). These images are representative of four independent experiments.

Figure 3 In situ zymography of ZBF-fixed tissue. Green fluorescence (FITC) shows gelatinolytic activity, whereas nuclei are shown in blue (DAPI). (A) ZBF-fixed human tongue squamous cell carcinoma (SCC) from routine surgical pathology. e, normal surface squamous epithelium; s, normal stroma; d, dysplastic surface epithelium; t, tumor island. (B) ZBF-fixed murine tongue with xenografted human SCC tumor. (C) ZBF-fixed murine tongue muscle.

were no significant differences in the amount of enzymes in DMSO and Triton X-100 extracts, as shown in Figure 4A. Likewise, there were no significant differences in the types of enzymes extracted, based on their molecular size (Figure 4A).

In addition to hydrogen bonding and hydrophobic interactions, gelatin-degrading enzymes are also known to bind stromal components through ionic interactions such as the binding of MMP-2 to heparin (Wallon and Overall 1997). Sodium chloride (1 M) was therefore used to extract gelatin-degrading enzymes. In contrast to DMSO and Triton X-100 extraction, more enzymes were obtained from fixed material when NaCl was used (Figure 4A). The yield from ethanol-fixed and frozen material in the NaCl extract was almost identical for all gelatin-degrading enzymes, except for the 72-kDa gelatinase (Figure 4A). From ZBF-fixed material, the yield was somewhat lower than from ethanol-fixed and frozen tissue.

In some experiments, SDS was used for extraction. This compound gave a high yield of proteins from frozen, ethanol-fixed, and ZBF-fixed tissue (not shown). However, the high intensity of protein bands in these experiments interferes with and hides extracted gelatinases in the zymography gels. Therefore, no further analyses were done with SDS-extracted material.

Identification of Extracted Enzymes

To identify the class to which the extracted proteases belonged, different protease inhibitors were used. The gelatin zymography gels revealed that pepabloc, a serine protease inhibitor, had little effect on the activity in the various extracts (Figure 5). However, the MMP inhibitor EDTA blocked most of the enzyme activity in all three extracts, and hence, the extracted enzymes seen in

gelatin zymography are mainly metalloproteases (Figure 5). To determine to what extent the extracted enzymes represent active forms, extracts were incubated with α 2-macroglobulin, a general, irreversible proteinase inhibitor (Borth 1992), prior to gelatin zymography. None of the bands seen in the zymography gels were affected by the inhibitor (data not shown). This indicates that either they were in their inactive pro forms, or that the extracts contained one or more inhibitors that prevented their reaction with α 2-macroglobulin. Pro-MMP-9 and pro-MMP-2 normally have an M_r of 92 kDa and 72 kDa, respectively. In addition, it is known that MMP-9 can form complexes with a higher M_r that are not dissolved in SDS (Winberg et al. 2000; Malla et al. 2008b). Western blots on non-reduced extracted material were therefore performed to determine which of the zymographic bands in gelatin zymography are produced by MMP-9 and MMP-2. When an antibody against MMP-9 was used, a band slightly lower than the 92-kDa pro-MMP-9 in the THP-1 standard was detected in both the Triton X-100 and DMSO extracts of the frozen material (Figure 6A). In addition, several bands with much lower M_r were seen, probably representing enzymatically inactive degradation products. In the Triton X-100 extract, a band with an M_r larger than 220 kDa was detected. In all extracts from fixed material, as well as in the NaCl extract of the frozen material, only a few very weak bands were observed. When an antibody against MMP-2 was used, a band at $\sim$ 66 kDa was detected in all extracts of the frozen material (Figure 6B). Because the Western blot was run under non-reducing conditions, this band corresponds to the latent pro form of MMP-2. In the same samples, bands with an M_r of $\sim$ 150, 30, and $<$ 20 kDa were evident. The band at 150 kDa may be a complex that cor-

Figure 4 Gelatin zymography. Representative gels from at least three independent experiments. (A) Undiluted samples of Triton X-100, DMSO, and NaCl extracts from unfixed, ZBF-fixed, and ethanol-fixed kidney. The molecular mass standard (St) contains pro-MMP-9 dimer (225 kDa), pro-MMP-9 monomer (92 kDa), and pro-MMP-2 (72 kDa). (B) Triton X-100 extracts of unfixed, ZBF-fixed, and ethanol-fixed kidney. The following dilutions of the samples were used: 4:4, no dilution; 3:4, three parts extract + one part water; 2:4, two parts extract + two parts water; 1:4, one part extract + three parts water. The molecular mass standard (St) contains pro-MMP-9 dimer (225 kDa) and pro-MMP-9 monomer (92 kDa).

responds to those in the zymography, whereas the lower M_r bands are most probably truncated, inactive forms of the enzyme. As for MMP-9, only a very few weak bands were detected in the fixed material.

Effect of Fixatives on Tissue Extraction

Fewer gelatin-degrading enzymes were extracted from the ZBF-fixed tissue compared with frozen and ethanol-fixed tissue, whereas in situ zymography revealed the most gelatinolytic activity in the ZBF-fixed material. The ZBF may preclude extraction of enzymes by binding them more strongly to the tissue. Alternatively, components of the fixative may inhibit enzyme activity. Extracts from frozen tissue were therefore mixed with the individual components of the ZBF. As can be seen in Figure 6, Zn salts reduced the "gelatinolytic cloud" that occurred from the top of the separating gel down to around 150 kDa. As mentioned above, dilution of samples not containing any Zn salts resulted in the appearance of a band at 200 kDa (Figure 4B). When Zn salts were added, this band appeared also in the undi-

Figure 5 Gelatin zymography $\pm$ inhibitors. Representative gels from at least three independent experiments. Undiluted samples of extracts from unfixed, ZBF-fixed, and ethanol-fixed kidney. The gels were washed and incubated without (control) or with pefabloc (1 mM) and EDTA (10 mM). The molecular mass standard contains pro-MMP-9 dimer (225 kDa), pro-MMP-9 monomer (92 kDa), and pro-MMP-2 (72 kDa).

luted sample, which indicates that Zn might impede an inhibitor. Furthermore, no effect was seen on the 92- and 72-kDa bands (Figure 7).

Discussion

It is well known that the general quality and quantity of nucleic acids and many proteins extracted from ethanol- and ZBF-fixed tissue are similar to those of snap-frozen samples, and superior to formalin-fixed tissue (Gillespie et al. 2002; Leiva et al. 2003; Wester et al. 2003; Hicks et al. 2006; Lykidis et al. 2007; Chaurand et al. 2008). However, to our knowledge, it has not previously been shown that enzymes retain their activity in fixed tissue.

In the present study, we show that the activity of gelatin-degrading enzymes is preserved in tissue fixed in ethanol or ZBF. The results from in situ zymography show that tissue fixed in ZBF and ethanol expressed significantly more-intense and sharper signals compared

Panel A: SDS-PAGE analysis of protein expression. The gel shows protein bands for Triton X-100, DMSO, and NaCl treatments. Molecular weight markers are indicated on the right (225, 92, 220, 120, 100, 80, 60, 50, 40, 30, 20 kDa). The Triton X-100 treatment shows a prominent band at approximately 92 kDa. The DMSO and NaCl treatments show a prominent band at approximately 220 kDa.

Panel B: SDS-PAGE analysis of protein expression. The gel shows protein bands for Triton X-100, DMSO, and NaCl treatments. Molecular weight markers are indicated on the right (200, 120, 100, 80, 60, 50, 40, 30, 20 kDa). The Triton X-100 treatment shows a prominent band at approximately 92 kDa. The DMSO and NaCl treatments show a prominent band at approximately 220 kDa.

Legend for Panel A:

	Triton X-100			DMSO			NaCl		
Unfixed	+	-	-	+	-	-	+	-	-
ZBF	-	+	-	-	+	-	-	+	-
Ethanol	-	-	+	-	-	+	-	-	+

Legend for Panel B:

	Triton X-100			DMSO			NaCl		
Unfixed	+	-	-	+	-	-	+	-	-
ZBF	-	+	-	-	+	-	-	+	-
Ethanol	-	-	+	-	-	+	-	-	+

(Ahmed et al. 2007). Although MMPs are mainly considered to be extracellular enzymes, intracellular activity of MMPs, and in particular MMP-2, has been observed in myocardial muscle, in the nucleus of apoptotic endothelial cells, and in neuronal dendritic spines (Chow et al. 2007; Schulz 2007; Sung et al. 2007; Sbai et al. 2008; Ruta et al. 2009). Further, gelatinolytic intracellular activity is frequently reported in carcinoma cells (Di Nezza et al. 2002; Nagel et al. 2004; Kato et al. 2005; Heikkilä et al. 2006). Our knowledge of the role of intracellular MMP activity is limited (Strongin 2006). Incubation of extracts with α 2-macroglobulin prior to SDS-PAGE gelatin zymography revealed that the extracts only contained inactive gelatinolytic enzymes. Still, these enzymes can be active in the tissue because inhibitors may be mixed with active enzymes during the extraction procedures. Further, although only pro forms of MMP-2 and MMP-9 were extracted, this does not exclude that these enzymes are active in the tissue, inasmuch as it has previously been shown that pro-MMP-9 bound to elastin is active against a fluorogenic substrate without proteolytic removal of the pro domain (Bannikov et al. 2002).

Our results show that the yield of gelatinolytic enzymes extracted from the tissue and analyzed by SDS-

Figure 7 Gelatin zymography $\pm$ ZBF components. Representative gel of three experiments using DMSO extract of unfixed kidney. Different components of the ZBF fixative were added as indicated, and the samples were either undiluted (–) or diluted two times (+). The molecular mass standard contains pro-MMP-9 dimer (225 kDa), pro-MMP-9 monomer (92 kDa), and pro-MMP-2 (72 kDa).

PAGE gelatin zymography was influenced by the composition of the extraction buffer. Gelatin-degrading enzymes are known to be associated with cell surface molecules and extracellular matrix components (Fridman et al. 2003; Van den Steen et al. 2006; Piccard et al. 2007; Malla et al. 2008b). Because these interactions involve hydrophobic, hydrogen, and ionic bonds, the use of different agents may be necessary to extract these enzymes from the tissue. Triton X-100 is a detergent used to dissolve and extract proteins from cell membranes, and hence breaks hydrophobic interactions, whereas NaCl breaks ionic interactions. DMSO is used to dissolve various hydrophobic compounds into the water phase. As shown in Figures 4A and 6, the yield of different enzymes from both fixed and unfixed tissue was dependent on the extraction method. Although more enzymes were extracted from unfixed tissue, a significant amount of enzymes was also extracted from fixed tissue, especially tissue fixed in ethanol, confirming that ethanol fixation and ZBF fixation preserve functional gelatinolytic enzymes. However, the reduced yield of gelatinolytic enzymes obtained from ethanol- and ZBF-fixed tissue compared with unfixed tissue shows that unfixed tissue should preferentially be used for extraction experiments. The extracts from ZBF-fixed tissue contained less gelatin-degrading enzymes compared with extracts from the ethanol-fixed tissue. This is in contrast to *in situ* zymography, where the intensity of the signals was strongest in the ZBF-fixed tissue. Addition of the different components of the ZBF to extracts from unfixed tissue had only minor effects on the activity, analyzed by SDS-PAGE gelatin zymography (Figure 7). This reveals that the limited amount of gelatin-degrading enzymes from ZBF-fixed tissue is most probably due to a ZBF-induced alteration of the tissue that gives a lower yield of extracted enzymes compared with frozen and ethanol-fixed tissue. Zinc ions in the ZBF may induce crosslinking of proteins, due to interactions with available amino acids such as cysteine, glutamate, aspartate, and histidine on the surface of tissue proteins, which can explain the lower yield of gelatin-degrading enzymes extracted from ZBF-fixed tissue.

Despite the strong immunohistochemical staining of MMP-2, very little of this enzyme was obtained with the various extraction methods. MMP-2 is mainly observed inside tubular epithelial cells, whereas very little is seen in the extracellular matrix. Although the tissue homogenization method used should result in the disruption of cells, the low amount of MMP-2 in the various extracts suggests that this enzyme is bound to cellular components through a mixture of various types of interactions. Immunohistochemical staining for MMP-9 in the mouse kidney was sparse, whereas a considerable amount was found in the extracts. This discrepancy may reflect that the few MMP-9-positive cells contained a high level of MMP-9. In addition, these cells may store MMP-9 in

vesicles that readily disrupt upon homogenization. Because no MMP-9 was extracted with NaCl, but only with DMSO and Triton X-100, the enzyme must be bound to one or several components in the cellular vesicles through hydrophobic and hydrogen bond interactions.

We lack precise information on how most fixatives work and how they affect the activity of different proteolytic enzymes, the balance between them, and their association with inhibitors. NBF induces crosslinking between macromolecules in the tissue, which results in irreversible inactivation of enzymes. NBF can therefore not be used as a fixative when investigating enzyme activity. The morphology of NBF-, ethanol-, and ZBF-fixed paraffin-embedded tissue sections is comparable. For immunohistochemical analyses, ethanol- and ZBF-fixed tissue is found to be advantageous, often eliminating the need for antigen-retrieving procedures commonly performed on NBF-fixed tissue (Beckstead 1994; Ismail et al. 2003; Hicks et al. 2006). It has also been found that nucleic acids are better preserved in ethanol- and ZBF-fixed material than in NBF-fixed material (Gillespie et al. 2002; Leiva et al. 2003; Wester et al. 2003; Lykidis et al. 2007). In the present study, we have shown that paraffin-embedded, ethanol- or ZBF-fixed tissue is also well suited for *in situ* zymography. This is a significant achievement, because the morphology of fixed tissue is superior to the morphology of frozen tissue, which to date has been used for *in situ* zymography. Due to the superior morphology, *in situ* zymography performed on ethanol- or ZBF-fixed tissue allows assessment of the precise localization of gelatin-degrading activity. ZBF is non-hazardous, easy to prepare, inexpensive, and requires no special arrangements for storage. Compared with NBF, ZBF and ethanol fixation allow larger flexibility concerning the methodological repertoire. This is important, not only for research, but also for modern clinical pathology.

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