Zimography is an effective method for detection of matrix metalloproteinase 2 (MMP-2) activity in cultured human fibroblasts

Rodolfo Assis LisboaI, Marcus Vinícius AndradeII, José Renan Cunha-MeloIII

IPhD, Department of Pathology, School of Medicine, UFMG, Belo Horizonte-MG, Brazil, Acquisition, analysis and interpretation of data, statistical analysis and manuscript writing.
IIPhD, Associate Professor, Department of Internal Medicine, School of Medicine, UFMG, Belo Horizonte-MG, Brazil. Interpretation of data, manuscript writing, critical revision, final approval of the version to be published.
IIIPhD, Full Professor, Department of Surgery, School of Medicine, UFMG, Belo Horizonte-MG, Brazil. Substantive scientific and intellectual contributions to the study, responsible for conception and design, interpretation of data, manuscript writing, critical revision, final approval of the version to be published.

ABSTRACT

PURPOSE: To describe a method to characterize the gelatinase activity of cultured human periodontal fibroblasts stimulated with Pam3Cys and E. coli LPS, ligands of TLR2 and TLR4 respectively, and by centrifugation of the cultures, simulating an orthodontic force.

METHODS: To study MMP-2 activity, primary cultures of human periodontal fibroblasts were stimulated with the addition of TLRs 2 and 4 ligands and the application of mechanical force by centrifugation at 141 x g for 30 min. Supernatant media was collected 24 hours later to perform protein quantification and zymography.

RESULTS: MMP-2 activity suffered an increase in cultures co-stimulated with TLRs 2 and 4 ligands alone or with the presence of mechanical force application compared to basal levels.

CONCLUSION: Zymography, one of the several methods to study MMPs activities, is a simple, qualitative and efficient method based on electrophoresis of bis-acrylamide gels copolymerized with a protein substrate.

Key words: Zymography. Matrix Metalloproteinase 2. Fibroblasts. Periodontal Ligament. Toll-Like Receptors.
Zimography is an effective method for detection of matrix metalloproteinase 2 (MMP-2) activity in cultured human fibroblasts

Introduction

Metalloproteinases (MMPs) are Zn\(^{2+}\) dependent endopeptidases responsible for the degradation of extracellular matrix on physiological and pathological conditions such as inflammation, neoplasms, wound healing, angiogenesis and matrix remodeling. Collectively, they are capable of degrading all kinds of extracellular matrix proteins and also process a number of bioactive molecules. These enzymes are produced and released from cells as a latent form (Pro-MMP) and are activated by the removal of the NH\(_2\)-terminal pro-peptide of approximately 10 kDa. Their family are composed by 25 members divided on sub-families: collagenases, gelatinases, matrilisyns, stromelisyns and membrane type MMPs. The gelatinase sub-family is composed by gelatinase A (MMP-2) and gelatinase B (MMP-9) both capable of metabolizing native and denatured collagen, gelatin, elastin, laminin, fibronectin and the basement membrane. Stromelysin (MMP-3) and matrilysin (MMP-7) also demonstrate broader substrate specificity like proteoglycans, gelatins, elastin, and glycoproteins.

Several methods have been reported on literature to study the expressions and activities of MMPs, as an example: ELISA, Western Blotting, Immunohistochemistry, fluorometric oligopeptide substrates, radiolabeled collagen and zymography. Zymography is a simple and sensitive qualitative method based on the electrophoresis of SDS-PAGE gels copolymerized with a protein substrate. Gelatin is used as a protein substrate to evaluate the proteolytic activity of MMP-2 and MMP-9, casein for MMP-3, MMP-7 and MMP-10, and collagen for MMP-1. In a complex mixture as conditioned cultured cells media, biological fluids, or purified preparations, thezymography allows the identification of MMPs in their active and latent forms, based on molecular weight.

Toll-like receptors (TLRs) are a family of pattern-recognition receptors responsible for triggering inflammatory responses to microbial invasion. They recognize and distinguish the pathogen-associated molecular patterns (PAMPs), which are highly conserved structures of microorganisms. TLRs can initiate the intracellular signaling cascade leading to inflammatory and immune gene transcriptions like cytokines and matrix metalloproteinases (MMPs).

The aim of the study is to demonstrate that the zymography can be used to evaluate the gelatinase activity of cultured human periodontal fibroblasts. The cells were stimulated with Pam\(_3\)Cys and E. coli LPS, ligands of TLR2 and TLR4 respectively, or by centrifugation of the cultures at 141 x g for 30 min, a simulation of orthodontic force, as previously described.

Methods

Fibroblast cultures

Human periodontal fibroblasts were obtained from primary cultures of periodontal ligament explants. Healthy donors with indication for exodontia of enclosed third molars were selected at the Dental School of the Minas Gerais Federal University (UFMG), Brazil and invited to participate in the study, donating their extracted teeth. The study was approved by the Ethical Committee for Research, UFMG, Brazil (COEP-UFMG, license no. ETIC 0078.0.203.000-11 – 14/06/2011) and informed consent were obtained from all patients.

Teeth which had been sectioned during the surgery and those who hadn’t more than half of the root formed were discarded due to difficulty to immobilize the crown for tissue extraction and lack of periodontal tissue.

Extracted teeth were immersed in DMEM culture medium (Gibco, Grand Island, NY), with 1% antibiotic–antimycotic (Gibco) and 10% FCS (Sigma, St. Louis, MO) and transported to the Lineu Freire-Maia Laboratory at the Medical School, UFMG, Brazil. Teeth were rinsed with 70% ethanol solution followed by washing out with 0.9% NaCl. Periodontal ligament were removed from the middle third roots and fragmented. The explants were transferred to a 25cm\(^2\) culture flask containing the same culture medium used on transportation. Cultures were incubated at 37°C and 5% CO\(_2\) until confluence in which the medium was changed every three days. Fibroblasts were trypsinized (0.25% trypsin; trypsin-EDTA, Gibco) and transferred to a 75cm\(^2\) culture flask. On the third passage, the cells were seeded on 48 well plates for treatment.

Culture stimulation

Cells were washed out with 0.9% saline and a serum-free DMEM medium was added prior to stimulation. Experimental and control groups were planned. On the experimental groups, cultures were stimulated with 1 µg/mL of Pam\(_3\)Cys or E. coli LPS or by centrifugation at 141 x g for 30 min or combination of the stimuli as follows: Control group (CT), Centrifugation group (CTc), TLR4 ligand (LPS), TLR2 ligand (P3C), TLR4 + centrifugation (LPSc), TLR2 + centrifugation (P3Cc), TLR2 + TLR4 (PL), TLR2 + TLR4 + centrifugation (PLc). The cells were stimulated for 24 hours in a 5% CO\(_2\) incubator and supernatant collected. Samples were stored at -20°C until the gelatin zymography assay.
Cell viability assay

After stimulation, cell viability was tested under optical microscopy after the addition of 0.4% Trypan blue (BioWhittaker, Walkersville, MD, cat. 17-942E). The numbers of stained and non-stained cells were determined to avoid bias caused by stimulation-induced cell death.

Protein assay

A BCA spectrophotometric (490-nm wavelength). Protein Assay Kit (Pierce, Rockford, IL), was used for total protein quantification of culture media samples.

Zymogram

Gelatin zymograms were performed as follows: 10.5% SDS-polyacrylamide separating gels with 2 mg/mL of gelatin (30% bis-acrylamide, 1.5 M tris-HCL pH 8.8, 10% ammonium persulfate, 0.04% TEMED, 10% SDS and distilled water to reach final volume) and 5% SDS-polyacrylamide staking gels (30% bis-acrylamide, 1 M tris-HCL pH 6.8, 10% ammonium persulfate, 0.1% TEMED, 10% SDS and distilled water to reach final volume) were prepared. Solutions were cast on Mini Protean (BioRad) with the addition of ammonium persulfate and TEMED to polymerize the gels (1 mm thickness). The gels were loaded with samples diluted in 1:1 non-reducing buffer (12.5% 0.5 M Tris–HCl pH 6.8, 10% glycerol, 4% SDS, and 0.05% bromophenol blue) and the electrophoresis was carried at 4 °C using a constant current of 35 mA at 90 V for approximately 5 h. After disassembling the apparatus, gels were washed 2 X in 2.5% triton-x 100 for 15 minutes, incubated on incubation buffer (Tris–HCl 50 mM, CaCl2 10 mM, NaCl 50 mM, pH 7.6) for 18 hours at 37°C, stained by 0.1% Coomassie brilliant blue R-250 solution (with 40% methanol and 10% acetic acid) for 4 h under gentle shaking and destained 2 X for 20 minutes with 25% ethanol and 8% acetic acid solution. Gels were scanned at 600 dpi and analysed for densitometry at Kodak Molecular Imaging Software 4.0.5.

The gels were washed out with distilled water and scanned (Genius ColorPage-Vivid Pro II at 600dpi) for densitometry (Kodak Molecular Imaging software v.4.0.1).

Results

Cell viability assay showed no significant difference between groups (Kruskal Wallis, p>0.05).

Non-stained bands were shown at a blue background gel revealing the gelatin digestion by MMPs at its respective molecular weight.

Sample derived from human periodontal fibroblast culture media collected 24 hours after stimulation revealed only activity for 72 kDa MMP-2 on gelatin zymography. MMP-9 was not detected as depicted in Figure 1.

Discussion

The method of tissue collection from healthy teeth (no association with dental caries and periodontal diseases) on a surgical environment for fibroblast culture allowed a low index of
Zimography is an effective method for detection of matrix metalloproteinase 2 (MMP-2) activity in cultured human fibroblasts

Cell viability was determined to check if centrifugation-induced cell death might interfere with the results. A decrease of more than 20% on cell viability below control levels has been shown to interfere with the reliability of the data11. In our study, no significant difference was found on cell viability ratio between control and experimental groups.

MMP-2 was the only MMP showing activity by gelatin zymography with an observed effect on a 72 kDa form. The 72 kDa MMP-2 has gelatinolytic activity thanks to the disruption between the Zn$^{2+}$ and the free thiol radical present in a cysteine conserved region of its prodomain12.

The centrifugation alone does not induce an increase in MMP2 activity. However, the simultaneous activation with toll-like receptors agonists, TLRs 2 and 4, induced a two times fold increase in MMP-2 activity when compared to basal levels. The data seem to indicate a potentiation of the isolated stimulation by either one of these ligands. In addition, cells treated by simultaneous addition of TLRs 2 and 4 followed by centrifugation, have shown MMP-2 activity twice as higher than isolated stimulation by centrifugation.

Zymography is one of the most sensitive and reliable method to study the proteolytic activity of MMPs. This method is able to detect picograms of the enzyme and to distinguish active and latent form from different MMPs3,5,8,13 based on their molecular weight. Additional advantages of this method are its simplicity and low cost.

Even considering, during the electrophoresis, that gel conditions cause protein denaturation, the MMP-2 activity as demonstrated by gel destaining may be explained. The enzymes are separated under denaturing by sodium dodecyl sulfate (SDS) at non-reducing conditions. The removal of the SDS, usually by Triton X-100 and the incubation in the proper buffer, promotes protein renaturation and activity, allowing the visualization of bands after the staining and destaining procedures3.

The observed potentiation of both TLR2 and TLR4 ligands on MMP-2 activity seems to indicate that Toll-like Receptors may play a roll in the modulation of inflammatory responses in the periodontal ligament fibroblasts. These receptors are significantly more expressed on periodontal tissue during inflammation14,15. In vitro, human periodontal fibroblasts showed increased expression of TLRs 2 and 4 after infection by Gram negative periodontal bacteria14. Also Porphyromonas gingivalis, a gram negative anaerobe bacteria, is associated with bone loss on periodontal disease is thought to produce LPS that activates TLRs 2 and 416-18.

Conclusion

The zymography is a reproducible and reliable method to detect gelatinase activity at least of MMP-2 in primary cultures of human periodontal ligament fibroblasts with potential clinical applications.

References

16. Ogawa T, Asai Y, Hashimoto M, Takeuchi O, Kurita T, Yoshikai Y,


Acknowledgment

Marcus Vinícius Andrade is supported by a NIH Grant 1 R01 TW 00612 (GRIP).

Correspondence:
Jose Renan Cunha-Melo
Departamento de Cirurgia, Faculdade de Medicina, UFMG
Avenida Professor Alfredo Balena, 190/ sala 295
30130-100 Belo Horizonte – MG Brasil
jrcmelo@medicina.ufmg.br

Received: October 23, 2012
Review: December 19, 2012
Accepted: January 22, 2013
Conflict of interest: none
Financial sources: FAPEMIG (Minas Gerais Research Foundation), CNPq (National Council of Technological and Scientific Development) and CAPES (Coordination of Improvement for Higher Academic Staff).

Research performed at Professor Lineu Freire Maia Laboratory, Department of Surgery, School of Medicine, Minas Gerais Federal University (UFMG), Belo Horizonte-MG, Brazil. Part of PhD degree thesis, Pathology Postgraduate Program, Medical School, UFMG. Tutor: Jose Renan Cunha-Melo.