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RESEARCH ARTICLE

Lipopolysaccharide (LPS) Induces Matrix Metalloproteinase-2 and -9 (MMP-2 and MMP-9) in Bovine Dermal Fibroblasts

Ahmet Akkoc[1,*], M. Mufit Kahraman[1], Alper Vatansever[1], Elcin Gunaydın[2] and Ezgi Akdesir[1]

[1]Uludag University, Faculty of Veterinary Medicine, Department of Pathology, 16059, Gorukle, Bursa, Turkey;
[2]Veterinary Control Central Research Institute, Breeding Disease Diagnosis Laboratory, 06020, Etlik, Ankara, Turkey
*Corresponding author: aakkoc@uludag.edu.tr

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A B S T R A C T

Bovine digital dermatitis (BDD) is one of the most important problems in dairy herds. The exact etiology is unknown but it is believed that bacteria have cardinal roles. Matrix metalloproteinases (MMPs) are a group of enzymes responsible for the degradation of extracellular matrix proteins. The plausible role of these enzymes in the formation of ulcerative lesions in various tissues in humans is widely known. Ulcers on the plantar skin of hind feet are the prominent lesions in early stage of BDD. The main objective of this study was to determine whether MMP-2 and -9 enzymes are enhanced following LPS challenge in bovine dermal fibroblasts. Skin samples were obtained from two Holstein-Friesian cows after slaughtering. Primary bovine dermal fibroblasts were isolated from foot skin and then exposed to 5 µg/ml LPS. As result, we found that LPS stimulates the release of MMP-2 and MMP-9 enzymes in bovine dermal fibroblasts. When compared with controls, immunoblots revealed significant increase in MMP-2 (by 56%, $P<0.005$) and MMP-9 (by 74%, $P<0.005$) enzymes. Zymographic analyses demonstrated LPS challenge resulted with the release of higher amount of active MMP-2 and –9 enzymes by 37% and 48%, respectively. These data indicate LPS may be an important factor in the pathogenesis of BDD by causing local MMP-2 and MMP-9 enzyme release.

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INTRODUCTION

Bovine digital dermatitis (BDD) is an important foot disease commonly observed in dairy cattle and is recognized as one of the major causes of lameness (Zinicola *et al*., 2015). Typical BDD lesions develop as ulcerated lesion and progress over weeks to painful, proliferative nodular tissue outgrowths on the plantar skin of hind feet (Döpfer *et al.,* 1997). The etiology of BDD has not yet been completely determined and a complex multifactorial etiology can be argued. The majority of evidence obtained from the field studies suggests involvement of Gram (-) bacteria and spirochetes (Sullivan *et al*., 2015; Zinicola *et al*., 2015).

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram (-) negative bacteria and has various effects on host, it stimulates cells to produce cytokines and Matrix Metalloproteases (MMPs) (Kent *et al.,* 1998; Woo *et al.,* 2004; Wong *et al.,* 2011; Li *et al*., 2015). The recent studies conducted in other species other than cattle indicate that LPS stimulation causes increased activities of MMP-2 and MMP-9 enzymes (Pagenstecher *et al.,* 2000; Kim *et al.,* 2012; Fuegler *et al.,* 2013). In a single study, gene expression profile of MMP-12 enzyme in response to LPS challenge was evaluated in bovine dermal fibroblasts. In that study, authors reported 1.48-fold increase in the expression of MMP-12 gene in cultured bovine fibroblasts. Matrix metalloproteinases are zinc-dependent proteases responsible for the degradation of extracellular matrix proteins such as gelatin, collagen and laminin (Nagase *et al.,* 2006). One of these extracellular matrix proteins, collagen, is mainly found in the structure of basement membrane, and disruption of the basement membrane is critical for ulcerations. MMPs are synthesized by a wide variety of cells including fibroblasts (Archile-Contreras *et al.,* 2010; Toriseva *et al.,* 2012; Saalbach *et al*., 2015) and have pivotal roles in many pathological processes such as ulcerative wounds and cancer metastasis in which basement membrane disruption is the principal event (Lempinen *et al.,* 2000;

Mirastschijski *et al.,* 2002; Gonzalez *et al.,* 2010; Rath *et al.,* 2010). MMP-2 and MMP-9 enzymes have been shown to participate in the pathogenesis of diseases including septic shock and endotoxemia (Lalu *et al.,* 2006). In veterinary literature, increased plasma levels of MMP-2 and MMP-9 enzymes were recorded in horses after intravenous LPS administration (Fuegler *et al.,* 2013). Higher serum MMP-9 concentration was reported in acutely septic shock cattle when compared with healthy counterparts (Bannikov *et al.,* 2011).

In humans, the contribution of MMPs in the formation of various ulcerative skin lesions has been demonstrated (Fray *et al.,* 2003) we hypothesized that LPS challenge in bovine dermal fibroblasts is resulted with increased MMPs. To test this hypothesis, we isolated dermal fibroblasts from the mostly affected part of bovine foot skin and exposed these cells to bacterial LPS. Then, we evaluated the presence of MMP-2 and MMP-9 enzymes and changes after LPS stimulation. According to the best of author's knowledge, this study is the first attempt to document the effects of LPS on MMP-2 and MMP-9 enzymes in bovine digital dermal fibroblasts.

MATERIALS AND METHODS

Culture of bovine digital dermal fibroblasts: Dermal fibroblasts were isolated from two healthy Holstein-Friesian cows after slaughtering (Cimet, Bursa, Turkey). Plantar aspect of hind feet was trimmed and full thickness skin biopsy samples were taken. Following mincing of them under sterile conditions small fragments of dermal samples were placed into 6 well-plates. Culture medium M199 (M199, Sigma, M3769) containing 10% FBS were added and the medium was changed every other day until cells reached at their confluence. The cells from third to sixth passages were used throughout the experiments.

Characterization of cultured cells: Isolated cells were stained by using streptavidin biotin peroxidase method. Cells were characterized according to their reactions against monoclonal mouse anti- vimentin (M0725, DAKO, Denmark) and cytokeratin (M0821, Dako). Briefly, isolated cells were seeded on the sterile round glass and fixed with cold methanol and stained with diaminobenzidine which was the chromogen. The specificity of anti-vimentin and cytokeratin antibodies for bovine cells was tested in full thickness bovine skin sample, beforehand. Homologous nonimmune serum instead of primary antibodies was utilized as negative controls.

LPS challenge: LPS from *Escherichia coli* O111:B4 was (L4391, Sigma Aldrich, St.Louis, MO, USA) diluted in M199 cell culture medium. After serum starvation for 24-hour, cells were cultured with M199 medium containing 5µg/ml of LPS for 48-hour in the experiment groups. At the end of each experiment, cell culture media were collected aseptically, centrifuged at 4417g for 5 minutes and supernatants were kept frozen at -20°C until the day of further analysis. Relative amount of tubulin proteins, indicative for cell death, were compared between the experiment and the control groups.

Gelatin zymography: The total protein concentration of each supernatant was determined by commercially available BCA protein assay kit according to manufacturer's protocol (BCA-1 Kit, Sigma). The proteolytic activities of MMP-2 and MMP-9 enzymes were evaluated by gelatin zymography as described previously (Kleiner and Stetler-Stevenson, 1994) with 10% polyacrilamide gel containing 0.1% gelatin (G9136, Sigma) as substrate. Computerized densitometry was used to evaluate relative enzymatic activity (GelQuant Pro program, DNR ChemiBis, Israel) and the results were expressed in arbitrary units (AU) and normalized as percentages of control samples.

Immunoblotting: The presence and relative amount of the MMP-2 and MMP-9 enzymes in supernates was determined with immunoblotting as previously described (Akkoc *et al*., 2011). Briefly, the samples (30 µg protein) were loaded on 10% SDS-PAGE gels and electrophoresed proteins were transferred to the polyvinyl difluoride (PVDF) membranes (BioRad, 162-0177). Then membranes were blocked with 5% skimmed milk powder for 1 hour in room temperature; washed membranes were incubated with bovine specific primary antibodies (MMP-2: AP00546PU-N, Acris, Herford, Germany, MMP-9:Aviva Systems Biology, CA, USA, Beta tubulin: Abcam, Cambridge, MA) overnight at +4 °C temperature on the shaker. Secondary antibody was applied for 2 hours, washed and protein bands were visualized with enhanced chemiluminescence system (107-5010, BioRad) using digital imaging system (DNR ChemiBis, Israel). Immunoreactive bands were compared densitometrically using the GelQuant Pro program (DNR ChemiBis, Israel). Areas under the absorbance curve were expressed as arbitrary units and normalized as percentages of control samples.

Statistical analysis: The statistical significance of differences in MMP-2 and MMP-9 enzymes between treated and untreated dermal fibroblasts was tested with Student' unpaired two-tailed t test; $P<0.05$ was considered significant.

RESULTS

Digital dermal fibroblasts were successfully isolated, characterized and cultured. The spindle to oval-shaped morphology (Fig.1) and immune characteristics of isolated cells were in accordance with that of fibroblasts. The cultured cells displayed vimentin positive and cytokeratin negative immune features (Fig. 2). Immunoblotting and zymographic analyses revealed that primary bovine dermal fibroblasts synthesize and secrete MMP-2 and MMP-9 enzymes in their microenvironment under physiologic conditions (5% CO_2 and 37ºC). After 48-hour of LPS challenge, significant increase in MMP-2 and MMP-9 enzyme levels in culture medium was found. In immunoblots, compared with control groups, MMP-2 (by 56%; $P<0.05$; Fig. 3) and MMP-9 (by 74%; $P<0.05$; Fig. 3) proteins were increased. Then we examined the activity of MMP-2 and MMP-9 enzymes by gelatin zymography since gelatin is the shared substrate of MMP-2 and MMP-9 enzymes. Without any chemical activation

step, supernatants of LPS-treated cells contained substantial amounts of active MMP-2 and MMP-9 enzymes. Only, a constitutive synthesis of pro-form of MMP-2 and MMP-9 enzymes was observed in control groups. Compared with control groups, LPS treatment resulted with increased release of active MMP-2 and MMP-9 enzymes by 37%; ($P<0.05$) (Fig. 2) and 48% ($P<0.05$; Fig. 4).

DISCUSSION

Fibroblasts are an important group of cells in dermal connective tissue; they produce extracellular matrix components and orchestrate extracellular matrix biology in the skin. They are also able to synthesize MMPs (Archile-Contreras *et al.*, 2010: Akkoc *et al.*, 2011). The steady and higher expression of MMPs by host cells, including fibroblasts, following various stimuli contributes to destruction and ulcer formation in various organs and tissues (Vaalamo *et al*., 1997). Increased expression and activity of MMPs can lead to the degradation of basement membranes and this disintegration can be evaluated as an essential step in the formation of ulcerative changes (Reiss *et al.*, 2010). Bovine digital dermatitis is an ulcerative and proliferative skin problem of the bovine foot; painful ulcerative lesions in the heel bulbs are common in M2 stage of infection (Zinicola *et al*., 2015). The exact pathogenesis of BDD is not clear and most of the data obtained from field studies substantiate bacterial involvements, mostly in a gram negative nature, in the development of the lesions (Sullivan *et al*., 2015). By the utilization of *in vitro* culture models, the challenge of target cells with the structural antigenic components of bacteria may provide invaluable information in the understanding of complex mechanisms of inflammation of this region. LPS is one of the most important antigenic members of bacterial cell wall and the evaluation of the effects of LPS on MMPs in bovine dermal fibroblasts may reveal one of the missing parts of BDD jigsaw.

The information on the expression of MMPs in bovine fibroblast is very limited; except the expression of MMPs in fibroblasts from bovine muscle and placental tissues (Walter *et al.*, 2001). In a recent study, LPS-stimulated increase in the expression of MMP-12 enzyme gene was reported in bovine dermal fibroblasts (Evans *et al*., 2014). In that study, authors suggested fibroblasts may be essential cells for the pathogenesis of bovine digital dermatitis. According to our knowledge, there is no report on the expression of MMP-2 and MMP-9 in bovine dermal fibroblasts either; therefore, the data obtained from our study except bovines were discussed with the results of other human or laboratory animal studies. For instance, in the results obtained from humans and rat studies, LPS was found to induce MMP-2 and/or MMP-9 secretion and activation in various cell lines (Lee *et al.*, 2003). In the present study, we observed increased release of active form of both enzymes in cultured bovine dermal fibroblasts. Stimulation of the synthesis and activity of MMP-2 enzyme was reported in response to LPS challenge in human periodontal ligament cells (Kim *et al.*, 2012). Correspondingly, MMP-9 enzyme was induced in astrocytes and corneal fibroblasts after LPS exposure (Wong *et al.*, 2011).

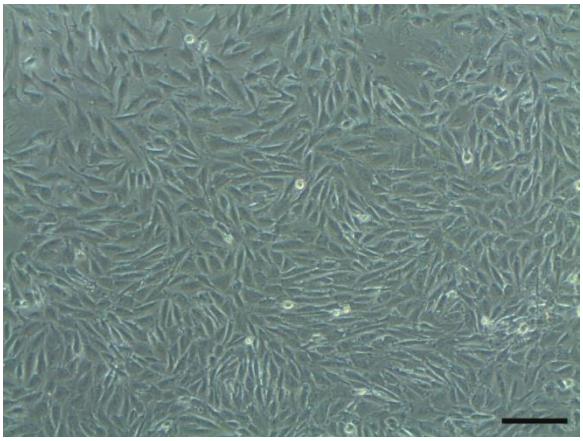

Fig. 1: Primarily isolated and cultured bovine dermal fibroblasts with spindle shaped morphology, confluent cells at third passage, inverted microscope image, Bar=50 µm.

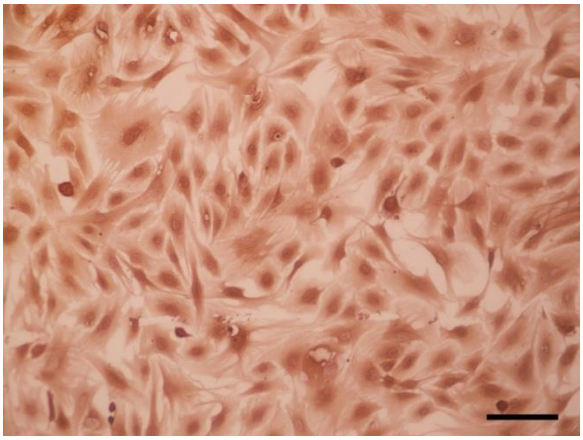

Fig. 2: Isolated cells were characterized by immunocytochemistry. Diffuse positive staining with anti-vimentin antibody. Positive cells displayed brownish coloration, streptavidin biotin peroxidase method, DAB chromogen, Bar=50 µm.

Matrix metalloproteinases have critical roles in many physiological and pathological processes, including normal tissue repair in wound healing or in the development of ulcerations (Swarnakar *et al.*, 2005). Excessive production of these proteases can lead to degradation of extracellular matrix components and lead to ulcerative changes in various organs including skin (Koji *et al.*, 2008). LPS-induced production and release of MMP-2 and MMP-9 enzymes in periodontal fibroblasts, and the contribution of these MMPs to local tissue destruction in periodontitis have been reported as well (Kent *et al.*, 1998). It was demonstrated that exogenous administration of MMP-9 enzyme delays skin wound healing in mouse model (Reiss *et al.*, 2010). Increased MMP-12 gene expression following LPS challenge was reported in bovine dermal fibroblasts (Evans *et al*., 2014). In that study, researchers have been solely investigated gene expression profile of MMP-12. They did not mention about neither protein level nor the activity of MMP-12 enzyme in their study. In the submitted study, by using immunoblotting and zymographic analyses, we found that LPS challenge resulted with increased MMP-2 and MMP-9 enzymes in bovine dermal fibroblasts. Hence, we can suggest that elevated amount of these enzymes may contribute the pathogenesis of BBD infection *in vivo*.

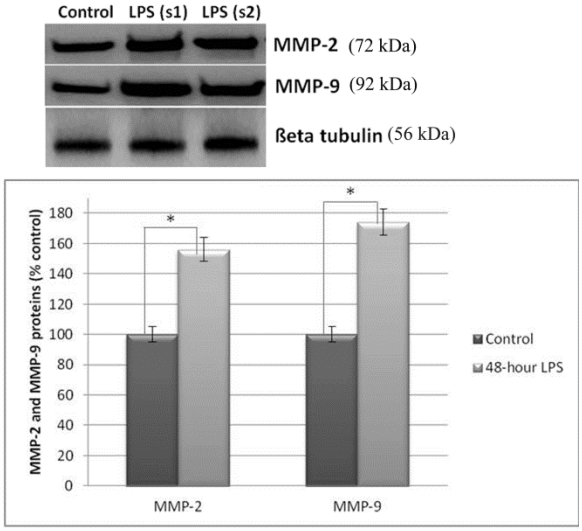

Fig. 3: Graph depicting MMP-2 and MMP-9 enzyme expression as percentage of control values. Tubulin was used as protein loading control. Thirty microgram of protein was loaded in each lane. The data shown are representative of three independent experiments from 2 cattle. Total MMP-2 (by 56%; P<0.05) and MMP-9 (by 74%; P<0.05) proteins were increased in LPS (5µg/ml) treated bovine dermal fibroblasts (s1: cells from first animal, s2: cells from second animal). *P<0.05 compared with control.

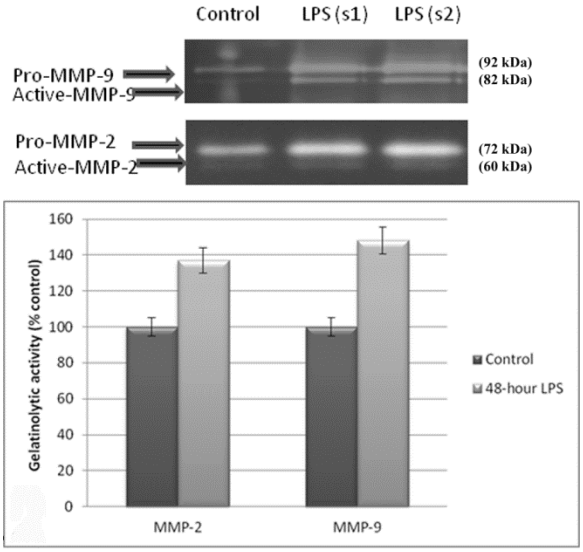

Fig. 4: Graph depicting gelatinolytic activity of MMP-2 and MMP-9 enzyme expression as percentage of control values. Thirty microgram of protein was loaded in each lane. The data shown are representative of three independent experiments from 2 cattle. Zymogram: gelatinolytic activity of MMP-9 and MMP-2 enzymes in control and LPS (5µg/ml) treated cells. MMP-2 (by 37%; P<0.05) and MMP-9 (by 48%; P<0.05) proteins were increased in LPS treated cells. *P<0.05 compared with control.

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Author's Contribution: Authors declared that AA and MMK designed the study. AV, EA and EG executed the experiments and analyzed cell culture mediums. AV, EG and EA were primarily responsible for cell culture and immunoblotting and zymographic analyses. Analysis results were evaluated and critically revised and approved for submitted version by all authors. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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