Hypoxia enhances secretion of matrix metalloproteinases in bovine dermal fibroblasts: an in vitro approach to bovine digital dermatitis

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ABSTRACT

Dermal fibroblasts are of great importance in skin homeostasis. They are responsible for the synthesis and remodelling of extracellular proteins by the way of synthesis of some specific kind of Matrix Metalloproteinases (MMPs). Imbalance between MMPs and their tissue inhibitors (TIMPs) are known to involve ulcerative wounds in many tissues, including skin. In veterinary medicine, bovine digital dermatitis (BDD) is an ulcerative skin disorder of dairy cattle. To date, Koch’s postulate has not yet been fulfilled for the pathogenesis. In present study, we tested in vitro role of hypoxia if it evokes a response in MMP-1, MMP-2 and MMP-13 enzymes and their inhibitors in bovine dermal fibroblasts. Dermal fibroblasts were exposed to hypoxia in various time periods (6, 12 and 24 h). Immunoblotting revealed 6-, 12- and 24-h hypoxia, which resulted in 43.5%, 75% and 65% increase in MMP-1, 76%, 61% and 55% increase in MMP-2 and 80%, 78% and 55% increase in MMP-13 enzymes levels, respectively. We observed slight increases in TIMP-1 and TIMP-2 enzymes, but differences between treatment and control groups did not show any statistical significance. Our findings indicate that hypoxia induces MMP-1, MMP-2 and MMP-13 enzymes and may contribute to pathogenesis of BDD through in the development of in vivo lesions.

Introduction

Bovine digital dermatitis (BDD) is an important foot disease commonly observed in dairy cattle (Evans et al. 2014). BDD presents several phases with different effects, even less obvious or painful. These lesions can be classified according to a scoring system comprising five stages of M0–M5, as developed by Döpfer et al. (1997). In the M2 stage of infection, typical ulcerations, which vary in size and are generally larger than 2 cm, are seen in bovine foot skin (Döpfer et al. 1997; Berry et al. 2012; Gomez et al. 2012). The majority of evidence obtained from the field studies and experimental infection models suggests spirochaete involvements in the pathogenesis of BDD (Döpfer et al. 2012; Gomez et al. 2012). However, Koch’s postulate has not been fulfilled yet for the etiopathogenesis of digital dermatitis (Pringle et al. 2008; Evans et al. 2009; Yano et al. 2009). The results of several studies on experimental transmission of BDD revealed that not only bacteria but individual susceptibility of the host and environmental changes are also inseparable parts of BDD infection (Gomez et al. 2012). Consequently, researchers hypothesized that digital dermatitis can occur as a result of exposure of digital skin of a cow to oxygen-depleted, wet, organic material containing causative organisms (Berry 2001). It has been suggested that BDD lesions can be reproduced experimentally in susceptible animals after inert wrapping to generate relative anaerobiosis in the hind feet of the cow before the inoculation of diseased material (Gomez et al. 2012). Accordingly, Gomez et al. (2012) successfully established an experimental infection model to induce acute ulcerative BDD lesions by the inoculation of diseased tissue homogenate and pure Treponema spp. to hind feet after wrapping of the legs to mimic conditions of prolonged moisture and reduced access to air and therefore by the induction of hypoxia induction. Thus, the hypoxia has been evaluated and evidenced as an important preconditioning factor in the etiopathogenesis of digital dermatitis (Gomez et al. 2012). Therefore, it is possible to conclude that the hypoxia facilitates the growth of anaerobic bacteria which are involved in the pathogenesis of BDD infection and can also engender important changes in cellular levels on the expression of various proteins (MMPs, growth factors, chemokines, etc.) which may be important in the skin biology of the foot (Evans et al. 2014). In many premises, bovine hind feet recurrently contacts with manure; and it is also known that continuous moisture and low oxygen tension are present on the hind feet of cattle if manure removal and hygiene is not adequate (Berry 2001). Heretofore, the bacterial strains identified from clinical BDD cases are mainly in anaerobic nature and they need reduced oxygen tension like hypoxia or anoxia in tissues to overgrowth and generate infection (Evans et al. 2009; Döpfer et al. 2012).

Matrix metalloproteinases (MMPs) are zinc-dependent proteases responsible for the degradation of extracellular matrix proteins such as gelatin, collagen, laminin and fibronectin. MMPs are synthesized by a wide variety of cells, including fibroblasts (Nagase & Woessner 1999; Toriseva et al. 2012; Evans et al. 2014). Their importance and contributions in both skin wound healing and formation of ulcerative lesions were
mainly emphasized in humans (Trengove et al. 1999; Rayment et al. 2008; Subramaniam et al. 2008). A study demonstrated that increased amount of these proteases contributes ulcerative changes in the skin (Ladwig et al. 2002). The balance between MMPs and TIMPs is an important equilibrium for common physiologic functions, and it can also be responsible for the nature of proteolytic events either in the direction of tissue remodelling or breakage during wound healing, resulting in ulceration. Inflammatory gene expression profiles of bovine dermal fibroblasts and keratinocytes after challenge with Treponemal wall fragments have been investigated in a recent in vitro study on BDD (Evans et al. 2014). In this study, upregulation of many genes, including MMP-12, has been reported in cultured dermal fibroblasts, but not in keratinocytes. Similarly, it was demonstrated that lipopolysaccharide (LPS) challenge in bovine dermal fibroblasts resulted in increased secretion of MMP-2 and MMP-9 enzymes (Akkoc et al. 2016).

The effects of hypoxia in the expression and release of different MMPs from various cell types in humans were widely investigated. After hypoxic treatment, elevated level of MMP-1, MMP-2 and MMP-13 proteins in various cultured cell types (including fibroblasts) in humans was reported (Yamanaka & Ishikawa 2000; Kan et al. 2003; Lolmede et al. 2003; Leufgen et al. 2005). According to the best of authors’ knowledge, there is only one study about the involvement of MMP enzymes in the pathogenesis of BDD in bovine dermal fibroblast (Evans et al. 2014). It is clear from their study that MMP-12 enzyme was strongly upregulated (up to nine folds) when bovine dermal fibroblasts exposed to various Treponemal cell sonicates (bacterial fragments). They also reported an increase in TIMP-1 and TIMP-3 gene expressions, but that increase could not been substantiated statistically.

In our research, we aimed to evaluate the response of MMP-1, MMP-2 and MMP-13 and their tissue inhibitors (TIMP-1 and TIMP-2) in an in vitro hypoxic cell culture model to mimic in vivo hypoxia, which is a prerequisite for anaerobic bacterial growth in vivo. To the best of the authors’ knowledge, this study is also the first to document the expression of MMP-1, MMP-2 and MMP-13 and TIMP-1 and TIMP-2 enzymes in bovine dermal fibroblasts upon exposure to hypoxia.

Material and methods

Culture of bovine digital dermal fibroblasts

The study was carried out after obtaining the written approval of the Institution’s Scientific and Ethical Committee and Animals Right’s Observation Committee (2009-03/2). Dermal fibroblasts were isolated and cultured from the hind leg skin samples of three healthy Holstein Friesian cows slaughtered in a local slaughterhouse. Standard cell culture conditions were followed according to previous studies from both cattle and humans (Mizuno & Glowacki 2005; Green et al. 2011; Evans et al. 2014). Briefly, after slaughtering, left hind feet of animals were collected and plantar aspect of feet were shaved with scissors and cleaned with 70% ethanol. A small, full-thickness skin biopsy sample (size: 0.5 x 0.5 x 0.5 cm) was taken by sterile scalpel blades and placed into sterile phosphate buffered saline (PBS) containing 2x antibiotic–antimycotic combination (penicillin, streptomycin, amphotericin B, 200 units/ml, 200 units/ml, 0.5 µg/ml, respectively (03-033-1, Biolnd, Israel). After transportation of tissues to the laboratory in cold chain, samples were washed 10 times with PBS and incubated overnight at +4°C in a refrigerator. Excessive fat tissues and epidermis were removed, and then remaining samples were chopped with sterile crossed scalpel blades in 100 mm petri dishes in sterile conditions. Small fragments of dermal samples (size: 3 x 3 x 3 mm) were placed into bottom of each well of six-well plates. The bottom of each well of six-well plates was drawn by sterile scalpel blades to improve the attachment of dermal samples before placing. To facilitate stronger attachment, explant tissues were incubated at 37°C for 10 minutes in an incubator and then two drops of foetal bovine serum (F4135, Sigma, St. Louis, MO, USA) were added on each dermal explants and incubated overnight at 37°C with 5% CO2 in an incubator (Juan, MC150, France). Following the incubation, FBS was replaced with M199 cell culture medium (M199, Sigma, M3769) containing 10% FBS. The cells from third to sixth passages were used in the experiments.

Characterization of cultured cells

Isolated cells were characterized according to a previous protocol (Evans et al. 2014). Briefly, cells were immunocytochemically stained using monoclonal anti-vimentin (M0725, Dako, Denmark) and anti-cytokeratin (M0821, Dako) antibodies.

Hypoxia experiments

A special multigas hypoxia incubator (MCO-5M, Sanyo, Japan) was used for the generation of hypoxic condition (hypoxia: 5% O2). Dermal fibroblasts (5 x 10^5 cells) seeded in each well of 24-well plates were exposed to 6-, 12- and 24-h hypoxia (5% CO2, 5% O2 and balance N2), previous studies (Lolmede et al. 2003; Mizuno & Glowacki 2005; Distler et al. 2007; Van et al. 2016). Parallel cultures were placed in normoxic conditions in a standard incubator with 5% CO2, 21% O2 and 74% N2. All experiments were performed in triplicates.

Confirmation of hypoxia

To confirm generation of cellular hypoxia, hypoxia inducible factor-α (HIF-1α, ab16066, Abcam, Cambridge, UK) was immunoblotted in cell lysates from normoxia and from experiment cell groups. Membranes were re-probed with anti-tubulin antibody (ab125267, Abcam) for loading control.

Measurement of total proteins

The total protein concentration of each sample was determined by commercially available BCA protein assay kit and suggested manufacturer’s protocol was followed (BCA-1 Kit, Sigma). Bovine serum albumin (BSA) (1 mg/ml) (82516, Sigma) was used as the protein standard. Absorbance values were obtained at 570 nm using conventional ELISA reader (Thermo Multiscan FC, Finland).
Enzyme-linked immunosorbent assay
The amounts of MMP-1, MMP-13, TIMP-1 and TIMP-2 enzymes were measured with commercially available anti-bovine ELISA kits (MMP-1: SEA097Bo, MMP-13: SEA099Bo, TIMP-1: RPA552Bo01, TIMP-2: RPA128Bo01, USCN, Wuhan, PRC). Anti-human ELISA kit was used for MMP-2 enzyme. For the analyses, protocol recommended by the manufacturer was followed. Absorbance values at 450 nm were read using microplate reader (Thermo Multiscan FC). Duplicate readings were averaged for each standard, control and samples and subtract the average zero standard optical density. Standard curves were designed with software program Curve Expert 1.3. With interpolation of the values, MMP-1, MMP-2, MMP-13 and TIMP-1 and TIMP-2 concentrations were calculated.

Gelatin and collagen zymography
The existence of active and pro-forms of MMP-1, MMP-2 and MMP-13 was evaluated by gelatin and collagen zymography as described previously (Rayment et al. 2008) with 10% polyacrylamide gel containing 0.1% gelatin (G9136, Sigma) and collagen as substrates.

Immunoblotting
The presence and relative amount of the enzymes in supernates were determined with immunoblotting. Briefly, 20 μg of protein was loaded in each lane on 10% SDS-PAGE gels and electrophoresed at constant 175 V for 75 minutes using Mini-Protein® II electrophoresis unit (BioRad, USA). After the transfer of proteins to polyvinyl difluoride membranes (BioRad, 162-0177) using a Transblot Cell (BioRad), membranes were probed with anti MMP-1, MMP-2 and MMP-13 antibodies. Following the incubation with appropriate secondary antibody, protein bands were visualized with enhanced chemiluminescence system (107-5010, BioRad) using digital imaging system (DNR ChemiBis, Israel) and results generated as fold increases by GelQuant Pro program. Areas under the absorbance curve were expressed as arbitrary units and normalized as percentages of normoxia samples.

Statistical analysis
Statistical analyses were performed using Sigma Plot, version 12.0, software. The data were presented as mean ± standard error of the means. Comparisons with regard to MMPs and TIMPs levels obtained from hypoxia and normoxia for each time point (6, 12 and 24 h) were made using one-way analysis of variance followed by post hoc Tukey test. p < .05 was considered significant. For the ELISA, statistical significance of the results was determined by using Student’s t test when 6-, 12- and 24-h hypoxia groups were compared with normoxia groups. The value of p < .05 was considered significant. Bar graphs (Figure 3B–D) depict MMP-1, MMP-2 and MMP-13 enzyme expressions as percentage of normoxia values.

Results
Cell culture
Digital dermal fibroblasts were successfully isolated, cultured and characterized and thus the shape and morphology of the isolated cells were in a similar manner as of fibroblast cells. The cells also displayed a positive reaction to anti-vimentin antibody and a negative reaction with anti-cytokeratin antibody.

Induction of hypoxia
Existence of HIF-1α protein was demonstrated successfully in hypoxic treatment groups and very weak protein lines were also observed in normoxia samples. The amount of β-tubulin protein levels in both normoxia and hypoxia groups did not show any difference, indicating no cell damage (Figure 1).

Gelatin and collagen zymography
The activities of MMP-1, MMP-2 and MMP-13 in cell culture supernates were demonstrated by substrate zymography. Zymographic analysis revealed that dermal fibroblasts in normoxia groups constitutively expressed pro-form of MMP-1, MMP-2 and MMP-13 enzymes. Active forms of MMP-1, MMP-2 and MMP-13 enzymes were also detected in normoxia- and hypoxia-treatment groups (Figure 2).

![Figure 1.](image-url) Confirmation of hypoxia by demonstration of HIF-1α protein in normoxic and hypoxic cells. Line C1 and C2 depicts normoxic supernates, and line-H1 and H2 (hypoxia) display increased expression of HIF-1α in hypoxic cells.

![Figure 2.](image-url) Zymographic analysis of matrix metalloproteinases (MMP-1, MMP-2 and MMP-13). Black arrows show pro-form of MMP-1, MMP-2 and MMP-13. White arrows indicate active forms of MMP-1, MMP-2 and MMP-13 enzymes. Active forms of MMP-1, MMP-2 and MMP-13 were also detected in normoxia- and hypoxia-treatment groups (Figure 2).
Immunoblotting

Bar graphs depict MMP-1, MMP-2 and MMP-13 enzyme expressions as percentage of normoxia values

MMP-1: Compared with normoxia groups, MMP-1 enzyme was 43.5% ($p < .05$), 76% ($p < .05$) and 80% ($p < .05$) increased in 6-, 12- and 24-h hypoxia groups, respectively (Figure 3(A,B)).

MMP-2: Compared with normoxia groups, MMP-2 enzyme was 75% ($p < .05$), 61% ($p < .05$) and 78% ($p < .05$) increased in 6-, 12- and 24-h hypoxia groups, respectively (Figure 3(A,C)).

MMP-13: Compared with normoxia groups, MMP-13 enzyme was 65% ($p < .05$), 55% ($p < .05$) and 55% ($p < .05$) higher in 6-, 12- and 24-h hypoxia groups, respectively (Figure 3(A,D)).

ELISA

Amounts of all MMPs were increased in all time periods when compared with normoxia groups. Slight TIMP-1 and TIMP-2 enzyme increase was recorded in 24-h hypoxia period; however, the differences between the experiment and normoxia groups were not statistically significant ($p > .05$). ELISA results were summarized in Table 1.

Discussion

The present study is the first to document in detail the release of matrix metalloproteinases (MMP-1, MMP-2 and MMP-13 and their tissue inhibitors (TIMP-1 and TIMP-2) in response to hypoxia in bovine cultured dermal fibroblasts. MMPs are responsible for tissue remodelling in many physiological and pathological processes; their cardinal roles in wound healing, ulcerative wound formation, invasion and metastasis of tumour cells have been well documented previously (Karakiulakis et al. 2007; Rayment et al. 2008; Toriseva et al. 2012).

In this study, the effects of hypoxia on MMPs in cultured bovine digital fibroblasts were investigated. Hypoxia, which is known one of the important triggering agents of MMPs, occurs in acute and chronic wounds (Kan et al. 2003; Modarressi et al. 2010; Chen et al. 2012; Deschene et al. 2012). Modarressi et al. (2010) has shown that hypoxia is of negative roles on skin wound healing by inhibiting fibroblast to myofibroblast differentiation. In this present study, MMP-1, MMP-2 and MMP-13 enzymes were included as they are known to be secreted mainly by dermal fibroblasts (Kan et al. 2003; Lam et al. 2005; Deschene et al. 2012). The possible role of bovine dermal fibroblast in the pathogenesis of BDD has been reported by Evans et al. (2014). They reported that some of target genes

Figure 3. Immunoblot demonstration of matrix metalloproteinases (MMP-1, MMP-2 and MMP-13) proteins in culture supernates from normoxia- and hypoxia-treated groups (6-, 12- and 24-h hypoxia groups). β-tubulin was used as positive internal and loading control. The data shown are representative of three independent experiments. When compared with normoxia groups, MMP-1 protein was 43.5% ($p < .05$), 76% ($p < .05$) and 80% ($p < .05$) increased in 6-, 12- and 24-h hypoxia groups, respectively (Figure 3B). MMP-2 protein was 75% ($p < .05$), 61% (by; $p < .05$) and 78% ($p < .05$) increased in 6-, 12- and 24-h hypoxia groups, respectively, when compared with normoxia (3C). Compared with normoxia, MMP-13 protein was 65% ($p < .05$), 53% ($p < .05$) and 55% ($p < .05$) higher in 6-, 12- and 24-h hypoxia groups, respectively (3D). Bars represent standard deviations.
strongly upregulated in dermal fibroblasts instead of keratinocytes in the existence of Treponema cell sonicates. In a previous study, increased amounts of MMP-2 and MMP-9 enzymes were shown in bovine dermal fibroblasts by LPS challenge (Akkoc et al. 2016).

Immunoblots revealed prominent 120 kDa protein bands that corresponded HIF-1α protein in all hypoxia-treatment groups. Weaker 120 kDa protein bands were also noticed in normoxia groups. Existence of lower levels of HIF-1α protein in normoxia groups can be explained by constitutive expression. HIF-1α protein expression was already demonstrated by western blotting and immunohistochemistry in the various tissues of normoxic mice (Stroka et al. 2001). Stroka et al. (2001) also emphasized that constitutive expression of HIF-1α undertook important roles in tissue homeostasis. In this context, weaker expression of HIF-1α in normoxia was found in accordance with the study mentioned above.

Exposure of bovine dermal fibroblasts to 6-, 12- and 24-h hypoxia led to MMP-1, MMP-2 and MMP-13 enzymes’ release in cell culture media. When MMP-1, MMP-2 and MMP-13 amounts in each hypoxia group (6, 12 and 24 h) were compared with normoxia groups, the enzymes were significantly elevated respectively (p < .05 in each group). A slight increase was found in the amount of TIMP-1 and TIMP-2 enzyme in hypoxia groups (6, 12 and 24 h) when compared with normoxia, but the difference between each hypoxia and normoxia groups did not show any statistical significance (p > .05). During the hypoxic stress, release of various MMPs (MMP-1, MMP-2 and MMP-13) into culture media from different types of cells (including dermal fibroblasts) from various organs and species has also been reported (Kan et al. 2003; Papakonstantinou et al. 2003; Deschene et al. 2012). Increased expression and release of MMP-1, MMP-2 and MMP-13 enzymes in human lung fibroblasts cultured in hypoxic condition were stated by Karakiulakis et al. (2007). Kan et al. 2003 and Papakonstantinou et al. 2003 also demonstrated that fibroblasts (including dermal fibroblasts) released TIMP-1 and TIMP-2 enzymes into culture media in response to hypoxia, and they also reported that the difference in TIMP-1 and TIMP-2 levels between normoxia and hypoxic cells were not statistically significant. A slight, but not statistically significant upregulation of TIMP-1 and TIMP-3 enzyme in bovine fibroblast has been demonstrated in a recent study on the pathogenesis of BDD (Evans et al. 2014). In this study, a slight increase was observed in TIMP-1 and TIMP-2 enzymes between the normoxia- and hypoxia-treated cells, but the differences were not statistically different (p > .05) and we thought that our findings on TIMP-1 and TIMP-2 enzymes were in accordance with studies mentioned above.

Zymographic analysis revealed that dermal fibroblasts in normoxia groups constitutively expressed pro-form of MMP-1 and MMP-2 enzymes and did not express MMP-13. Previous studies have also reported that MMP-1 and MMP-2 enzymes were constitutively expressed by human dermal fibroblasts (Kan et al. 2003; Lam et al. 2005). The expression of MMP-13 enzyme is not detected in normal skin fibroblasts (Ravanti et al. 1999; Toriseva et al. 2012), but excessive expression of MMP-13 by dermal fibroblasts in ulcerative skin lesions has been reported (Vaalamo et al. 1997). Hence, our findings were in accordance with previous studies mentioned above.

Earlier studies reported that high levels of MMPs are involved in the generation of ulcerative skin wounds (Ladwig et al. 2002; Rayment et al. 2008). It is also known that exogenous administration of MMP-9 delays wound healing in skin (Reiss et al. 2010). BDD is defined as an ulcerative dermatitis and characterized by painful ulcerative lesions in the M2 stage of infection (Döpfer et al. 1997). Increased secretion of MMP-2 and MMP-9 enzymes in response to LPS challenge in bovine dermal fibroblasts was shown in a previous study (Akkoc et al. 2016). Strong upregulation in the expression of MMP-12 gene after challenge with Treponema species was revealed in bovine dermal fibroblasts in an in vitro model of BDD, and the authors argue that fibroblasts (instead of keratinocytes) may be the pivotal cells in the pathogenesis of BDD lesions (Evans et al. 2014). Parallel to the results of Evans et al. (2014) and Akkoc et al. (2016), we suggest that hypoxia-induced release of MMP-1, MMP-2 and MMP-13 enzymes in bovine dermal fibroblasts may contribute to the formation of ulcerative lesions due to their detrimental effects on extracellular matrix proteins. Therefore, investigation of MMPs and TIMPs profiles in this subset of cells can be critical for the pathogenesis. The inhibition of these enzymes by known synthetic inhibitors such as batimastat, marimastat or tetracyclines may bring new therapeutic applications in clinical BDD cases.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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