Effect of low oxygen conditions on matrix metalloproteinase-9 production of macrophages subjected to cyclic stretching: involvement of ERK and Rho kinase pathways

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Received: 31 October 2016; Revised: 10 January 2017; Accepted: 10 February 2017

Abstract

Macrophages infiltrated in the walls of abdominal aortic aneurysms (AAA) are subjected to cyclic stretching due to pulsatile deformation of blood vessels and low oxygen conditions caused by intraluminal thrombus. These conditions could induce aberrant changes in macrophage functions and lead local weakening of AAA walls. We previously reported that the combination of 10% cyclic stretching and 2.2% O₂ conditions caused an increase in matrix metalloproteinase-9 (MMP-9) production in macrophages as well as changes in morphological responses to cyclic stretching. In the present study, we investigated the effects of oxygen concentrations on MMP-9 productions and morphological changes of macrophages subjected to cyclic stretching. Macrophages differentiated from THP-1 cells were subjected to 10% cyclic stretching under 5% or 1% O₂ conditions for 24 h, and gelatinolytic activity of MMP-9 in the conditioned medium was assessed by zymography. Cells showed spread and rounded shape under static conditions and elongated and oriented to the direction of stretching after exposure to cyclic stretching, and there were no obvious effects of oxygen concentrations in cell morphology. An O₂ concentration of 5% did not change MMP-9 productions of macrophages in static culture and subjected to cyclic stretching compared to normal cell culture condition of 20% O₂. In contrast, 1% O₂ condition stimulated MMP-9 production of macrophages both under static culture and cyclic stretching conditions. We also found that treatments of inhibitors for extracellular signal-regulated kinase (ERK) and Rho associated protein kinase (Rho kinase) suppressed the increased MMP-9 productions of macrophages by 1% O₂ condition. These results suggest that lower oxygen conditions such as 1% O₂ stimulate MMP-9 production in macrophages through signaling pathways involving ERK as well as Rho kinase-mediated actin cytoskeletal contractility.

Key words: Macrophage, Cyclic stretching, Hypoxia, Abdominal aortic aneurysm, Matrix metalloproteinase

1. Introduction

Abdominal aortic aneurysms (AAA) are local dilation of abdominal aorta resulted from chronic weakening of the vessel walls and associated with a risk of rupture and sudden death. Identification of processes of formation and enlargement of aneurysms would valuable to predict the AAA rupture or to delay its progression. Studies for AAA tissues have found extensive inflammatory infiltrates such as macrophages and T lymphocytes (Bobryshev, et al., 1998) (Pearce and Koch, 1996), and increased expression of MMPs (Thompson, et al., 1995), which is a family of enzymes that proteolytically degrade various components of the extracellular matrix. Since MMP-9, the most abundant gelatinolytic MMP and secreted in high levels in AAA tissue (Thompson, et al., 1995), colocalized with infiltrated macrophages (Kazi, et al., 2005), functions of macrophages under AAA walls condition have been examined in association of formation and enlargement of aneurysms.

Macrophages infiltrated in AAA would be subjected to cyclic stretching due to pulsatile deformation of blood vessels
and also exposed to low oxygen conditions caused by intraluminal thrombus (ILT) (Vorp, et al., 2001) (Vande Geest, et al., 2006). According to the hypothesis that these conditions induce aberrant changes in macrophage functions as well as morphology, we have investigated the effect of cyclic stretching and low oxygen conditions on macrophages. As a result, we reported that production of MMP-9 from macrophages increased under the combination of 10% cyclic stretching and 2.2% O₂, mimicking an oxygen condition in ILT-accumulated AAA walls (Vorp, et al., 2001), compared to static and 5% stretching conditions (Oya, et al., 2011). We also showed that 2.2% O₂ condition affects morphological changes induced by cyclic stretching compared to normal culture condition of 20% O₂ (Oya, et al., 2013). Since cyclic stretching under 20% O₂ does not change MMP-9 production of macrophages, oxygen conditions are believed to have a critical role in macrophage functions and morphology.

In the present study, we examined the effects of oxygen concentrations on MMP-9 productions and morphological changes of macrophages subjected to cyclic stretching. In addition, we assessed roles of intracellular signaling factors, extracellular signal-regulated kinase (ERK) and Rho associated protein kinase (Rho kinase), in macrophage responses by using their specific inhibitors PD98059 and Y27632, respectively. ERK is a mitogen-activated protein kinase controlling major cell functions in response to extracellular stimulations and has been reported to be involved in cellular responses to low oxygen condition (Swinson, et al., 2004) (Gao, et al., 2009) as well as MMP-9 expression (Huang, et al., 2012). Rho kinase is widely known as a principal mediator regulating actin cytoskeleton contractility and dynamics in response to mechanical signals and has also been shown to be activated by low oxygen conditions (McMurtry, et al., 2003) (Bailly, et al., 2004).

2. Methods

2.1 Cell culture

The human acute monocytic leukemia THP-1 (Riken BioResource Center, Japan) were cultured in RPMI1640 (Wako, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, USA) and 1 unit/ml penicillin/streptomycin (Wako) at 37°C with 5% CO₂ in a humidified incubator (Wakenyaku, Japan).

2.2 Cyclic stretching experiment

Prior to cell seeding, silicone membrane of the stretch chambers (4cm², Strex, Japan) were immersed in 10 N sulfuric acid (Wako) for 2 h, rinsed extensively with autoclaved distilled water, and coated with 0.1 mg/ml collagen type I (Nitta Gelatin, Japan). THP-1 suspension of 1 mL was then poured into the stretch chamber at density of 5 × 10⁴ cells/ml. Differentiation of THP-1 to macrophages were induced by culturing cells with 20 µM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 3 days and confirmed by CD14 expression (data not shown), according to our previous studies (Oya, et al., 2011) (Oya, et al., 2013).

After PMA treatment, the culture medium was replaced with fresh macrophage-SFM (Invitrogen, USA), and cells were exposed to 10% cyclic stretching at 1 Hz for 24 h under normal (20%O₂) or hypoxic low oxygen conditions (1% or 5% O₂). For low oxygen conditions, cyclic stretching was performed in an oxygen-regulated incubator (Wakenyaku). For the experiments involving signal inhibitions, the culture medium containing 25 µM PD98059 (Wako) or 10 µM Y27632 (Wako) were used.

2.3 Gelatin zymography

After exposure to cyclic stretching, conditioned medium was collected and concentrated using Vivaspin (Sartorius Stedim Biotech, Germany). The concentrated medium was mixed with 2x sample buffer and subjected to electrophoresis in an 7.5% SDS polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gels were washed with Zymogram Renaturing Buffer (Invitrogen), incubated at 37°C for 18 h to 24 h in Zymogram Developing Buffer (Invitrogen), and then stained for 1 h in 0.25% Coomassie brilliant blue (Wako). Gelatinolytic activities appearing as clear bands in the gels were quantified using ImageJ software (National Institutes of Health, USA).

2.4 Quantification of cell morphology

Cells in the stretch chambers were fixed in 4% paraformaldehyde (Wako) for 20 min, permeabilized with 0.1% Triton X-100 (Wako) for 5 min, and stained with Alexa Fluor 546 phalloidin (Invitrogen) and Hoechst 33342 for 20 min at RT.
Fluorescent images of cells were obtained with an inverted fluorescent microscope (Olympus, Japan), and analyzed to determine morphological parameters using ImageJ. Cell morphology was evaluated on the basis of two parameters: inverse aspect ratio and orientation angle of cell. The inverse aspect ratio was defined as the ratio between the minor and major axes of an ellipsoid equivalent to the cell outline. The inverse aspect ratio is ranged from 0 to 1, and the larger the value, the more circular the object is. The orientation angle of cell was defined as the angle between the major axis of the ellipsoid and the direction of cyclic stretching. Cells with orientation angle of 0° to 30° were assumed to orient to the stretching direction in this study.

2.5 Statistics

Results are expressed as mean ± SD determined from at least three independent experiments. Student's t-test and the Mann-Whitney U test were used for comparison between groups. \( P < 0.05 \) was considered to be statistically significant.

3. Results

3.1 Morphology of macrophages exposed to cyclic stretching under low oxygen conditions

Fig. 1 shows fluorescence images of actin filaments and nuclei of macrophages exposed to cyclic stretching under 20%, 5%, and 1% \( \text{O}_2 \) conditions. Cells showed spread and rounded shape under static conditions, and they exhibited elongation and orientation to the direction of stretching after exposure to cyclic stretching. Thick actin filament bundles traversing the longitudinal axis were observed in cyclically stretched cells, regardless of oxygen concentration. These morphological changes of macrophages coincide with our previous study (Oya, et al., 2013).

![Fig. 1](image-url) Representative fluorescence images of actin filaments (red) and nuclei (green) in macrophages subjected to cyclic stretching for 24 h under 20%, 5%, and 1% \( \text{O}_2 \) conditions.
Results of morphological analyses of macrophages are shown in Fig. 2. Inverse aspect ratios for macrophages were significantly decreased by exposure to cyclic stretching, but were not affected by oxygen concentrations (Fig. 2A). The percentage of cells oriented to the direction of stretching increased by exposure to cyclic stretching under 20% O₂ condition (Static 37.7%, Stretched 52.0%) (Fig. 2B). Although the percentage of cells oriented was slightly decreased compared to 20% O₂ conditions, many cells oriented in the direction of cyclic stretching under lower oxygen conditions (5% and 1%) after exposure (5%O₂: Static 30.9%, Stretched 43.5%; 1%O₂: Static 34.4%, Stretched 44.3%).

Fig. 2  Results of morphological analyses of macrophages subjected to cyclic stretching for 24 h under 20%, 5%, and 1% O₂ conditions. (A) Inverse aspect ratios of macrophages. Results are expressed as mean ± SD. (B) Histograms of angles of cell orientation. 20%O₂: n = 284 cells (Static), n = 296 (Stretched); 5%O₂: n = 362 (Static), n = 375 (Stretched); 1%O₂: n = 387 (Static), n = 318 (Stretched).

Fig. 3 Results of gelatin zymography. (A) MMP-9 was detected by gelatin zymography in media conditioned with macrophages subjected to cyclic stretching for 24 h under 20%, 5%, and 1% O₂ conditions. (B) MMP-9 was quantified by band density of zymograms. Results are from four independent chambers.

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3.2 MMP-9 production of macrophages exposed to cyclic stretching under low oxygen conditions

Gelatin zymography of macrophage-conditioned medium detected only MMP-9 production but not MMP-2 (Fig. 3A), which agreed with our previous study (Oya, et al., 2011). Levels of MMP-9 were quantified by the relative intensity of bands (Fig. 3A) and expressed as the percentages of MMP-9 for cells statically cultured under normal 20% O₂ condition (Fig. 3B). The levels of MMP-9 increased under 1% O₂ condition in macrophages both cultured statically and...
exposed to cyclic stretching. In contrast, MMP-9 levels did not change after exposure to cyclic stretching under 20% and 5% O2 conditions.

3.3 Effects of ERK and Rho kinase inhibitors on macrophages

To examine intracellular signaling in macrophages, cells were exposed to cyclic stretching under 1% O2 with medium containing PD98056 (ERK inhibitor) or Y27632 (Rho kinase inhibitor). Cells treated with PD98056 or Y27632 also showed thick actin filament bundles oriented to the direction of cyclic stretching in cells (Fig. 4A). While there were no significant effects of inhibitor-treatment on aspect ratios of cells under static conditions, the inverse aspect ratios were significantly lower for PD98059-treated and higher for Y27932 compared to non-treated cells after exposure to cyclic stretching (Fig. 4B). Cells treated with inhibitors showed cyclic stretching-induced reorientation (PD98059: Static 29.5%, Stretched 64.0%; Y27632: Static 43.2%, Stretched 50.8%), which was similar to non-treated cells after exposure (Fig. 4C). Treatment of PD98059 or Y27632 suppressed the increase in macrophage MMP-9 productions caused by 1% O2 both under static and cyclic stretching conditions, which was no significant different from that under 20% O2 condition (Fig. 5).

4. Discussion

Macrophages in aneurysmal walls are exposed to cyclic stretching due to heartbeat and low oxygen stress caused by intraluminal thrombus. Production of MMPs by macrophages under these conditions are believed to be implicated in weakening of arterial walls (Vorp, et al., 2001) (Kazi, et al., 2005) (Murdoch, et al., 2005) (Oya, et al., 2011). In the present study, we focused on the effect of oxygen concentrations on MMP production of macrophages subjected to cyclic stretching. As a result, the increased productions of MMP-9 from macrophages were observed at 1% O2 both under static and cyclic stretching conditions, while MMP-9 from macrophages under 5% O2 condition were not changed compared to 20% O2. Our previous study showed that only the combined condition of cyclic stretching and 2.2% O2 caused an increase in MMP-9 production from macrophages, but either condition of 2.2% O2 or cyclic stretching did not induce changes in macrophage MMP-9 production (Oya, et al., 2011). Although cell culture is generally conducted under 20% O2 condition, oxygen concentration ranges in vivo from 3% to 12% (Csete, 2005). It is possible that less than 3% O2 condition be a cue for induction of changes of MMP-9 production of macrophages, and further low oxygen concentration such as 1% O2 itself stimulates MMP-9 expression even under static condition. A previous study demonstrated that expression of tissue inhibitor of metalloproteinases (TIMPs), known as inhibitors of MMPs, from monocytic cells are also decreased by 1% and lower oxygen concentrations (Lahat, et al., 2011). Much lower oxygen condition may enhance degradation functions of macrophages in diseased vessels and cause local weakening of the arterial walls.

We further examined the intracellular signaling pathways, which stimulate MMP-9 production under the 1% O2 condition, by using an ERK inhibitor PD98059 and Rho kinase inhibitor Y27632. In the results, we show that ERK and Rho kinase are involved in the increased production of MMP-9 caused by the 1% O2 condition. Hypoxia inducible factor (HIF)-1α is a well-known signaling factor responsible for changes in cell functions under hypoxic conditions, and HIF-1α is suggested to have a regulatory role in the expression of MMP-9 (Swinson, et al., 2004) (Gao, et al., 2009). Since ERK acts as an upstream signal for HIF-1α expression (Mottet, et al., 2002) (Westra, et al., 2010), activation of ERK may occur under the 1% O2 condition, which leads the increased productions of MMP-9 in the present study. We also have a result that the increases in MMP-9 production by 1% O2 were suppressed by the inhibition of Rho kinase, which is a downstream effector of the small GTPases Rho family RhoA and regulates actin cytoskeletal contractility via myosin light chain phosphorylation (Deguchi, et al., 2011). Previous studies have demonstrated that tension in actin cytoskeleton plays a regulatory role in activation of ERK (Hirata and Sokabe, 2015) and low oxygen conditions induce activation of the Rho-Rho kinase pathway (McMurtry, et al., 2003) (Bailly, et al., 2004). Although the detailed mechanisms by which low oxygen conditions induce Rho kinase activation are still unclear, our results suggest that even under statically cultured condition, an activation of Rho-Rho kinase signaling is caused by 1% O2 in macrophages, which resulted in an increase in MMP-9 production. Taking into account these considerations, we hypothesize that an increase in MMP-9 production of macrophages under 1% O2 condition is led by activation of ERK, which in part involves an increase in actin cytoskeleton tension mediated by Rho kinase (Fig. 6).
This hypothesized pathway shown in Fig. 6 may also account for our previous results, in which we demonstrated that only a combined condition of cyclic stretching and 2.2% O\(_2\) increased MMP-9 production and HIF-1α expression in macrophages, whereas 2.2% O\(_2\) under static culture condition did not cause these changes (Oya, et al., 2011) (Oya, et al., 2013). Although we do not have evidence for changes in tension of actin cytoskeleton in macrophages by cyclic stretching, it has been shown that cyclic stretching causes changes in actin cytoskeleton tension and actin-stretch induced ERK activation (Ikeda, et al., 1999) (Numaguchi, et al., 1999). This suggest that oxygen concentration of 2.2% is not sufficient for induction of HIF-1α expression and the combination with a signaling associating with actin cytoskeleton tension may be necessary for increases in HIF-1α expression and a downstream production of MMP-9 in macrophages.

Previous studies have shown that cyclic stretching causes parallel orientation of macrophages with the direction of stretching (Matsumoto, et al., 1996) (Matheson, et al., 2006). Miyazaki and Imamura also showed that actin accumulation in the peripheral of cells in the direction of stretching occurs prior to cyclic stretching-induced reorientation of macrophages (Miyazaki and Imamura, 2006). Our results for macrophage morphology are consistent with the previous studies. The other type of cells such as vascular endothelial cells, fibroblasts, and cardiomyocytes, in contrast, are known to orient perpendicular to the direction of stretching (Salameh, et al., 2010) (Huang, et al., 2012) (Greiner, et al., 2013), and Y27632 treatment causes parallel orientation of cells to the stretch direction (Kaunas, et al., 2005). Lee et al also showed that after exposure to cyclic stretching, osteosarcoma U2SO cells treated with Y27632 were oriented parallel to the direction of stretching, while non-treated cells were aligned perpendicular to the direction (Lee, et al., 2010). Based on live cell observations of actin fiber dynamics, they suggested that actin fiber formation parallel to the direction of stretching may occur to maintain or compensate tension of actin fibers reduced by the inhibition of Rho-kinase activity, resulting in cell orientation parallel to the direction of stretching. Such "tensional homeostasis" may induce orientation of macrophages exposed to cyclic stretching. However, many other types of cells exhibit thick actin fibers even under static condition, but the organized bundle of actin filaments are not observed in statically cultured macrophages. In addition, we also found that the treatment of macrophages with PD98059 increased stretch-induced macrophage elongation, though treatment with PD98059 did not affect stretch-induced morphological changes of endothelial cells and cardiomyocytes (Ikeda, et al., 1999) (Salameh, et al., 2010). These also suggest that macrophages may have distinct mechanisms for morphological changes in response to cyclic stretch from the other type cells. Although further studies will be needed to address the mechanisms, we found in the present study the ERK and Rho-Rho kinase signaling associate elongation of macrophages due to cyclic stretching. Since ERK is suggested to oppose the Rho-Rho kinase signaling (Mavria, et al., 2006), inhibition of ERK by the PD98059 treatment may result in activation of Rho-Rho kinase signaling, leading to increased elongation of macrophages, and inhibition of Rho kinase by Y27632 may suppress macrophage elongation induced by cyclic stretching.

Acknowledgements

The present study was supported in part by Grants-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (Nos. 15K01304 and 16K15837) and the interdepartmental research fund of Kawasaki University of Medical Welfare.

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