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Activation of MMP-2 by *Clostridium difficile* Toxin B in Bovine Smooth Muscle Cells

Teruhiko Koike,* Masafumi Kuzuya,*[,1] Toshinobu Asai,* Shigeru Kanda,* Xian Wu Cheng,* Kunitomo Watanabe,† Yoshiko Banno,‡ Yoshinori Nozawa,§ and Akihisa Iguchi*

**Department of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan; †Institute of Anaerobic Bacteriology, and ‡Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500-8705, Japan; and §Department of Environmental Cell Responses, Gifu Institute of Biotechnology, Mitake, Gifu 505-0116, Japan*

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Matrix metalloproteinase-2 (MMP-2) plays critical roles in cell migration through the breakdown of the extracellular matrix. Cell movements require dynamic actin reorganization, which is controlled by Rho family GTPases. In order to examine the relation between MMP-2 regulation and actin reorganization, we used several inhibitors of Rho family GTPases. Treatment of smooth muscle cells with *Clostridium difficile* toxin B known to inactivate Rho family GTPases activated MMP-2. However, neither C3 transferase, a Rho inhibitor, nor Y-27632, a specific inhibitor of Rho-kinase, induced MMP-2 activation. Treatment with C3 transferase and Y-27632 caused morphological changes into the round and stellate shape, respectively, by inhibition of actin stress fiber formation. In addition, toxin B treatment induced expression and processing of MT1-MMP, a major activator of MMP-2. Taken together, we suggest the involvement of Rho family GTPases, although inhibition of neither Rho nor Rho-kinase is sufficient, in the activation of MMP-2 through expression and activation of MT1-MMP. © 2000 Academic Press

Key Words: **matrix metalloproteinase-2 (MMP-2); Rho; toxin B; C3 transferase; Y-27632; MT1-MMP; actin cytoskeleton.**

Matrix metalloproteinase-2 (MMP-2), also called gelatinase A, contributes to normal biological processes such as wound healing and angiogenesis as well as pathological processes such as cardiovascular diseases and tumor metastasis (1). MMP-2 is secreted as pro-MMP-2 by various types of cells. Pro-MMP-2 is activated by membrane type-1 matrix metalloproteinase (MT1-MMP) at the cell surface (2). Tissue inhibitors of matrix metalloproteases (TIMPs) are major endogenous regulators of MMP activities. TIMP-2, pro-MMP-2, and MT1-MMP form a complex, which is important in the cell surface activation of pro-MMP-2 by MT1-MMP (2).

MMP-2 is induced by growth factors, chemical agents, and oncogenic transformation (1). Drugs that disrupt actin stress fiber such as cytochalasin D also activate MMP-2 (3). Alterations in cytoskeleton and subsequent cell shape changes exert specific effects on the expression of various genes. Reorganization of cytoskeletal structure seems to correlate with MMP-2 induction and activation. However, the mechanism linking cytoskeletal change to MMP-2 expression and activation remains to be defined.

Cell migration is regulated by turnover of actin polymerization and focal adhesions, which is the dynamic processes controlled by Rho family GTPases (Rho, Rac, Cdc42) (4, 5, 6). Activation of Rho induces stress fiber formation and focal adhesion complexes, whereas Rac and Cdc42 mediate signals to form lamellipodia and filopodia, respectively. Rho-kinase is the downstream target of Rho and plays a critical role in the formation of stress fiber and focal adhesions (7).

MMP-2 is regulated spatially and temporally during cell migration, but it is not clear whether there is any regulation of MMP-2 by Rho family GTPases. In order to investigate the relation between actin fiber reorganization and the regulation of MMP-2, we examined the effects of toxin B (8), a bacterial enzyme known to inactivate Rho family GTPases (Rho, Rac, Cdc42), C3 transferase, a specific inhibitor of Rho, and Y27632, a specific inhibitor of Rho-kinase, on MMP-2 activation (9).

MATERIALS AND METHODS

Cell culture. Bovine smooth muscle cells (BSMCs) were isolated from calf aortic media by explant method and cultured with Dul-

[1] To whom correspondence should be addressed. Fax: +81-52-744-2371. E-mail: kuzuya@med.nagoya-u.ac.jp.

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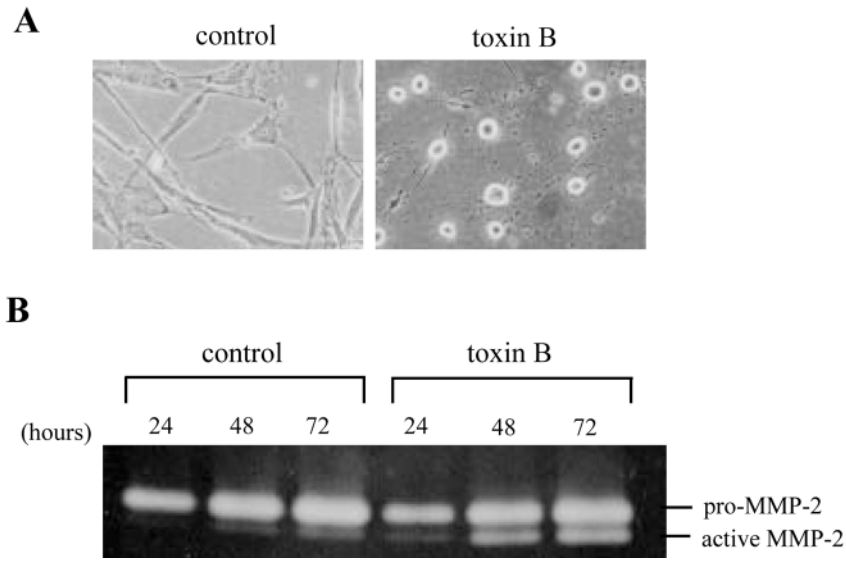

FIG. 1. Effect of toxin B on the morphological change and the activation of MMP-2. BSMCs were treated with 10 ng/ml of toxin B for 24, 48, and 72 h in serum free DMEM. (A) Photographs were taken under a phase-contrast microscope in the presence or absence of 10 ng/ml of toxin B at 24 h incubation. Magnification, × 200. (B) Medium of each time point was collected and subjected to zymography.

becco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum (FCS) and antibiotics.

Electrophoresis and blotting. Serum-free medium conditioned by BSMCs was analyzed by gelatin zymography as described (10). For immunoblotting with anti-TIMP-1 polyclonal antibody (Ab) (Sigma, T8187) and anti-TIMP-2 polyclonal Ab (Sigma, T8062), conditioned medium was concentrated by Centricon YM10 (Millipore). Proteins were separated through SDS–PAGE and transferred onto PVDF membrane as described (11). Membranes were reacted with anti-MT1-MMP polyclonal Ab (Sigma, M3927), anti-TIMP-1 polyclonal Ab, or anti-TIMP2 polyclonal Ab. Immunoreactive bands were visualized using horseradish peroxidase-conjugated protein A and ECL system (Amersham Corp.).

Preparation of GST fusion proteins. GST fusion proteins were prepared as described (12).

Scrape loading. BSMCs growing on 60-mm dishes in serum-containing medium were washed in Dulbecco's phosphate buffered saline (Nissui Pharmaceutical) and scraping buffer (114 mM KCl, 15 mM NaCl, 5.5 mM $MgCl_2$, 10 mM Tris–HCl). Cells were then gently scraped in 0.25 ml of scraping buffer in the presence of GST fusion proteins, and resuspended in DMEM containing 10% FCS as described previously (13). Conditioned medium was replaced with FCS free medium after 24 h. Cells were further incubated for the indicated times and conditioned medium collected was subjected to gelatin-zymography. The morphological change induced by C3 transferase was stable for at least 72 h.

Reagents. Toxin B from *Clostridium difficile* was purified as described previously (14). Y-27632 was kindly provided by Welfide Corporation (Osaka, Japan) (9). GST fusion protein of C3 transferase was kindly provided by Professor Alan Hall (MRC Laboratory for Molecular Cell Biology, University College London).

RESULTS AND DISCUSSION

In order to examine the involvement of Rho family GTPases in MMP-2 regulation, we treated BSMCs with toxin B, which inactivates Rho family GTPases including Rho, Rac, and Cdc42 (8), and analyzed its effect on MMP-2 activation. Toxin B treatment drastically induced rounding of BSMCs, indicating disruption of the actin cytoskeleton (Fig. 1A). BSMCs maintained their morphological change during stimulation and stayed attached to the dish. Gelatin zymographic analysis of conditioned medium from control culture revealed that major gelatinolitic activity was observed at molecular mass of 72 kDa, which corresponded to pro-MMP-2 (Fig. 1B). We found that toxin B treatment induced processing of pro-MMP-2 to a molecular weight 62 kDa, an active form of MMP-2, within 48 h of incubation and the effect was sustained for 72 h (Fig. 1B). The same effects of toxin B on morphological change and MMP-2 activation were observed in human skin fibroblasts, indicating that this effect of toxin B is not restricted to smooth muscle cells (data not shown).

To examine this phenomenon further, we utilized C3 transferase and Y-27632, specific inhibitors of Rho and Rho-kinase, respectively. Rho-kinase is a major target protein of Rho and regulates formation of stress fibers and focal adhesions (7). Several lines of evidence have shown that alterations in the organization of the actin cytoskeleton modulate MMP profiles in certain cells (3, 15, 16). Treatment of BSMCs with C3 transferase clearly induced a rounded morphology similar to toxin B treatment (Fig. 2A). However, despite disruption of stress fiber and cell shape change in C3 transferase-treated cells, activation of MMP-2 was not detected (Fig. 2B). The incubation of BSMCs with Y-27632 induced a stellate rather than rounded morphology (Fig. 3A), but it did not induce the activation of MMP-2 (Fig. 3B). The failure of both C3 transferase and Y-27632

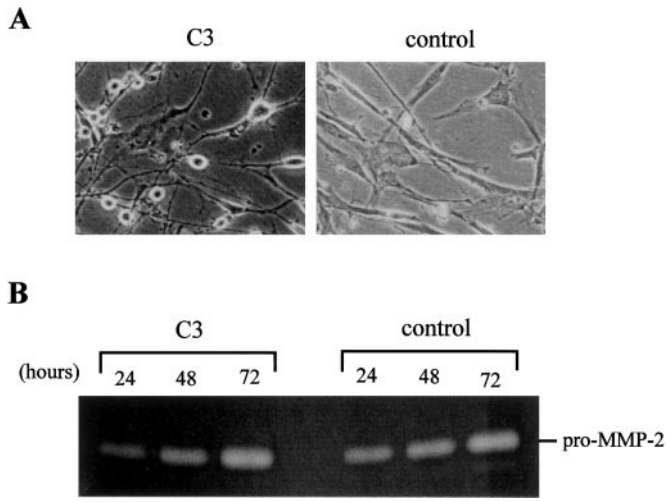

FIG. 2. Effect of C3 transferase on the morphological change and the activation of MMP-2. BSMCs were loaded with 4 μg/ml of GST-C3 transferase or GST as a control. (A) Photographs were taken under a phase-contrast microscope at 24 h incubation. Magnification, $\times$ 200. (B) Medium of each time point was collected and subjected to zymography.

treatment to activate MMP-2 indicates that disruption of actin stress fiber by the inhibition of Rho pathway is not, at least, sufficient to induce MMP-2 activation. On the other hand, treatment of cytochalasin D, a widely used inhibitor of actin polymerization, also induced cell rounding (Fig. 3A), and it strongly activated and induced expression of MMP-2 as previously described (3) (Fig. 3B). Although cell rounding due to the disruption of actin stress fiber formation seems to correlate with MMP-2 activation in toxin B- and cytochalasin D-treated cells, results obtained from the inhibition of Rho pathway do not support this hypothesis. Furthermore, these results raise the possibility that Rho family GTPases other than Rho might play a role in the regulation of MMP-2 activation.

To determine whether induction of MMP-2 activation by toxin B treatment reflects changes in the expression of BSMC MT1-MMP, we analyzed cell lysates by immunoblotting with antibody to the MT1-MMP. Immunoblots of the extracts of BSMCs treated with toxin B demonstrated the increased immunoreactive bands of 68-kDa, 63-kDa, comparable to the reported weights of pro-MT1-MMP and activated MT1-MMP, respectively, as well as 55-kDa, corresponding to its degradation product (Fig. 4A) (17, 18). These findings are in agreement with the previous report that proteolytic processing of MT1-MMP associates with MMP-2 activation (19). In fact, increase of three bands as well as processing of MT1-MMP was observed in BSMCs treated with concanavalin A, which is known to trigger augmented secretion and proteolytic activation of MMP-2 in various cells (data not shown). Increased density of all three bands in treatment with toxin B indicates an increase in the total amount of MT1-MMP by this treatment. Neither increase nor processing of MT1-MMP was observed without toxin B treatment up to 72 h (data not shown).

Since it has been demonstrated that an excess of TIMP-2 inhibits MMP-2 activation (1, 2), we determined TIMPs production from BSMCs treated with toxin B. No change was observed in TIMP-1 produc-

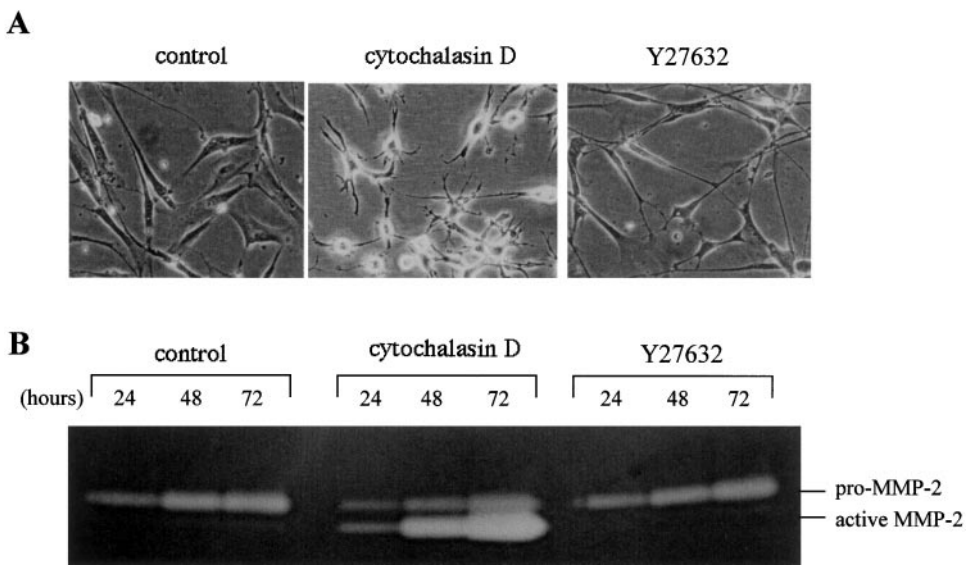

FIG. 3. Effect of cytochalasin D and Y-27632 on the morphological change and the activation of MMP-2. BSMCs were incubated in the presence or absence of 40 μM of cytochalasin D or 10 μM of Y-27632 for the indicated times. (A) Photographs were taken under a phase-contrast microscope at 24 h incubation. Magnification, $\times$ 200. (B) Medium of each time point was collected and subjected to zymography.

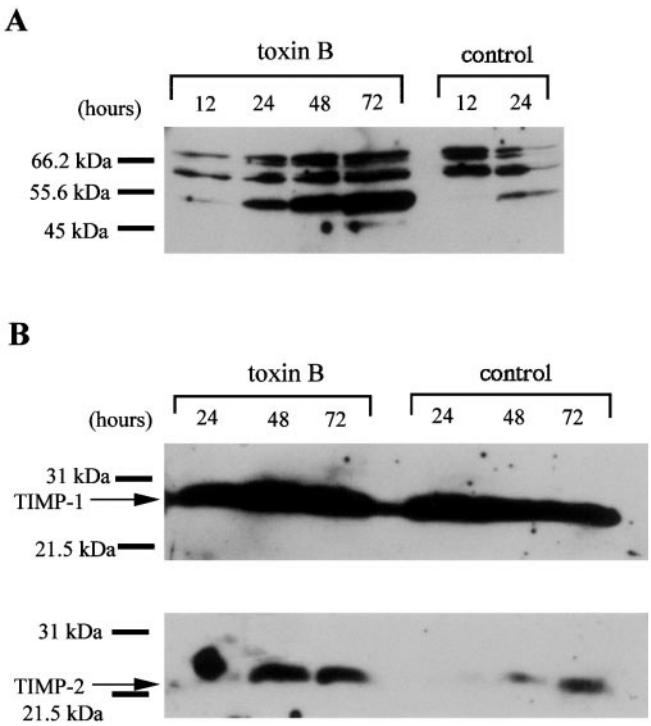

FIG. 4. Effect of toxin B on the expression of MT1-MMP1, TIMP-1, and TIMP-2. BSMCs were treated with 10 ng/ml of toxin B for the indicated times. (A) Cell lysates were subjected to electrophoresis and immunoblotted with anti-MT1-MMP Ab. (B) Conditioned medium concentrated by Centricon was subjected to electrophoresis and immunoblotted with anti-TIMP-1 or anti-TIMP-2 Ab. Molecular mass markers are indicated on the left.

tion, whereas the TIMP-2 level increased in toxin B treatment (Fig. 4B), thereby suggesting that toxin B-induced MMP-2 activation was not due to the inhibition of TIMP-2 production.

Warny *et al.* recently reported that *Clostridium difficile* toxin A, which similarly inhibits Rho family GTPases, activated MAP kinase pathway in monocytes (20). They showed that toxin A-induced IL-8 transcription and cell necrosis in monocytes were the downstream event of MAP kinase activation that might be independent of the Rho inactivation. Therefore, it is possible that our finding of the effect of toxin B on MMP-2 activation might be mediated by cell signaling pathways which are independent of the inhibition of Rho family GTPases.

Rho family GTPases and MMPs are regulated spatially and temporally during cell migration. Thus, it is likely that Rho family GTPases regulate MMPs in a coordinate manner with adhesion and actin cytoskeletal reorganization. Kheradmand *et al.* recently showed MMP-1 expression by Rac1 activation (21). MMP-1 gene expression is induced, in an autocrine manner, by interleukin-1α that is regulated by reactive oxygen species generated by Rac1 activation. It is thus conceivable that activation of MMP-2 by the *Clostridium difficile* toxin B involves the similar mechanism. To our knowledge, this is the first report to demonstrate that the inactivation of Rho family GTPases by toxin B treatment activates MMP-2 through induction and activation of MT1-MMP.

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