Jpn. J. Cancer Res. 88, 281–288, March 1997

Bacillus Calmette-Guérin Enhances Production and Secretion of Type IV Collagenases in Peripheral Blood Mononuclear Cells

Yukio Kageyama, Satoru Kawakami, Yasuhisa Fujii, Kazunori Kihara and Hiroyuki Oshima

Department of Urology, Tokyo Medical and Dental University School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113

Intravesical administration of bacillus Calmette-Guérin (BCG) is an effective and widely accepted treatment for superficial bladder cancer. Rapid progression of the disease after BCG therapy, however, has been reported in some cases refractory to the treatment. We examined whether BCG treatment and coexistence of peripheral blood mononuclear cells (PBMCs) alter the invasive potential of bladder cancer cells. Production and secretion of two type IV collagenases, matrix metalloproteinase (MMP) 2 and MMP 9, by PBMCs from five healthy donors or bladder cancer cells (T24, JTC 30, and JTC 32) were evaluated by gelatin zymography, western blot analysis, and northern blot analysis. Invasion of bladder cancer cells was also examined using reconstituted basement membrane (Matrigel). BCG (5, 50, and 500 μg/ml) had no effect on secretion of MMP 2 and MMP 9 by bladder cancer cells, but increased the production and secretion of MMP 9 by PBMCs in a dose-dependent manner. The coexistence of PBMCs increased invasion of T24 cells and BCG further enhanced the invasion. Thus, BCG promotes invasion of bladder cancer cells under certain conditions. An increase in the secretion of MMP 9 by PBMCs may account in part for the effect.

Key words: BCG vaccine — Bladder neoplasm — Invasiveness — Lymphocyte — Monocyte

Intravesical administration of bacillus Calmette-Guérin (BCG) is an effective and widely used treatment modality for superficial bladder cancer. BCG treatment has been demonstrated not only to eradicate the cancer cells but also to reduce recurrence after transurethral resection of the tumor.[1,2] In spite of the excellent treatment results, however, bladder cancers refractory to BCG therapy have been reported to show rapid progression.[3] Some of our cases with bladder cancer also showed extensive muscle invasion after BCG therapy.

Type IV collagenase is a metalloproteinase which cleaves type IV collagen, a main component of basement membranes. There are studies indicating a correlation between the production of type IV collagenase by cancer cells and invasive potential in various types of malignancies, including bladder cancer.[4–10] The enzyme has also been demonstrated to be produced by non-malignant cells such as fibroblasts or peripheral blood mononuclear cells (PBMCs)[11–14] and these cells were suggested to promote invasion of cancer cells under certain conditions.[15–18]

In this study, we evaluated the effect of BCG on the production of two type IV collagenases, matrix metalloproteinase (MMP) 2 (72 kDa type IV collagenase: gelatinase A) and MMP 9 (92 kDa type IV collagenase: gelatinase B), by bladder cancer cells and PBMCs. Migration of bladder cancer cells through reconstituted basement membrane was also examined to see whether the administration of BCG and existence of PBMCs alter the invasive potential of the cancer cells.

MATERIALS AND METHODS

Reagents BCG (Tokyo 172 strain) was purchased from Japan BCG Co., Tokyo. Recombinant human tumor necrosis factor α (rTNF-α) was purchased from Dainippon Pharmaceutical Co., Tokyo. Recombinant human interferon γ (rIFN-γ) was purchased from Shionogi Pharmaceutical Co., Tokyo. Mouse anti-human MMP 2 monoclonal antibody (mAb) and mouse anti-human MMP 9 mAb were purchased from Cosmo Bio, Tokyo.

Preparation of PBMCs PBMCs were prepared from five healthy volunteers using Ficoll-Paque, a lymphocyte isolation reagent (Pharmacia Biotech AB, Uppsala, Sweden), and incubated in RPMI 1640 medium with 10% fetal calf serum (FCS) for 2 h at 37°C. Floating cells (lymphocytes) and adherent cells (monocytes) were separately collected, identified by Giemsa staining, and mixed again (10 parts of lymphocytes and 1 part of monocytes) for further experiments.

Bladder cancer cell lines Three bladder cancer cell lines T24,[19] JTC 30,[20] and JTC 32[20] were used. JTC 30 and JTC 32 were established from primary tumors of patients with well differentiated and anaplastic bladder cancer, respectively. T24 was cultured in α MEM medium (Life Technologies, Grand Island, NY) containing 10% FCS, and JTC 30 and JTC 32 were cultured in DM 170 medium (Kyokuto Pharmaceutical Industrial Co., Tokyo) with 10% FCS.

Gelatin zymography Bladder cancer cells or PBMCs were cultured in serum-free media at 37°C for 2 h. BCG,

phytohemagglutinin, rTNF-α, rIFN-γ, or anti-human rTNF-α mAb was added to selected cultures. Conditioned media of the cultures were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis with 4% stacking gel and 10% running gel containing 0.1% gelatin (Sigma Chemical Co., St. Louis, MO) at 40 mA for 2.5 h at room temperature. The gels were washed twice in 2.5% Triton X-100 and then in 10 m*M* Tris HCl (pH 8.0), and incubated in 50 m*M* Tris-HCl (pH 8.0) containing 0.5 m*M* $CaCl_2$ and 1 m*M* $ZnCl_2$ for 48 h at 37°C. The bands with gelatinase activity were visualized by staining the gels in 1% Coomassie Brilliant Blue R 250 and quantitated with image analyzing software, NIH image.

Western blot analysis Conditioned media (20 μl) or lysate of the cultured PBMCs (50 μg) were subjected to SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Bedford, MA). Conditions of electrophoresis were the same as those in gelatin zymography except that gelatin was omitted from the running gels. The membranes were blocked overnight with 5% skim milk, exposed to mouse anti-human MMP 2 mAb or mouse anti-human MMP 9 mAb for two hours at room temperature, and processed with a Vectastain ABC staining kit for mouse IgG (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The bands reactive to the primary antibodies were visualized with 0.25 mg/ml 3,3′-diaminobenzidine in phosphate-buffered saline (PBS) containing 0.03% H_2O_2.

Northern blot analysis Total RNA was isolated from PBMCs using Isogen, an RNA isolation reagent (Wako Pure Chemical Industry, Tokyo). Thirty micrograms of the total RNA was electrophoresed in 1% agarose gels containing formaldehyde and transferred to nylon membranes. The membranes were hybridized with an α-^{32}P-labeled cDNA clone for human type IV collagenase (Clone ColIVA) (American Type Culture Collection, Rockville, MA) and analyzed with an image analyzer (BAS 2000) (Fuji Film Industry, Tokyo). The membranes were rehybridized with an α-^{32}P-labeled probe for human beta actin (Wako Pure Chemical Co., Tokyo) as an internal standard.

Cytotoxicity assay Ten thousand bladder cancer cells were seeded in triplicate in 24-well plates and incubated for 24 h at 37°C. Then the medium was changed to serum-free medium with (5, 50, or 500 μg/ml) or without BCG. PBMCs (5×10^5 lymphocytes and 5×10^4 monocytes) were added in selected wells. After incubation for an additional 24 h, the plates were washed with PBS three times and the cultures were continued in media supplemented with 10% FCS for one week at 37°C. The cultured bladder cancer cells were then fixed with methanol and stained with Giemsa's solution. Cytotoxicity was evaluated by measuring the number of cells with a Monocelater (Olympus Co., Tokyo).

***In vitro* invasion assay** Migration of the T24 cells was evaluated using reconstituted basement membrane containing type IV collagen (Matrigel Biocoat Invasion Chamber) (Becton Dickinson Labware, Bedford, MA). T24 cells (10^5) in 200 μl of serum-free medium (α MEM) containing 0.1 % FCS with or without BCG (500 μg/ml) or conditioned media of PBMCs culture (10^5 lymphocytes and 10^4 monocytes) with BCG (500 μg/ml) were placed in the upper wells of the Matrigel chambers. In some wells, 50 μg/ml of anti-MMP 9 mAb was added to the culture media and PBMCs (10^5 lymphocytes and 10^4 monocytes) were added to selected samples. Ten micrograms/ml of fibronectin (Iwaki Glass Co., Ltd., Tokyo) in the serum-free medium containing 0.1% FCS was added to the lower wells of the chambers and the system was incubated for 24 h at 37°C. Then cells on the upper surface of the membranes were removed by gently wiping with cotton swabs. Cancer cells which had migrated through the membranes were stained with Giemsa's solution and counted on a light microscope.

Statistical analysis All statistical analyses were made by using Student's *t* test.

RESULTS

Gelatin zymography of the conditioned media of the PBMCs culture revealed marked gelatinase activity at 92 kDa. Weaker gelatinase activities were also observed at 170 kDa and 72 kDa. The 92-kDa and 170-kDa gelatinase activities were significantly enhanced in a dose-dependent manner by the presence of BCG in the culture medium (Figs. 1 and 2). The gelatinase activities increased time-dependently on BCG (500 μg/ml) treatment and reached a plateau at 2 h (Fig. 3).

In western blot analysis of the conditioned media of the PBMCs culture, the 92-kDa gelatinase was reactive only to the anti-MMP 9 mAb, while the 170-kDa gelatinase was positively stained by both anti-MMP 2 and anti-MMP 9 mAbs. Positive bands were seen only when PBMCs were incubated in the presence of BCG. The band of 72 kDa was not detectable by either of the mAbs, irrespective of the presence of BCG (Fig. 1).

Western blot analysis of the lysate of cultured PBMCs using anti-MMP 9 mAb revealed signals at 92 kDa which were increased by up to 460% in a dose-dependent manner by BCG treatment. In northern blot analysis with total RNA from the PBMCs, mRNA for type IV collagenase was also increased dose-dependently by BCG treatment (Fig. 4).

The 170-kDa, 92-kDa, and 72-kDa gelatinases were also demonstrated in gelatin zymography of the conditioned media obtained by separate culture of lymphocytes or monocytes from Donor 1. BCG increased the activities of 170-kDa and 92-kDa gelatinases only in the conditioned media of lymphocyte cultures (Fig. 5).

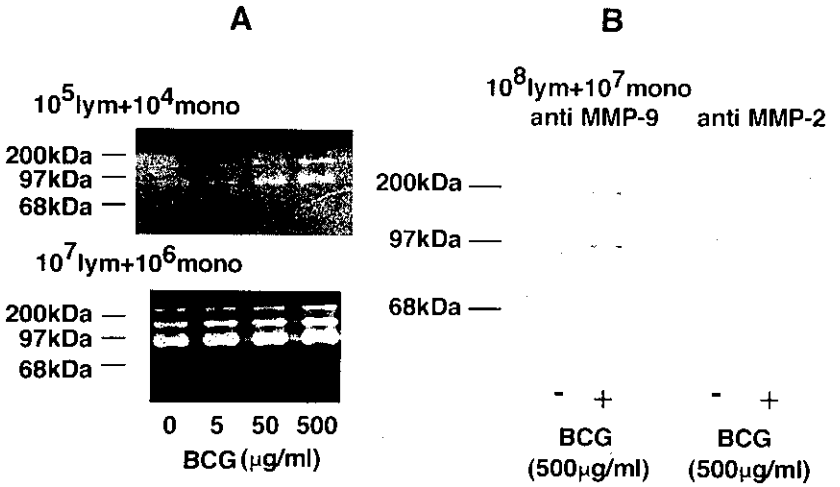

Fig. 1. Gelatin zymography (A) and western blot analysis (B) of conditioned media obtained from cultures of peripheral blood mononuclear cells (PBMCs) from donor 1. Administration of bacillus Calmette-Guérin (BCG) increased gelatinase activity of 92 kDa and 170 kDa in a dose-dependent manner. Two bands of 92 kDa and 170 kDa were stained with anti-matrix metalloproteinase (MMP) 9 antibody after the treatment with BCG. The band of 170 kDa was also stained by anti-MMP 2 antibody. "lym" and "mono" denote lymphocyte and monocyte, respectively.

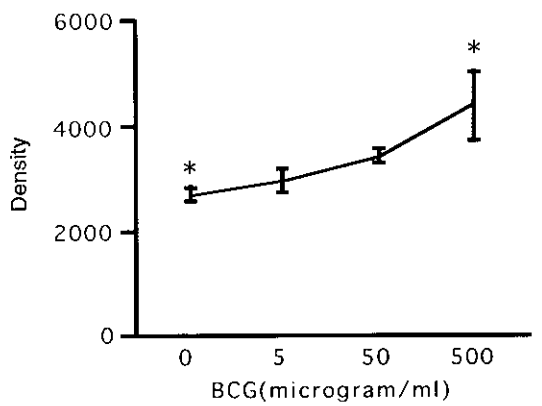

Fig. 2. Relative 92-kDa gelatinase activity in a duplicate experiment with 10^7 lymphocytes and 10^6 monocytes from donor 1. Bacillus Calmette-Guérin (BCG) increased the activity in a dose-dependent manner. * $P<0.05$.

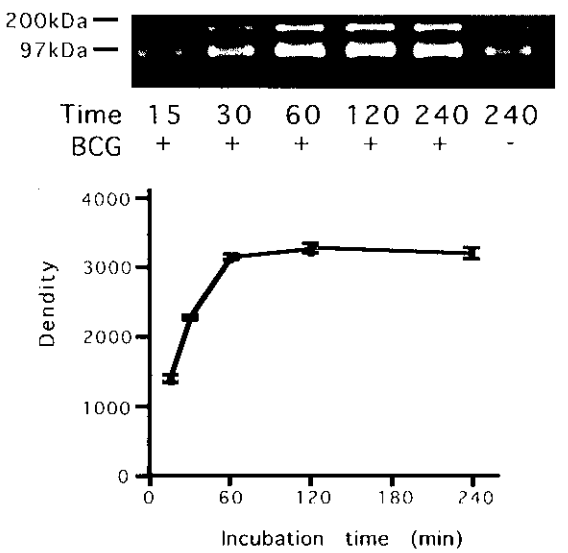

Fig. 3. Gelatin zymography of conditioned media obtained from cultures of peripheral blood mononuclear cells (PBMCs) (10^7 lymphocytes and 10^6 monocytes) from donor 1. Bacillus Calmette-Guérin (BCG) time-dependently increased the gelatinase activity at 92 kDa and 170 kDa. The results of a duplicate experiment are shown.

Addition of rTNF-α in the culture of PBMCs (donor 1) dose-dependently increased 72-kDa gelatinase activity, while dose-dependent decreases in 170-kDa and 92-kDa gelatinase activities in the conditioned media were observed. The decreased gelatinase activities recovered after the addition of 500 μg/ml of BCG to the culture. Treatment of PBMCs with an anti-human rTNF-α neutralizing mAb did not change these gelatinase activities. The two gelatinase activities were not affected by rIFN-γ, regardless of the presence or absence of BCG.

Non-specific stimulation of PBMCs with phytohemagglutinin did not affect either of the gelatinase activities (Fig. 6).

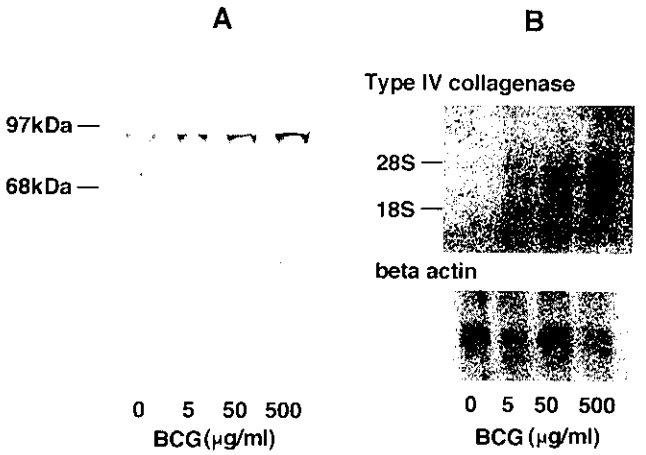

Fig. 4. Western blot analysis (A) of cell lysate (50 μg protein) and northern blot analysis (B) of total RNA (30 μg) obtained from peripheral blood mononuclear cells (PBMCs) of donor 1. Bacillus Calmette-Guérin (BCG) increased the amount of 92-kDa gelatinase which is reactive to anti-matrix metalloproteinase (MMP) 9 monoclonal antibody in a dose-dependent manner. mRNA for type IV collagenase was also increased dose-dependently by BCG treatment.

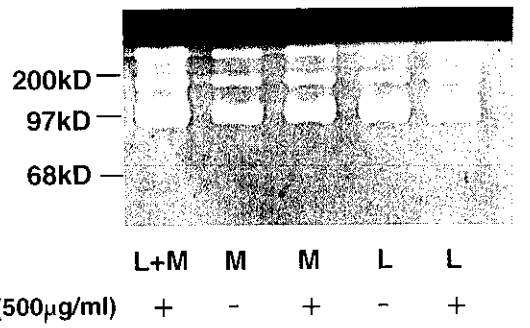

Fig. 5. Gelatin zymography of conditioned media of lymphocytes and monocytes (donor 1) cultured separately. Although gelatinases were secreted by both lymphocytes and monocytes, bacillus Calmette-Guérin (BCG) mainly increased the gelatinase activity of lymphocytes. L and M denote lymphocytes and monocytes, respectively.

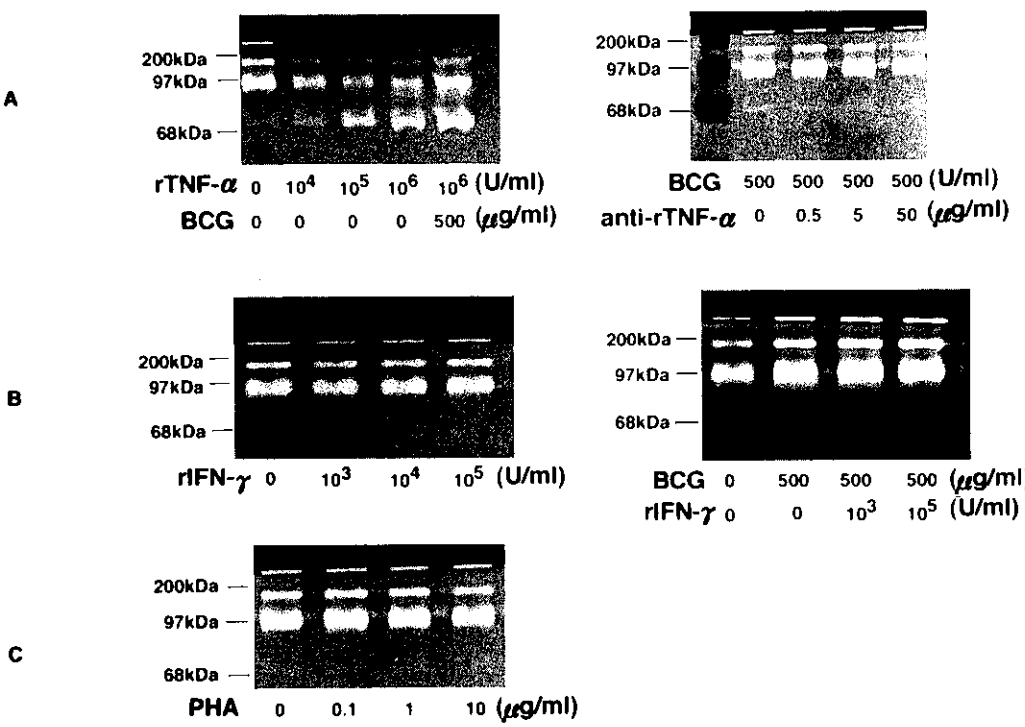

Fig. 6. Effect of various stimulants on gelatinase activity of peripheral blood mononuclear cells (PBMCs) (10^7 lymphocytes and 10^6 monocytes) from donor 1. Recombinant human tumor necrosis factor (rTNF)-α increased the 72-kDa gelatinase activity and decreased the 92-kDa and 170-kDa gelatinase activities in a dose-dependent manner. Anti-TNF-α antibody did not affect the gelatinase activities increased by bacillus Calmette-Guérin (BCG) treatment (A). Recombinant human interferon (rIFN)-γ did not alter the basal or BCG-enhanced gelatinase activities (B). Non-specific stimulation with phytohemagglutinin (PHA) had no effect on the basal gelatinase activity (C).

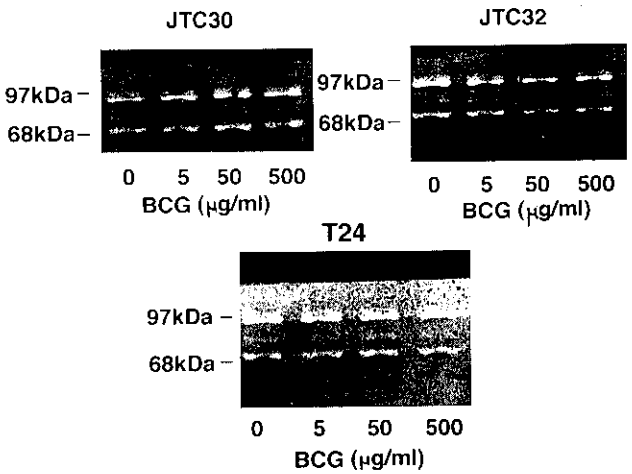

Fig. 7. Gelatin zymography of conditioned media of bladder cancer cell line cells (10^5 cells). All three cell lines (T24, JTC 30, and JTC 32) secreted gelatinases of 92 kDa and 72 kDa. Addition of BCG had no effect on basal gelatinase activities.

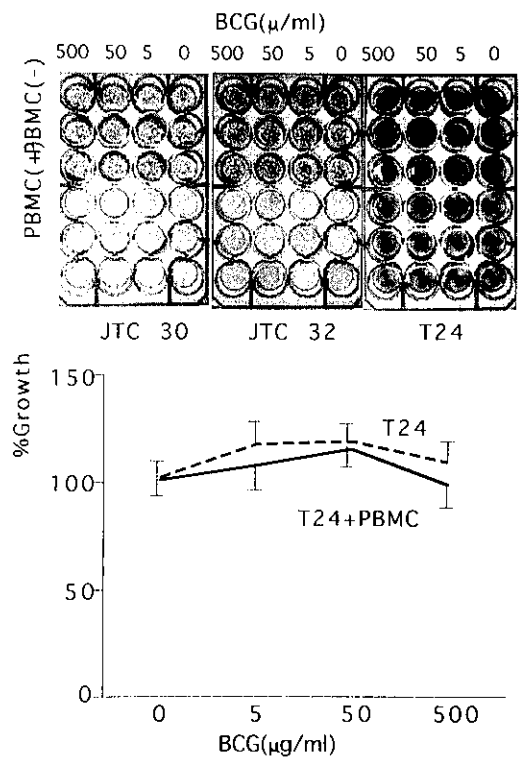

Fig. 8. Effects of BCG and peripheral blood mononuclear cells (PBMCs) on viability of three bladder cancer cell line cells (10^4 cells at the start of the assays). Culture with PBMCs (5×10^5 lymphocytes and 5×10^4 monocytes) for 24 h severely impaired the viability of JTC 30 and JTC 32 regardless of bacillus Calmette-Guérin (BCG) treatment, whereas BCG and/or PBMCs did not alter the viability of T24. Vertical lines in the graph represent standard deviations.

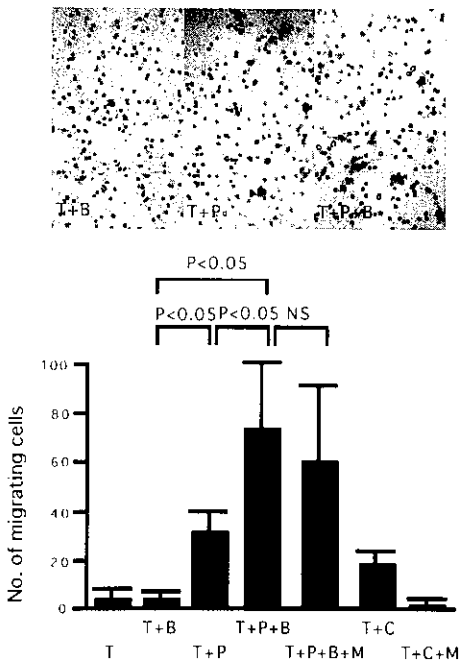

Fig. 9. *In vitro* invasion assay of T24. T24 cells (10^5) were added to the upper wells of the invasion chamber and cells that migrated through the reconstituted basement membrane were counted. Although only a few T24 cells showed migration under the basal conditions, the presence of peripheral blood mononuclear cells (PBMCs) (10^5 lymphocytes and 10^4 monocytes from donor 1) promoted the migration. Addition of bacillus Calmette-Guérin (BCG) further enhanced the invasion of T24. Although the effect was not statistically significant, anti-matrix metalloproteinase (MMP) 9 monoclonal antibody (mAb) partially suppressed the enhanced migration of T24 cells induced by BCG. Conditioned media of PBMCs culture with BCG also enhanced T24 cell migration, which was markedly suppressed by anti-MMP 9 mAb. T, B, P and M denote T24, BCG (500 μg/ml), PBMCs and anti-MMP 9 mAb, respectively. C, Culture medium was substituted for conditioned medium of PBMCs culture with BCG. Vertical lines in the bar graph represent standard deviations.

Conditioned media of three bladder cancer cell line cell cultures all contained gelatinase activities of 92 kDa and 72 kDa, which were not affected by addition of BCG to the culture (Fig. 7). Coexistence of PBMCs markedly impaired the viability and growth of both JTC 30 and JTC 32, regardless of the presence or absence of BCG, while T24 cells were not affected by BCG or PBMCs (Fig. 8).

In *in vitro* invasion assay using T24 (Fig. 9), only a few cells migrated through the reconstituted basement membranes when T24 cells alone were cultured. Addition of BCG alone did not increase the number of migrating cells. Co-culture of T24 and PBMCs obtained from

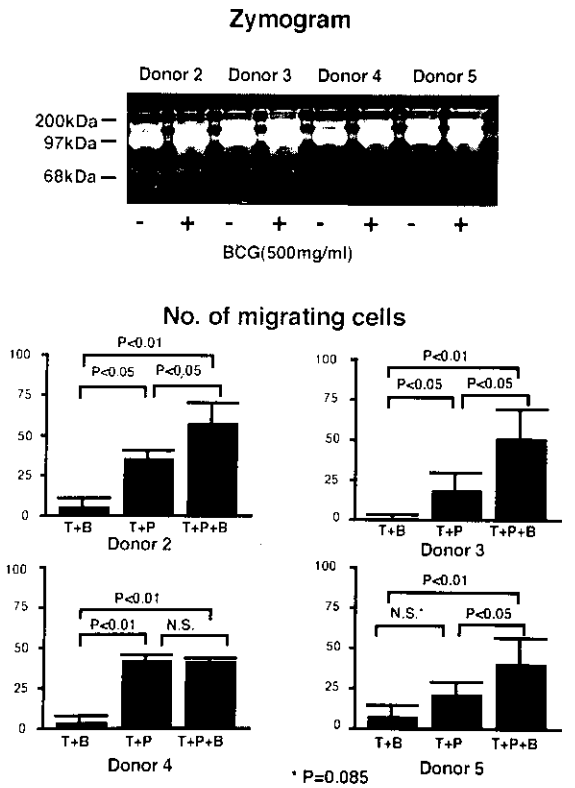

Fig. 10. Results of gelatin zymography and *in vitro* invasion assay of T24 using peripheral blood mononuclear cells (PBMCs) (10^5 lymphocytes and 10^4 monocytes) of donors 2–5. Bacillus Calmette-Guérin (BCG) increased the 92-kDa gelatinase activity in all of the samples. Coexistence of PBMCs increased migration of T24 in all of the experiments and BCG further enhanced the invasion in three of them. Vertical lines in the bar graphs represent standard deviations.

Table I. Effects of BCG on 92-kD Gelatinase Activity

	BCG (−) mean density (SD)[a]	BCG (+) mean density (SD)[a]	
Donor			
2	123.5 (6.4)	176.5 (5.0)	$P<0.05$
3	111.0 (5.7)	173.5 (13.4)	$P<0.05$
4	177.0 (4.2)	192.0 (8.4)	NS ($P=0.16$)
5	181.5 (6.4)	221.0 (15.6)	$P<0.05$

a) Calculated from one duplicate experiment of gelatin zymography.

Donor 1 significantly increased the migration of T24 cells (6.9 times). The presence of BCG with PBMCs further increased migrating T24 cells to 15.9 times those under the basal condition (Fig. 9). Although the effect was not statistically significant, anti-MMP 9 mAb partially suppressed the enhanced migration of T24 cells induced by BCG. Conditioned media of PBMCs culture with BCG also enhanced T24 cell migration, which was markedly suppressed by anti-MMP 9 mAb.

Addition of BCG enhanced 92-kDa gelatinase activity in the conditioned media of cultures of PBMCs prepared from Donors 2–5 to 108–156% (mean 132%). Migration of the T24 cells through reconstituted basement membrane was also increased by co-culture with the PBMCs by 2.7–14.3 times (mean 8.5 times). Administration of BCG (500 μg/ml) further enhanced the migration by 5.1–39.2 times (mean 16.6 times), except for the experiment with PBMCs obtained from Donor 4 (Fig. 10, Table I).

DISCUSSION

In the current study, the conditioned media of PBMCs cultures were demonstrated to contain 170-kDa, 92-kDa and a small amount of 72-kDa gelatinase activities. The results of western blot analysis suggest that the 92-kDa gelatinase is MMP-9, which is consistent with other reports demonstrating that MMP-9 is a main component of gelatinase secreted by lymphocytes.[11, 12] The reactivity of the 170-kDa gelatinase to both anti-MMP 2 and anti-MMP 9 mAbs suggests that the compound is a dimer of MMP 2 and MMP 9.

The presence of BCG increased the 92-kDa gelatinase activity in the conditioned media, as well as 92-kDa gelatinase in the lysate and mRNA for type IV collagenase of the PBMCs, in a dose-dependent manner. The results indicate that BCG increases production of the 92-kDa gelatinase in PBMCs. Since the gelatinase activities in the conditioned media were not affected by phytohemagglutinin, sensitization or stimulation of PBMCs with bacterial bodies appears to increase the production and secretion of the 92-kDa and 170-kDa gelatinases specifically. Further, lymphocytes play a major role in the enhanced secretion of the enzymes in response to BCG, while both lymphocytes and monocytes are able to secrete the 92-kDa and 170-kDa gelatinases independently.

The current results suggest that TNF-α and IFN-γ are not involved in the enhancement of 92-kDa and 170-kDa gelatinase activities in the conditioned media of PBMCs culture by BCG, at least within the first two hours of incubation. PBMCs, however, have been reported to secrete TNF-α and IFN-γ after stimulation with BCG.[21–23] TNF-α has been reported to enhance production of MMP 2 or MMP 9,[24–29] whereas IFN-γ has been demonstrated to suppress these enzymes.[30–33]

Of the three bladder cancer cell lines, JTC 30 and JTC 32 were not able to survive when co-cultured with PBMCs. The viability of T24 cells, however, was not affected by the existence of PBMCs and/or BCG. Therefore, T24 was used for *in vitro* invasion assays, which

demonstrated that PBMCs promoted migration of T24 cells through reconstituted basement membranes and that BCG further enhanced this effect, which was partially suppressed by anti-MMP 9 mAb. Conditioned media of PBMCs culture with BCG also increased the migration of T24 cells, which was markedly suppressed by anti-MMP 9 mAb. The current results indicate involvement of 92-kDa and 170-kDa gelatinase production by PBMCs and its enhancement by BCG in the enhanced migration of T24 cells in the presence of PBMCs and/or BCG.

The rate of disease progression of bladder carcinoma *in situ* refractory to BCG instillation therapy during five years has been reported to be 95%, which is markedly higher than that (50%) in untreated cases.[2, 34] Close monitoring of the patients, therefore, is needed after BCG therapy, and if recurrence is recognized, curative treatments including total cystectomy should be considered without delay. In conclusion, we have elucidated one of the mechanisms of the rather uncommon malignant progression of the bladder carcinoma resistant to BCG intravesical instillation therapy, though invasion of cancer cells through basement membrane is a complex process and may involve many factors.

(Received October 11, 1996/Accepted December 25, 1996)

REFERENCES

1) Herr, H. W., Wartinger, D. D., Fair, W. R. and Oettgen, H. F. Bacillus Calmette-Guérin therapy for superficial bladder cancer: 10-year followup. *J. Urol.*, **147**, 1020–1023 (1992).
2) Herr, H. W., Schwalb, D. M., Zhang, Z. F., Sogani, P. C., Fair, W. R., Whitmore, W. J. and Oettgen, H. F. Intravesical bacillus Calmette-Guérin therapy prevents tumor progression and death from superficial bladder cancer: ten year follow-up of a prospective randomized trial. *J. Clin. Oncol.*, **13**, 1404–1408 (1995).
3) Harland, S. J., Charig, C. R., Highman, W., Parkinson, M. C. and Riddle, P. R. Outcome in carcinoma *in situ* of bladder treated with intravesical Bacille Calmette-Guérin. *Br. J. Urol.*, **70**, 271–275 (1992).
4) Davies, B., Waxman, J., Wasan, H., Abel, P., Williams, G., Krausz, T., Neal, D., Thomas, D., Hanby, A. and Balkwill, F. Levels of matrix metalloproteases in bladder cancer correlate with tumor grade and invasion. *Cancer Res.*, **53**, 5365–5369 (1993).
5) Kawamata, H., Kameyama, S., Kawai, K., Tanaka, Y., Nan, L., Barch, D. H., Stetler-Stevenson, W. G. and Oyasu, R. Marked acceleration of the metastatic phenotype of a rat bladder carcinoma cell line by expression of human gelatinase A. *Int. J. Cancer*, **63**, 568–575 (1995).
6) Kubota, S., Mitsudomi, T. and Yamada, M. Invasive human fibrosarcoma DNA mediated induction of 92 kDa gelatinase/type IV collagenase leads to an invasive phenotype. *Biochem. Biophys. Res. Commun.*, **181**, 1539–1547 (1991).
7) Stetler-Stevenson, W. G. Type IV collagenases in tumor invasion and metastasis. *Cancer Metastasis Rev.*, **9**, 289–303 (1990).
8) Sreenath, T., Matrisian, L. M., Stetler, S. W., Gattoni, C. S. and Pozzatti, R. O. Expression of matrix metalloproteinase genes in transformed rat cell lines of high and low metastatic potential. *Cancer Res.*, **52**, 4942–4947 (1992).
9) Shah, V., Kumar, S. and Zirvi, K. A. Metastasis of human colon tumor cells *in vitro*: correlation with the overexpression of plasminogen activators and 72 kDa gelatinase. *In Vivo*, **8**, 321–326 (1994).
10) Emmert, B. M., Roth, M. J., Zhuang, Z., Campo, E., Rozhin, J., Sloane, B. F., Liotta, L. A. and Stetler, S. W. Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. *Am. J. Pathol.*, **145**, 1285–1290 (1994).
11) Leppert, D., Waubant, E., Galardy, R., Bunnett, N. W. and Hause, S. L. T cell gelatinases mediate basement membrane transmigration *in vitro*. *J. Immunol.*, **154**, 4379–4389 (1995).
12) Weeks, B. S., Schnaper, H. W., Handy, M., Holloway, E. and Kleinman, H. K. Human T lymphocytes synthesize the 92 kDa type IV collagenase (gelatinase B). *J. Cell. Physiol.*, **157**, 644–649 (1993).
13) Zhou, H., Bernhard, E. J., Fox, F. E. and Billings, P. C. Induction of metalloproteinase activity in human T-lymphocytes. *Biochim. Biophys. Acta*, **1177**, 174–178 (1993).
14) Montgomery, A. M., Sabzevari, H. and Reisfeld, R. A. Production and regulation of gelatinase B by human T-cells. *Biochim. Biophys. Acta*, **1176**, 265–268 (1993).
15) Baba, K., Kuwano, H., Kitamura, K. and Sugimachi, K. Carcinomatous invasion and lymphocyte infiltration in early esophageal carcinoma with special regard to the basement membrane. An immunohistochemical study. *Hepatogastroenterology*, **40**, 226–231 (1993).
16) Foidart, J. M., Olette, M., Birembaut, P. and Noel, A. Breast cancer invasion: the key role of normal cells of host tissues. *Bull. Acad. Natl. Med.*, **178**, 533–542 (1994).
17) Jiang, W. G., Hiscox, S., Hallett, M. B., Mansel, R. E. and Puntis, M. Regulation of motility and invasion of cancer cells by human monocytic cells. *Anticancer Res.*, **15**, 1303–1310 (1995).
18) Slootweg, P. J., de Pagter, M., de Weger, R. and de Wilde, P. Lymphocytes at tumor margins in patients with head and neck cancer. Relationship with tumor size, human lymphocyte antigen molecules, and metastasis. *Int. J. Oral Maxillofacc. Surg.*, **23**, 286–289 (1994).
19) Bubenik, J., Baresova, M., Viklicky, V., Jakoubkova, J.,

Sainerova, H. and Donner, J. Established cell line of urinary bladder carcinoma (T24) containing tumour-specific antigen. *Int. J. Cancer,* **11,** 765–773 (1973).
20) Kakuya, T., Yamada, T., Yokokawa, M. and Ueda, T. Establishment of cell strains from human urothelial carcinoma and their morphological characterization. *In Vitro,* **19,** 591–599 (1983).
21) Kurisu, H., Matsuyama, H., Ohmoto, Y., Shimabukuro, T. and Naito, K. Cytokine-mediated antitumor effect of bacillus Calmette-Guérin on tumor cells *in vitro. Cancer Immunol. Immunother.,* **39,** 249–253 (1994).
22) McAveney, K. M., Gomella, L. G. and Lattime, E. C. Induction of TH1- and TH2-associated cytokine mRNA in mouse bladder following intravesical growth of the murine bladder tumor MB49 and BCG immunotherapy. *Cancer Immunol. Immunother.,* **39,** 401–406 (1994).
23) Shin, J. S., Park, J. H., Kim, J. D., Lee, J. M. and Kim, S. J. Induction of tumor necrosis factor-alpha (TNF-alpha) mRNA in bladders and spleens of mice after intravesical administration of bacillus Calmette-Guérin. *Clin. Exp. Immunol.,* **100,** 26–31 (1995).
24) Kawashima, A., Nakanishi, I., Tsuchiya, H., Roessner, A., Obata, K. and Okada, Y. Expression of matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) induced by tumor necrosis factor alpha correlates with metastatic ability in a human osteosarcoma cell line. *Virchows Arch.,* **424,** 547–552 (1994).
25) Ries, C., Kolb, H. and Petrides, P. E. Regulation of 92-kD gelatinase release in HL-60 leukemia cells: tumor necrosis factor-alpha as an autocrine stimulus for basal and phorbol ester-induced secretion. *Blood,* **83,** 3638–3646 (1994).
26) Mackay, A. R., Ballin, M., Pelina, M. D., Farinia, A. R., Nason, A. M., Hartzler, J. L. and Thorgeirsson, U. P. Effect of phorbol ester and cytokines on matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase expression in tumor and normal cell lines. *Invasion Metastasis,* **12,** 168–184 (1992).
27) Okada, Y., Tsuchiya, H., Shimizu, H., Tomita, K., Nakanishi, I., Sato, H., Seiki, M., Yamashita, K. and Hayakawa, T. Induction and stimulation of 92-kDa gelatinase/type IV collagenase production in osteosarcoma and fibrosarcoma cell lines by tumor necrosis factor alpha. *Biochem. Biophys. Res. Commun.,* **171,** 610–617 (1990).
28) Mann, E. A., Hibbs, M. S., Spiro, J. H., Bowick, C., Wang, X. Z., Clawson, M. and Chen, L. L. Cytokine regulation of gelatinase production by head and neck squamous cell carcinoma: the role of tumor necrosis factor-alpha. *Ann. Otol. Rhinol. Laryngol.,* **104,** 203–209 (1995).
29) Rawdanowicz, T. J., Hampton, A. L., Nagase, H., Woolley, D. E. and Salamonsen, L. A. Matrix metalloproteinase production by cultured human endometrial stromal cells: identification of interstitial collagenase, gelatinase-A, gelatinase-B, and stromelysin-1 and their differential regulation by interleukin-1 alpha and tumor necrosis factor-alpha. *J. Clin. Endocrinol. Metab.,* **79,** 530–536 (1994).
30) Schirren, C. G., Majewski, S., Hunzelmann, N., Heckmann, M. and Krieg, T. The effect of interferon-gamma on the invasiveness of HT-180 cells. *Br. J. Dermatol.,* **126,** 331–336 (1992).
31) Hujanen, E. S., Vaisanen, A., Zheng, A., Tryggvason, K. and Turpeenniemi, H. T. Modulation of M (r) 72,000 and M (r) 92,000 type-IV collagenase (gelatinase A and B) gene expression by interferons alpha and gamma in human melanoma. *Int. J. Cancer,* **58,** 582–586 (1994).
32) Shapiro, S. D., Campbell, E. J., Kobatashi, D. K. and Welgus, H. G. Immune modulation of metalloproteinase production in human macrophages. Selective pretranslational suppression of interstitial collagenase and stromelysin biosynthesis by interferon-gamma. *J. Clin. Invest.,* **86,** 1204–1210 (1990).
33) Gohji, K., Fidler, I. J., Tsan, R., Radinsky, R. von E. A., Tsuruo, T. and Nakajima, M. Human recombinant interferons-beta and -gamma decrease gelatinase production and invasion by human KG-2 renal-carcinoma cells. *Int. J. Cancer,* **58,** 380–384 (1994).
34) Wolf, H., Melson, F., Pedersen, S. E. and Nielsen, K. T. Natural history of carcinoma *in situ* of the urinary bladder. *Scand. J. Urol. Nephrol.,* **157** (Suppl.), 147–151 (1994).