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Expression of matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) induced by tumour necrosis factor α correlates with metastatic ability in a human osteosarcoma cell line

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Abstract We have examined the correlation between matrix metalloproteinase (MMP) expression and metastatic properties of a low metastatic osteosarcoma cell line, osteosarcoma takase (OST), under stimulation by tumour necrosis factor α (TNFα). In vivo, OST cells exhibited significantly increased colonization in the lungs of nude mice in a dose-dependent manner when they were treated by TNFα prior to injection. In vitro, TNFα enhanced tumour cell invasion through the reconstituted basement membrane in a transwell chamber up to 2.5-fold. Gelatin zymography and sandwich enzyme immunoassays demonstrated marked production of MMP-9 [92-kDa gelatinase/type IV collagenase (gelatinase B)] but not MMP-2 [72-kDa gelatinase/type IV collagenase (gelatinase A)], MMP-3 (stromelysin-1) or MMP-7 (matrilysin). Motility of the tumour cells and adhesion to cultured endothelial cells were slightly increased by the TNFα treatment up to 1.6-fold and 1.4-fold, respectively, while the growth rate was decreased. These results suggest that upregulation of MMP-9 together with enhanced motility and endothelial adhesion contribute to the increased metastatic ability of OST cells induced by TNFα treatment.

Key words Osteosarcoma · Invasion · Metastasis · Matrix metalloproteinase · Tumour necrosis factor α

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Introduction

Many investigators have reported that type IV collagenolytic matrix metalloproteinases (MMPs) including MMP-2 [(72/kDa gelatinase/type IV collagenase (gelatinase A)] [30], MMP-9 [(92-kDa gelatinase/type IV collagenase (gelatinase B)] [1, 33], MMP-3 (stromelysin-1) [17] and MMP-7 (matrilysin) [25] play a key part in invasion of tumour cells. Sato et al. [27] have recently demonstrated that among these MMPs malignant mesenchymal tumour cells express MMP-9 constitutively. However, several lines of evidence have indicated that the expression by tumour cells can be modified by 12-o-tetradecanoylphorbol-13-acetate (TPA) [20], interleukin-1 [6], epidermal growth factor [18] and tumour necrosis factor alpha (TNFα) [23]. Our previous studies on human sarcoma cell lines have shown that among six cytokines/growth factors examined TNFα induces and stimulates MMP-9 production exclusively [23]. We have further demonstrated that enhanced MMP-9 production by TNFα in TPA-differentiated U937 monoblastoid cells correlates well with in vitro cellular invasion of the cells [32]. In these studies, however, in vivo metastatic ability was not examined. TNFα is a pluripotential mediator influencing the growth, differentiation and function of a broad range of cells [12, 29]. Thus, treatment of tumour cells by TNFα may induce changes in the growth rate, motility and adhesiveness to endothelial cells in addition to MMP-9 production, all of which could affect their metastatic ability in vivo.

In this study we have examined the effects of TNFα on invasion and metastasis of a well-characterized low metastatic cell line, osteosarcoma takase (OST) cells [16, 23], using in vivo lung colonization assay, and further studied the possible mechanism involved in the increased metastatic ability of the cells by TNFα treatment.

Materials and methods

Materials were obtained as follows: acrylamide and gelatin from Wako Chemicals (Tokyo, Japan); antibiotics, fetal calf serum (FCS), lactalbumin hydrolysate from Gibco Laboratories (Grand Island, N.Y., USA); Transwell chambers from Costar (Cambridge, Mass., USA); Matrigel from Becton Dickinson Labware (Bedford, Mass, USA); polyclonal rabbit anti-human TNFα antibodies from Cytokine Research Products (Cambridge, Mass. USA); normal rabbit immunoglobulin fraction from DAKO (Glostrup, Denmark). Human recombinant TNFα was kindly provided by Research Laboratories, Dainippon Pharmaceutical Company (Osaka, Japan).

OST cells established from human osteosarcoma were kindly donated by Dr. Katsuro Tomita, Department of Orthopaedic Surgery, School of Medicine, Kanazawa University [16]. They were maintained in a culture of Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. For the analyses of cell growth rates, OST cells (5×10^4 cells per 35 mm dish) were cultured in DMEM/10% FCS with TNFα (0, 400 units/ml). The cell numbers were counted with haemocytometer.

OST cells (2×10^5 cells per 60 mm dish) were cultured with TNFα (0, 400 units/ml) for 48 h in DMEM/10% FCS and photographed with an inverted microscope equipped with phase-contrast optics. For electron microscopy, the cells cultured on Lab-Tek slides (Miles Laboratories, Naperville, Ill., USA) were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 15 min at 4° C, and post-fixed for 60 min with 2% osmium tetroxide in the same buffer. The samples were embedded in Epon 812-filled gelatin capsules after dehydration with ethanols. Ultrathin sections were made with an LKB Ultrotome using a diamond knife, stained with 5% uranyl acetate and 6.5% lead citrate, and examined under a Hitachi H-500 electron microscope (75 kV).

For the quantitative analyses of MMP-3, MMP-9 and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), OST cells (1.3×10^6 cells per 60 mm dish) were cultured in DMEM/10% FCS. Culture media were then replaced with DMEM/0.2% lactalbumin hydrolysate containing TNFα (0, 100, 400 units/ml) and harvested after culturing for 70 h. Concentrations of MMP-3, TIMP-1 and TIMP-2 in the culture media were measured by the sandwich enzyme immunoassays as described previously [7, 10, 22]. Levels of MMP-9 were also assayed by a similar sandwich enzyme immunoassay using monoclonal antibodies previously described [24].

Zymography in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.2% gelatin or 0.2% casein was performed according to the method of Hibbs et al. [8]. Briefly, the samples were mixed with SDS sample buffer in the absence of reducing agent, incubated at 37° C for 20 min, and electrophoresed on 8% or 12.5% polyacrylamide gel at 4° C. After electrophoresis, the gels were washed in 2.5% Triton-X 100 for 1 h to remove SDS and incubated for 15 h at 37° C in 50 mM TRIS/hydrochlonic acid, pH 7.6/0.15 M sodium chloride/10 mM calcium chloride/0.02% sodium azide and then stained with 0.1% Coomassie brilliant blue R250.

OST cells treated with TNFα (0, 100, 400 units/ml) for 24 h were assessed for their metastatic ability in nude mice by injecting the cells (5×10^5/mouse) into the tail vein of 5-week-old BALB/C nu/nu mice. The mice were sacrificed 2 weeks later by cervical dislocation and the lungs were removed. Each lung lobe was cut into slices to make the largest cut surface and subjected to paraffin-embedded blocks. The number of metastatic foci in the lungs was determined by counting tumour cell colonies in the paraffin sections stained with haematoxylin and eosin. A total of 32 animals was used for the analysis.

Transwell chambers fitted with polyvinylpyrolidone-free polycarbonate filters with 12 μm-sized pores (12 mm diameter) were used. Each filter was coated with 300 μl of Matrigel in DMEM diluted 20-fold (90 μg) and dried under a hood to make a thin continuous barrier on the top of the filter. The membrane of Matrigel was washed with 300 μl of DMEM at 22° C for 90 min before the assay was started.

The invasion assay method developed by Repesh [26] was modified and utilized to detect the invasive activity of OST cells. The cells were labelled for 24 h with [methyl tritiated] (^{3}H)-thymidine (10 μCi/ml) in DMEM containing 10% FCS and then treated with TNFα (0, 4, 40, 400, 4000 units/ml) for 24 h. They were seeded onto the reconstituted basement membrane of the upper chamber of Transwell at a concentration of 1×10^6/ml in 500 μl of DMEM/10% FCS and cultured at 37° C in carbon dioxide (CO_2) incubator. Cells that passed through the reconstituted membrane into the lower chamber were collected by centrifugation of the culture medium obtained after incubating the lower side of the filter with 0.2% trypsin. Radioactivity was counted for ^{3}H using a liquid scintillation counter (LSC-3100, Aloka) after extraction of DNA from the collected cells [9].

In order to study the specific effect of TNFα , invasion assay was performed using the labelled-OST cells treated for 24 h with TNFα , which had been incubated with polyclonal rabbit anti-human TNFα antibodies or normal rabbit immunoglobulin (150 μg of immunoglobulin against 500 units of TNFα) at 37° C for 30 min.

To measure cell motility, OST cells were first labelled with (methyl ^{3}H)-thymidine (3 μCi/ml) for 24 h and treated with TNFα (0, 100, 400 units/ml) for 24 h prior to the assay. The cells (3.8×10^5 cells/chamber in 500 μl of DMEM/10% FCS) were subjected to motility assay using Transwell chambers of 12 μm pores. After incubation for 24 h at 37° C in a CO_2 incubator, the cells that had passed through polycarbonate filter into the lower chamber were collected. Radioactivity was counted in a similar way as described in the invasion assay.

The adhesion assay was performed according to the method described by Dejana et al. [5] but slightly modified. Briefly, the endothelial cells (EC) derived from human umbilical vein were grown to confluence in 24 well-plates. OST cells in culture were washed 3 times with Hanks' balanced salt solution (HBSS) and incubated in serum-free RPMI-1640 medium containing TNFα (0, 100, 400 units/ml) for 24 h. The cells were labelled with ^{51}Cr (1 μCi/ml) for 15 h before the end of TNFα treatment. They were then detached from the culture dishes with phosphate buffered saline containing 50 mM ethylenediaminetetraacetic acid and suspended at a concentration of 1.0×10^6 cells/ml in HBSS containing 0.25% bovine serum albumin (BSA) and TNFα (0, 100, 400 units/ml). The tumour cell suspension (300 μl, 3×10^5 cells) was added to each well of the cultured EC and incubated for 30 min at 37° C. At the end of the incubation, the supernatant was carefully aspirated and the wells were washed 3 times with 1 ml of HBSS/0.25% BSA to remove nonadherent tumour cells. The adhesiveness of OST cells to EC (%); 100× cell lysate/cell lysate+supernatant+rinse) was calculated by measuring radioactivity in the supernatant, rinse and cell lysate obtained by incubation of the wells for 60 min at 23° C with 250 μl of 1 M sodium hydroxide/1% SDS.

Results were compared using Student's unpaired *t*-test. A *P*-value less than 0.05 was considered significant.

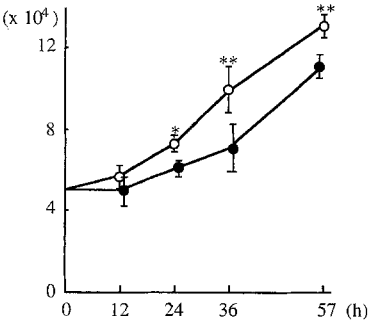


Fig. 1 Effect of tumour necrosis factor α (TNFα) on cell growth of osteosarcoma takase (OST) cells. Cell growth curves were drawn with the average numbers of cells±standard deviation (SD) in each of the three wells. *Open circle,* control cell growth curve; *closed circle,* TNFα (400 units/ml)-treated cell growth curve; $^*P<0.01$; $^{**}P<0.05$

Results

The growth rate of TNFα-treated OST cells was significantly lower than that of untreated cells (Fig. 1). Under the phase-contrast microscope, OST cells without TNFα treatment showed cuboidal appearances with a sheet-like growth pattern, whereas the TNFα-treated cells showed stellate features. They were not tightly attached to each other. Electron microscopically, the endoplasmic reticulum and well-developed Golgi apparatus were more prominent in the cytoplasm of TNFα-treated OST cells than in that of untreated OST cells, and TNFα-treated OST cells had many cell-processes compared with controls.

Quantitative analyses of MMP-3, MMP-9, TIMP-1 and TIMP-2 in the culture media were performed using sandwich enzyme immunoassays. As shown in Fig. 2, MMP-9 production was induced by treatment with TNFα and was significantly increased in a dose-dependent manner. However, levels of MMP-3 were below detection sensitivity. No significant effect of TNFα on TIMP production was seen with the exception of TIMP-1 production by treatment with 400 units/ml TNFα with which the TIMP-1 level was increased 1.3-fold (Fig. 2). Gelatin zymography of the same media confirmed the data that MMP-9 production was induced and stimulated by TNFα treatment, whereas MMP-2 was constitutively produced (data not shown). On casein zymography (12.5% total AG), no enzyme activities were found in these samples, indicating that they contain no or little MMP-7.

A suspension of OST cells (0.2 ml, 5×10^5 cells) was injected into the tail vein of each nude mouse. Although

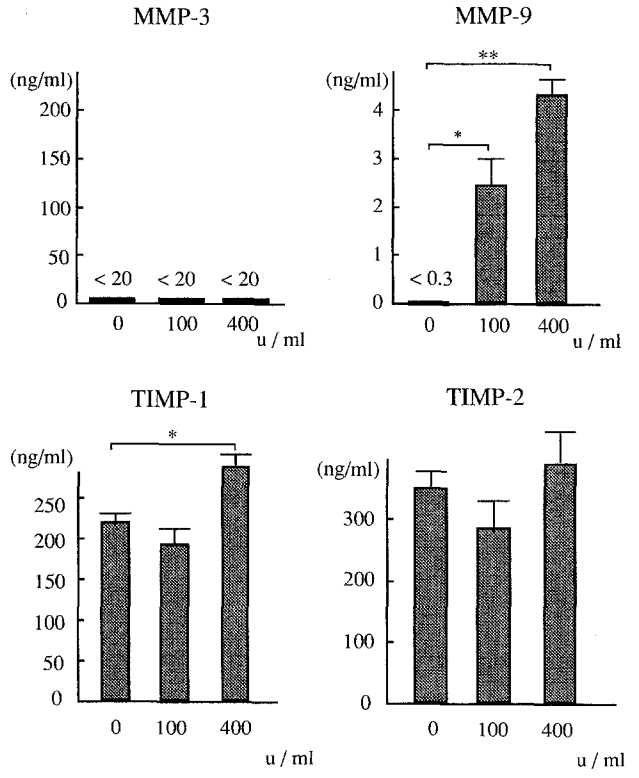
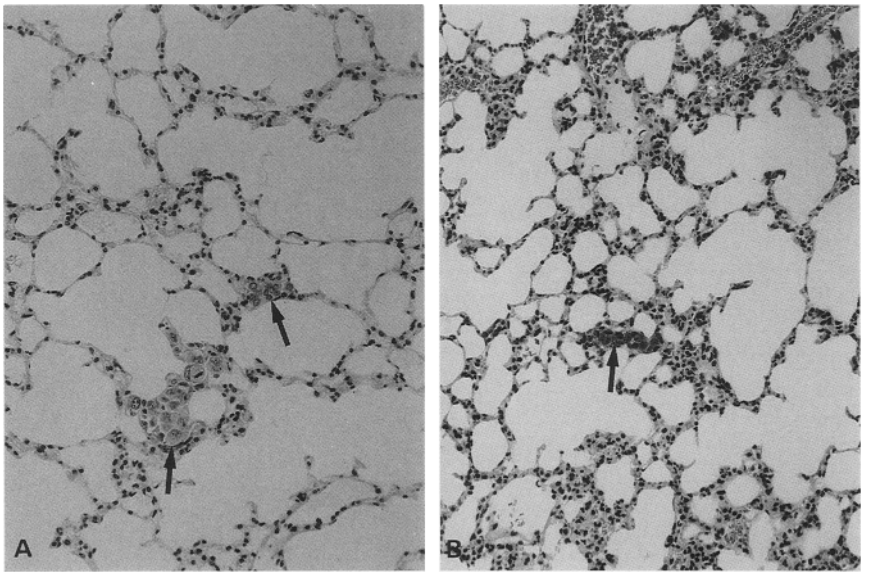
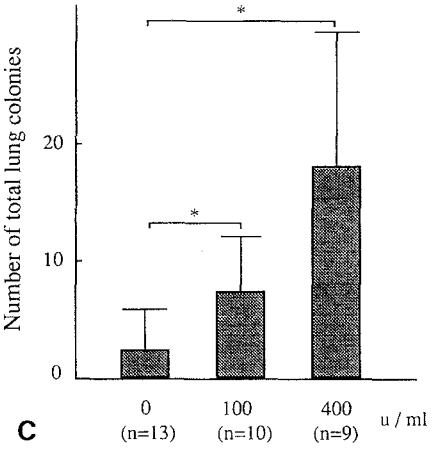


Fig. 2 Quantitative analyses of matrix metalloproteinase (MMP)-3, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 in the culture media of OST cells treated with TNFα (0, 100, 400 units/ml). Concentrations of the proteinases and inhibitors in the conditioned media from OST cells were measured by sandwich enzyme immunoassays. The assay was performed in triplicate. * $P<0.01$; ** $P<0.0001$

Fig. 3A–C Lung colonization assay of OST cells. OST cells treated with or without TNFα (0, 100, 400 units/ml) were injected into the lateral tail vein of nude mice as described in materials and methods. **A, B** Light microscopy of the lungs with micrometastases. Lungs of the nude mice 2 weeks after injection of 5×10^5 cells treated with TNFα (400 units/ml; **A**) or untreated cells (**B**) were examined in paraffin sections stained with haematoxylin and eosin. *Arrows* indicate metastatic foci in the lungs. × 185. **C** Data of lung colonization assay. Number of metastatic foci in the lungs was determined by counting tumour cell colonies. The mean number of lung colonies ±SD is shown. * $P<0.05$





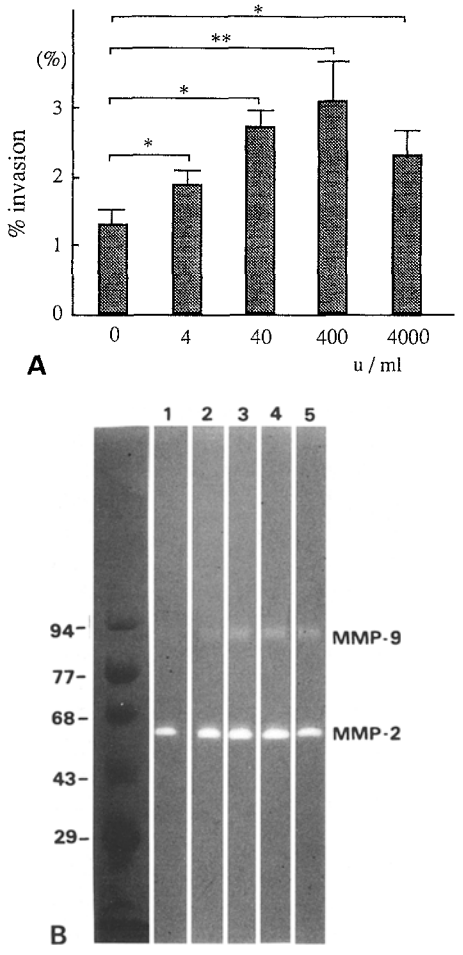


Fig. 4A, B In vitro invasion assay. **A** [^{3}H]-labelled OST cells were treated with different concentrations of TNFα (0, 4, 40, 400, 4000 units/ml) for 24 h and subjected to the invasion assay as described in materials and methods. The assay was performed in triplicate. Invasion is shown as a percentage (%) of radioactivity of the cells migrated into the lower chamber to that of the total cells seeded. Mean % invasion ±SD is shown. * $P<0.05$; ** $P<0.01$. **B** Gelatin zymography of the media from the upper chambers of the invasion assay. The samples (10 µl) were electrophoresed on 8% sodium dodecyl sulfate/polyacrylamide gel containing 0.2% gelatin under the non-reducing conditions and the gel was processed as described in materials and methods. Lanes 1–5, the samples from the assay of OST cells treated with 0, 4, 40, 400 or 4000 units/ml TNFα, respectively. The relative molecular masses ($\times 10^{-3}$) of standard proteins are indicated

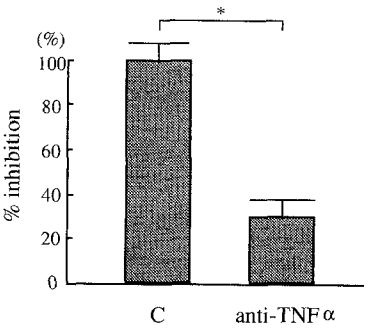


Fig. 5 Inhibition of in vitro invasion by anti-TNFα antibodies. TNFα was first incubated with polyclonal rabbit anti-human TNFα antibodies or normal rabbit immunoglobulin (C) (150 µg of immunoglobulin against 500 units of TNFα) at 37° C for 30 min and used to treat [^{3}H]-labelled OST cells for 24 h. The in vitro invasion assay was performed in triplicate as described in materials and methods and relative invasiveness was compared. Mean % invasion ±SD is shown. * $P<0.001$

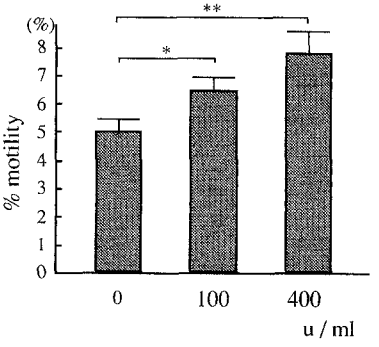


Fig. 6 Motility assay. The [^{3}H]-labelled OST cells were treated with TNFα (0, 100, 400 units/ml) for 24 h and subjected to the motility assay. The cells were harvested from the lower chambers and radioactivities were counted as described in materials and methods. The values (% motility) are mean ±SD of quadruplicate. * $P<0.01$; ** $P<0.001$

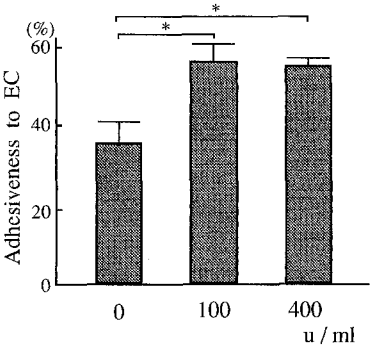


Fig. 7 Adhesion assay. OST cells treated with TNFα (0, 100, 400 units/ml) for 24 h and labelled with ^{51}Cr (1 µCi/ml) for 15 h were cultured on endothelial cells (*EC*) for 30 min and % increment ±SD of tumour cell adhesion was calculated as described in materials and methods. The assay was done in quadruplicate. * $P<0.01$

most metastatic foci in the lungs could not be recognized macroscopically 2 weeks after the injection, they were countable by microscopy. The number of metastatic foci appeared to be larger in the mice having received TNFα-treated OST cells than the control mice injected with untreated cells (Fig. 3A, B). However, no apparent differences in morphology of the metastatic cells were seen between the lungs of the mice with TNFα-treated OST cells and those with untreated cells. Figure 3C shows a dose-dependent increase in the number of metastatic foci with TNFα-treated OST cells. None of these mice died as a result of the injection or metastasis until the examination was finished.

The ability of OST cells treated with or without TNFα to invade the reconstituted basement membrane was measured by in vitro invasion assay. TNFα significantly enhanced the cellular invasion up to 2.5-fold in a dose-dependent manner with a peak concentration of 400

units/ml (Fig. 4A). Increased invasive activity of the TNFα-treated OST cells was abolished to a basal level with the cells treated with TNFα being neutralized by anti-human TNFα antibodies (Fig. 5). Parallel analysis on gelatin zymography for the gelatinolytic activities in the culture media from the upper chamber demonstrated the dose-dependent production of MMP-9 due to treatment with TNFα (Fig. 4B). The activity corresponding to MMP-2 was also detected in all the samples, but TNFα did not affect production.

The motility of TNFα-treated OST cells increased slightly in a dose-dependent manner with a maximum value of 1.6-fold compared with the untreated cells (Fig. 6).

TNFα-treated cells adhered more actively to cultured EC (144%) compared with control cells (Fig. 7). Adhesion reached a plateau at a concentration of 100 units/ml.

Discussion

Our data on the sandwich enzyme immunoassays and gelatin zymography have demonstrated that MMP-9 production by OST cells is induced and stimulated by TNFα in a dose-dependent manner. This is consistent with our previous finding that induction of MMP-9 production by TNFα-treated OST cells is achieved at a transcriptional level [23]. In the present study, we have further shown that neither induction nor enhanced production of MMP-2, MMP-3 and MMP-7 occurs by TNFα treatment. In addition, TNFα did not stimulate TIMP-2 production, while TIMP-1 production was slightly increased following TNFα treatment. Previous studies suggested that biosynthesis of both TIMPs by tumour cell lines [30] and human alveolar macrophages [28] is regulated in a different manner. In fact, Shapiro et al. [28] reported that TIMP-2 production by human fibroblasts was unaffected by TNFα treatment.

It has been demonstrated that the expression of MMP-9 closely correlates with the metastatic potential of tumour cell lines [1, 33] and the acquisition of the invasive phenotype of fibroblasts transfected with HT1080 fibrosarcoma genomic DNA [11]. Similar correlation is also reported with physiological cells showing invasive properties such as polymorphonuclear leucocytes [8, 21], macrophages [2] and cytotrophoblasts [13]. Our recent studies on U937 monoblastoid cells have demonstrated that in vitro invasion of the cells involves the type IV collagenolysis by MMP-9 overproduced by TNFα treatment [32]. The present study further indicates that even sarcoma cells with low metastatic ability become more invasive as a result of the induction and stimulation of MMP-9 production. OST cells stimulated by TNFα showed slightly increased motility and developed many cell processes. Therefore, it seems likely that the enhanced in vitro invasiveness of OST cells is ascribed to the increased ability in both the production of MMP-9 and movement.

The present study demonstrates that TNFα treatment of human osteosarcoma cells increases their metastatic ability in nude mice. This appears to contract with the originally-proposed function of TNFα in malignant tumour cells: endotoxin-elicited tumour necrosis [3]. However, accumulated data indicate that TNFα performs a wide range of actions on cells and tissues [12, 29]. In fact, recent investigations have shown that TNFα enhances experimental metastasis in tumour cell lines [14, 19], and that Chinese hamster ovary cells transfected with human TNFα gene invade peritoneal surfaces aggressively and metastasize in nude mice [15]. These studies, however, showed no data on the precise mechanisms of the increased metastatic ability by TNFα . The initial event in the lung metastasis by our in vivo lung colonization assay is tumour cell adherence to the endothelial cells of the lung blood vessels. Since our data on the adhesion assay showed slightly increased adhesiveness of the TNFα-treated OST cells to the endothelial cells, this may contribute to tumour cell embolization. Overproduction of MMP-9 and increased motility by TNFα would further facilitate formation of the metastatic foci in the lungs. Recent studies using tumour cells transfected with TIMP-1, TIMP-2 or MMP-7 DNA indicated that down-regulation of the activities of type IV collagenolytic MMPs by TIMP or up-regulation of MMP-7 alone result in enhanced metastasis of the tumour cells in vivo [4, 25, 31]. Although our data did not provide conclusive evidence on which alteration of OST cells induced by TNFα is responsible for the increased metastasis, such experimental studies utilizing DNA transfection technique suggest that induction and stimulation of MMP-9 production may be a key determinant for the enhanced metastasis of OST cells. Further studies using the tumour cells transfected with MMP-9 DNA would settle this question.

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