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# VCP (p97) Regulates NFκB Signaling Pathway, Which Is Important for Metastasis of Osteosarcoma Cell Line

Tatsuya Asai,[1] Yasuhiko Tomita,[1,3] Shin-ichi Nakatsuka,[1] Yoshihiko Hoshida,[1] Akira Myoui,[2] Hideki Yoshikawa[2] and Katsuyuki Aozasa[1]

*Departments of [1]Pathology and [2]Orthopedics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871*

**In order to identify genes associated with metastasis, suppression subtractive hybridization (SSH) was performed using murine osteosarcoma cell line Dunn and its subline with higher metastatic potential, LM8. SSH revealed expression of the gene encoding valosin-containing protein (VCP; also known as p97) to be constitutively activated in LM8 cells, but it declined in Dunn cells when the cells became confluent. Because VCP is known to be involved in the ubiquitination process of Inhibitor-κBα (IκBα), an inhibitor of nuclear factor-κB (NFκB), whether VCP influences NFκB activation or not was examined by using VCP-transfected Dunn cells (Dunn/VCPs). When stimulated with tumor necrosis factor-α (TNFα), Dunn/VCPs showed constantly activated NFκB, although in the original Dunn cells and control vector transfectant (Dunn/Dunn-c) NFκB activation ceased when the cells became confluent. Western immunoblot analysis showed an increase of phosphorylated IκBα (p-IκBα) in the cytoplasm of confluent Dunn/Dunn-c cells compared to that of Dunn/VCPs. Therefore, decrease of p-IκBα degrading activity might be responsible for the decrease in NFκB activation. *In vitro* apoptosis assay demonstrated increased apoptosis rates of Dunn/Dunn-c cells after TNFα stimulation compared to those of Dunn/VCPs and LM8 cells. *In vivo* metastasis assay showed increased incidences of metastatic events in Dunn/VCP-1 inoculated male C3H mice compared to those in Dunn/Dunn-c inoculated mice. These findings suggested that VCP expression plays an important role in the metastatic process. Anti-apoptotic potential in these cells owing to constant NFκB activation via efficient cytoplasmic p-IκBα degrading activity may explain the increased metastatic potential of these cells.**

Key words: Valosin-containing protein (VCP) — Nuclear factor-κB (NFκB) — Suppression subtractive hybridization (SSH) — Metastasis — Osteosarcoma

Metastasis involves multiple processes,[1,2] and the pattern of metastasis is distinct in each cancer cell type.[1,2] Osteosarcoma is the most common malignant bone tumor with a high metastatic potential mainly to the lung.[3,4] In order to understand the mechanisms involved in pulmonary metastasis of osteosarcoma, LM8 subline of murine osteosarcoma cell line Dunn was established.[5] LM8 was obtained through 8 rounds of *in vivo* selection according to the procedure of Poste and Fidler,[6] and showed a high metastatic incidence to the lung after subcutaneous inoculation into the back space of mice. No pulmonary metastasis was found in mice inoculated with the original Dunn cells.[5]

Suppression subtractive hybridization (SSH) is a PCR-based cDNA subtraction technique to construct differential gene expression libraries.[7] By SSH, we found the gene for valosin-containing protein (VCP; also known as p97) to be prominently expressed in LM8 cells. VCP, a member of the ATPases associated with various cellular activities (AAA) superfamily, is implicated in a large number of biological functions, such as fusion of the endoplasmic reticulum[8] and the reassembly of Golgi cisternae.[9] Furthermore, VCP physically associates with Inhibitor-κBα (IκBα) complexes both *in vivo* and *in vitro*, and is co-purified with the mammalian 26S proteasome, and thus might be involved in the ubiquitin-dependent proteasome degradation pathway of IκBα.[10]

IκBα belongs to the IκB family (IκBs) of inhibitors of the activation of a transcription factor, nuclear factor-κB (NFκB).[11] In unstimulated cells, NFκB is localized in the cytoplasm in a complex with IκBs, which mask its nuclear-localization signal (NLS) and prevent its translocation to the nucleus.[12,13] Upon stimulation, the entire NFκB complex become hyperphosphorylated. Phosphorylation of IκBα signals for subsequent ubiquitination and degradation, allowing inhibitor-free NFκB complex to translocate to the nucleus.[14–16] Further, NFκB activation is inhibited by expression of a dominant-inhibitor IκBα mutant.[17] These findings prompted us to postulate that the NFκB signaling pattern might be altered by the increase of cytoplasmic VCP concentration.

NFκB is a transcription factor which acts as a protective factor against apoptosis, as well as a mediator of immune

[3] To whom correspondence should be addressed.
E-mail: yt@molpath.med.osaka-u.ac.jp

and inflammatory responses.[18] Several studies have shown that NFκB is both necessary and sufficient to prevent apoptosis induced by tumor necrosis factor-α (TNFα), radiation, and chemotherapeutic agents.[19, 20] Furthermore, constitutive activation of NFκB is observed in Hodgkin's disease[21] and breast cancer,[22] which suggested that NFκB may contribute to the survival of the tumor cells.

In the present study, higher pulmonary metastatic incidence of VCP-transfected cells than that of original Dunn cells was shown by *in vivo* metastasis assay. Because NFκB signaling is increased in VCP-transfected Dunn osteosarcoma cells, our results suggest that VCP might be involved in the metastatic potential of cancer cells by influencing the anti-apoptotic NFκB signaling pathway.

## MATERIALS AND METHODS

**Animals** Male inbred C3H mice aged 5 weeks were purchased from Japan SLC (Shizuoka) for *in vivo* pulmonary metastasis assay.

**Cell lines and cell culture** Cloned murine osteosarcoma cell lines, Dunn and LM8, and human osteosarcoma cell lines, HOS, MG-63, and Saos-2 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma) in an air incubator with 5% $CO_2$ at 37°C.

**SSH** Messenger RNA (mRNA) was isolated from confluently growing Dunn and LM8 cells by using Oligotex-dT30 (TaKaRa, Kusatsu). Subtractive hybridization was performed by a PCR-based method using the PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Both cell lines were used alternatively as testers and drivers to produce libraries of candidate genes that were selectively expressed in Dunn or LM8 cells, respectively. Each clone obtained by SSH was confirmed to show modulated expression by dot-blot analysis and/or northern blot analysis.

**Isolation of *VCP* and plasmid construction** The full-length mouse *VCP* cDNA was prepared with RT-PCR using 1 μg of mRNA from Dunn cells and a set of primers (*VCP* forward primer: 5′-ACTGGATCCATGGCCTCTG-GAGCCGATTCAAAAGG-3′ and *VCP* reverse primer: 5′-CTGTTCAGACTGAGGAATGGAGCAGGCC-3′). The PCR product was further cloned into the expression vector pIRESneo (Clontech) by using the GATEWAY cloning system (Invitrogen, Carlsbad, CA). The DNA sequence of the plasmid was confirmed by using an ABI dye terminator sequencing kit (Perkin Elmer, Foster City, CA).

**Northern blot analysis** Total RNA of Dunn and LM8 was prepared from cells at approximately 70% confluency (sub-confluent growth conditions) and 100% confluency with Trizol (Invitrogen). Ten micrograms of each sample was separated on 1% agarose-formaldehyde gel electrophoresis, transferred to Hybond-$N^+$ nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) in 10× SSC, and immobilized by UV cross-linking. The hybridization probe prepared from a gel-purified PCR fragment was denatured and random-labeled using large Klenow fragment of DNA polymerase and [α-$^{32}$P]dCTP. The blot was hybridized in a solution containing 20 m*M* Pipes, 800 m*M* NaCl, 50 m*M* sodium phosphate, 5% sodium dodecyl sulfate (SDS), 50% deionized formamide, and 100 μg/ml of heat-denatured salmon sperm DNA at 65°C for 24 h. After the hybridization, the blot was washed three times in 1× SSC, 5% SDS at 50–60°C. The washed membrane was autoradiographed at −80°C overnight.

**RT-PCR analysis of VCP** Ten micrograms of DNase I-treated total RNA was used for reverse transcription (RT) with Superscript II (Invitrogen). An aliquot representing 25 ng of input RNA was amplified by PCR with AmpliTaq Gold DNA polymerase (Perkin Elmer). For murine *VCP* amplification, 1 cycle of 95°C for 10 min then 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 2 min were performed with *VCP*-forward and reverse primers. For human *VCP* amplification, we used 1 cycle of 95°C for 10 min followed by 24 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min with primers 5′-TGGCA-GATGATGTGGACCTGGAACA-3′ and 5′-CAGCTTG-GCGGGCCTTGTCAAAGAT-3′. For human and murine *G3PDH* amplification, the conditions were 1 cycle of 95°C for 10 min followed by 24 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min with primers 5′-ACCA-CAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCT-GTTGCTGTA-3′. Amplified products were electrophoresed through 1% Nusieve 3:1 agarose gel (Biowhittaker Molecular Applications, Rockland, ME), stained with ethidium bromide, and photographed.

**Antibodies** The monoclonal antibody to VCP (p97) was purchased from Progen Biotechnik (Heidelberg, Germany), polyclonal antibodies to NFκB (sc-109 and sc-109x) and monoclonal antibody to phosphorylated IκBα (p-IκBα) (sc-8404) from Santa Cruz Biotech (Santa Cruz, CA), and polyclonal antibody to actin (A2066) from Sigma. Anti-p97, sc-8404, and A2066 were used for western immunoblotting, sc-109 for immunofluorescence staining, and sc-109x for electrophoretic mobility-shift assays (EMSAs). Anti-mouse IgG antibody or anti-rabbit IgG antibody linked with horseradish peroxidase purchased from Cell Signaling Tech. (Beverly, MA), and fluorescein-labeled anti-rabbit IgG antibody from Vector Laboratories (Burlingame, CA) were used as secondary antibodies.

**Western immunoblot analysis** Total cellular proteins were solved in a buffer containing 10% glycerol, 1 m*M* phenylmethanesulfonyl fluoride (PMSF), 1% Triton X-100 and 40 m*M* HEPES, pH 7.4. The protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). An aliquot of 20 μg from each sample

was used for the western immunoblot analysis. The extracts were boiled for 10 min, then discontinuous SDS-polyacrylamide gel electrophoresis was performed according to the standard protocols.[23] The separated proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After blockage of nonspecific binding sites with 5% nonfat milk in PBST (phosphate-buffered saline, 0.05% Tween 20), blots were incubated with anti-p97, washed, incubated again with the secondary antibody, and washed again, then the antibody binding was visualized using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA).

**Nuclear and cytoplasmic protein extraction from TNFα-induced cells** Nuclear and cytoplasmic extracts were prepared as described previously.[24] In brief, the cells cultured either sub-confluently or confluently as described above were trypsinized and re-suspended in DMEM with 0.5% FBS at a concentration of $1\times10^7$/ml, treated with TNFα (Sigma) for 30 min at a concentration of 5 ng/ml, washed with PBS, then resuspended in 400 μl of buffer A (10 m*M* Hepes pH 7.8, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1% Triton X-100, 1 m*M* dithiothreitol (DTT), 0.5 m*M* PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin), and allowed to swell on ice for 20 min. After centrifugation for 5 min at 6000*g*, the supernatent was collected and adjusted to 100 m*M* KCl, 20% glycerol, then used as a sample of cytoplasmic extract in the western immunoblot analysis for p-IκBα. The nuclear pellet was resuspended in 100 μl of buffer C (50 m*M* Hepes pH 7.8, 420 m*M* KCl, 0.1 m*M* EDTA, 5 m*M* $MgCl_2$, 2% glycerol, 1 m*M* DTT, 0.5 m*M* PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin), incubated on ice for 30 min, and centrifuged for 15 min at 15 000*g*, then the supernatant was subjected to the following EMSAs analysis.

**EMSAs** A probe was generated by using a pair of complementary oligonucleotides containing a specific binding site for NFκB transcription factor: 5′-AGCTTGGGGACTTTCCACTAGTACG-3′ and 5′-AATTCGTACTAGTGGAAAGTCCCCA-3′.[25] The oligonucleotides were boiled for 5 min, allowed to anneal by cooling gradually on the benchtop, then end-labeled using large Klenow fragment of DNA polymerase and [α-$^{32}$P]dCTP. EMSAs were performed as described previously[26] with ~2000 dpm of labeled oligonucleotide and 10 μg of nuclear extracts. The specificity of the binding was tested by competition analysis with an additional 100-fold molar excess of cold probe, leaving no shifted band on the gel, and super-shift analysis using anti p-65 to super-shift the protein-DNA complexes.

**Immunofluorescence microscopy** The cells were cultured confluently on a 8-chamber slide, treated with TNFα for 30 min at a concentration of 5 ng/ml, washed with PBS, then fixed in methanol at −20°C for 10 min. After fixation, the slides were washed in PBS, pre-incubated in 5% normal goat serum for 1 h, then incubated with anti-p65. After washing with PBS three times, the slides were

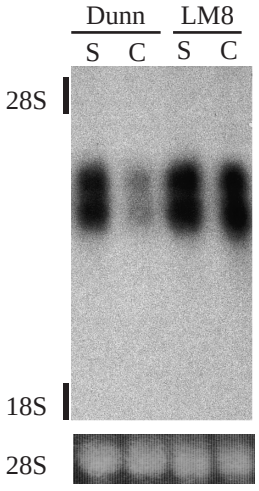


Fig. 1. Northern blot analysis of *VCP* in sub-confluent (S) and confluent (C) Dunn and LM8 cells. Dunn cells showed a marked decline in *VCP* expression when the cells became confluent, although LM8 cells showed a constant *VCP* expression. The 28S rRNA was used as a loading control.

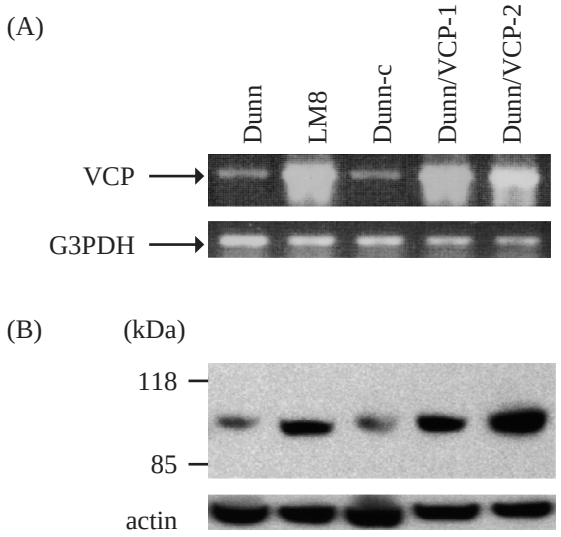


Fig. 2. (A) RT-PCR and (B) western immunoblot analyses of VCP in confluent Dunn, LM8, Dunn-c, and Dunn/VCP-1 and 2 cells. LM8 and Dunn/VCPs showed increased VCP expression levels compared to those of Dunn/Dunn-c in both RNA and protein. RT-PCR of the *G3PDH* gene was run as a control. After stripping, the western immunoblot membrane was reprobed with anti-actin.

incubated with fluorescein-labeled anti-rabbit IgG antibody, washed again, then examined under a fluorescence microscope.

**RT-PCR analysis of *c-IAP1* (cellular inhibitor-of-apoptosis 1)** After TNFα treatment for 30 min at a concentration of 5 ng/ml, total RNA extraction and RT-PCR were performed. For amplification of *c-IAP1*, 1 cycle of 95°C for 10 min followed by 30 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min were performed with primers 5′-TCATCTAGTAGTCTGCCAGGAAT-3′ and 5′-GGAACAGACACAGTCCTGTAGCT-3′.

**Apoptosis assay** The cells were cultured confluently. After TNFα treatment for 30 min at a concentration of 5 ng/ml, the cells were cultured in DMEM with 0.5% FBS for 12 h. The cells, either floating or attached, were collected, washed with PBS, and stained with trypan blue. Then the numbers of living (clear) and apoptotic (blue) cells were counted.

***In vivo* metastasis assay** C3H male mice aged 5 weeks were used to estimate the *in vivo* metastatic potential to the lung. Tumor cells ($1\times10^7$) were suspended in 0.2 ml of DMEM and inoculated subcutaneously into the back space of mice on day 0. Lungs were removed 4 weeks later to evaluate metastatic tumor nodules macroscopically using a magnifying glass and then routinely processed for histological examination; 5 μm sections of 15% formalin-fixed, paraffin-embedded lung specimens were cut stepwise, stained with hematoxylin-eosin and evaluated microscopically to confirm the presence of pulmonary metastasis.

**Statistics** The significance of the differences between the experimental groups was calculated by using the $\chi^2$ test or Mann-Whitney's *U* test.

## RESULTS

***VCP* identification as a gene over-expressed in LM8** Six-hundred and forty clones were isolated by SSH. Dot blot hybridization revealed that 92 of these were differently expressed between Dunn and LM8 cells. After sequencing analysis, 23 clones out of the 92 were chosen as candidates that might be involved in the metastatic event. Finally, *VCP* was selected as a gene to be investigated for functional association with metastatic activity.

Differential expression of *VCP* between Dunn and LM8 cells was further confirmed by northern blot analysis (Fig. 1). *VCP* expression was observed in both Dunn and LM8 cells when the cells were sub-confluent and exponentially growing. However, *VCP* expression in Dunn cells showed

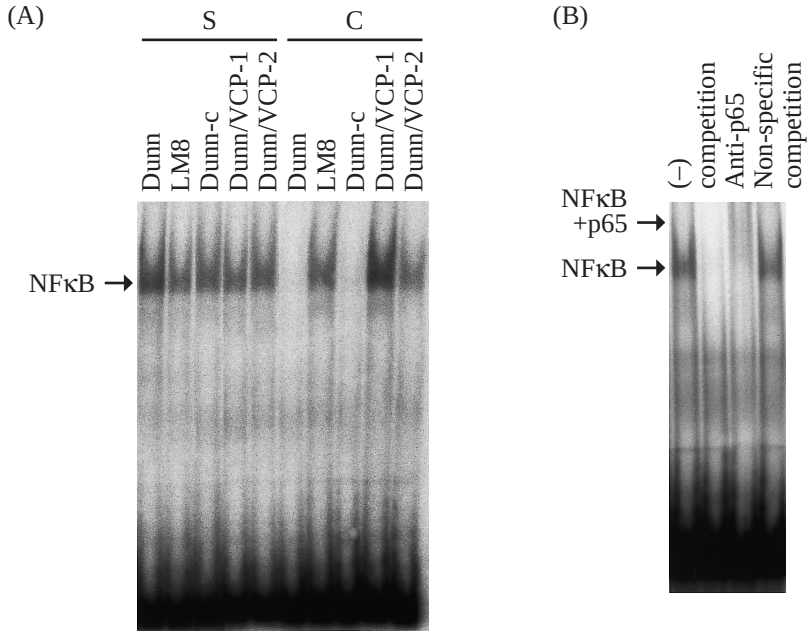


Fig. 3. Nuclear expression of NFκB in cells stimulated with TNFα. (A) Nuclear extracts were prepared from sub-confluent (S) and confluent (C) cells after stimulation with TNFα for 30 min. A 10 μg aliquot of each sample subjected to EMSAs for binding to the $^{32}$P-labeled NFκB oligomer. Free probes are shown below. (B) Additional 100-fold molar excess of cold probe (competition) completely inhibited the binding to labeled probe, although an additional 100-fold molar excess of unrelated sequence (non-specific competition) had no effect on DNA binding. Addition of anti p-65 shifted the protein-DNA complexes. Both analyses were performed on each cell type, and representative results are shown in (B).

a steep decrease as they became confluent and proliferation-arrested. On the contrary, *VCP* expression in LM8 cells was maintained even after the cells became confluent. Therefore, the difference in *VCP* expression was mostly observed when the cells became confluent and ceased proliferation.

**Construction of a Dunn subline constitutively active for VCP** Because VCP is involved in various cellular activities including fundamental functions for cell survival,[8–10] and the mutant form of VCP induces apoptosis in a dominant-negative manner,[27] we decided to evaluate the relationship between VCP expression pattern and metastasis by introducing sublines of Dunn cells constitutively active for VCP and comparing them with the original Dunn cells. Two lines were established from Dunn cells stably transfected with pIRESneo-VCP (Dunn/VCP-1 and 2). As a control, one line transfected with pIRESneo (Dunn-c) was also established. Constitutively active expression of VCP in Dunn/VCPs was confirmed by RT-PCR and western blot analyses (Fig. 2).

**VCP expression regulates NFκB activation** To assess the influence of VCP expression on NFκB signaling, EMSAs of NFκB was performed (Fig. 3). Transient NFκB activation by TNFα was markedly reduced in confluent Dunn/Dunn-c cells compared to that in the sub-confluent populations. Nevertheless, NFκB activation was maintained in Dunn/VCPs and LM8 even in the confluent condition. These findings together with the result of western blot analysis, showing the increase of cytoplasmic p-IκBα in confluent Dunn/Dunn-c cells (Fig. 4), indicate that NFκB activation in confluent cells is impaired by the excess amount of p-IκBα in the cytoplasm. Decrease of VCP expression in these cells (Fig. 4) is suggestive of a decrease of degrading activity of p-IκBα.

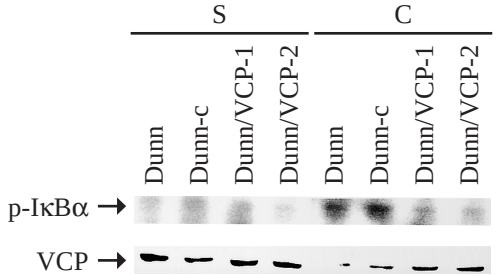


Fig. 4. Cytoplasmic expressions of p-IκBα and VCP in cells stimulated with TNFα. Cytoplasmic extracts were prepared from sub-confluent (S) and confluent (C) cells after stimulation with TNFα for 30 min. A 20 μg aliquot from each sample was used for western immunoblot analysis of p-IκBα. After stripping, the membrane was reprobed with anti-VCP (p97). Dunn/Dunn-c cells showed both increase of cytoplasmic p-IκBα and decrease of cytoplasmic VCP in the confluent state.

A difference in NFκB activation was confirmed by fluorescent immunohistochemical analysis, which showed nuclear localization of NFκB in Dunn/VCP-1 cells, but not in Dunn/Dunn-c cells in the confluent condition (Fig. 5).

Association of VCP with NFκB activation was further analyzed by RT-PCR analysis of c-IAP1, a gene which has an anti-apoptotic role and is known to be induced by NFκB. Dunn/Dunn-c cells showed reduced expression of c-IAP1 when the cells were in the confluent condition, whereas Dunn/VCPs cells showed a constant expression level of *c-IAP1* even in the confluent condition (Fig. 6).

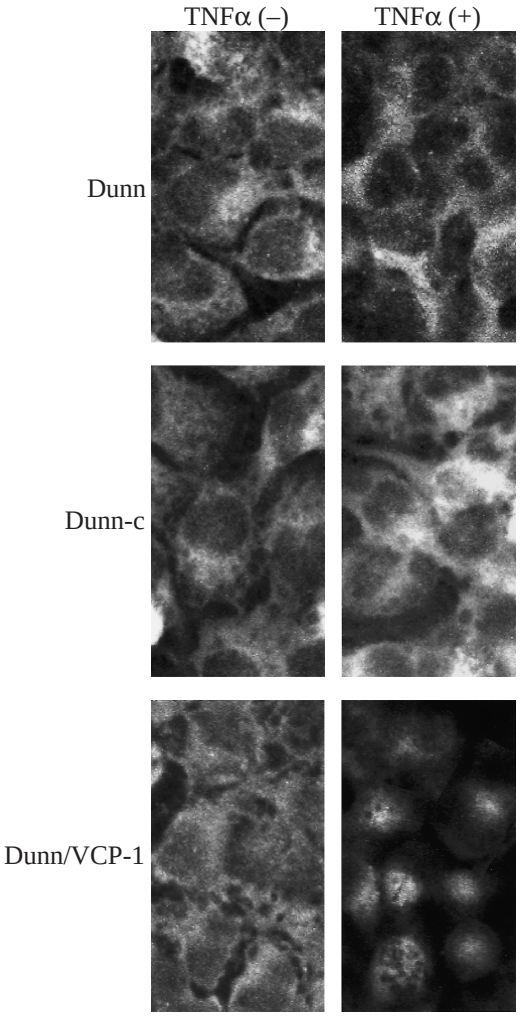


Fig. 5. Fluorescent immunohistochemical analysis of NFκB in cells stimulated with TNFα. Confluent cells were treated with TNFα for 30 min on a chamber slide. After fixation, the slides were processed for fluorescent immunohistochemical analysis for p-65. Nuclear localization of NFκB was observed in Dunn/VCP-1 cells, but not in Dunn/Dunn-c cells in the confluent state.

**VCP is anti-apoptotic against TNFα stimulation** The function of VCP against apoptosis was investigated by *in vitro* apoptosis assay. Dunn/Dunn-c cells showed increased apoptosis rates compared to those of Dunn/VCPs and LM8 cells ($P<0.01$) (Fig. 7).

**Difference of metastatic potential between Dunn/Dunn-c and Dunn/VCPs cells by *in vivo* metastasis assay** *In vivo* metastasis assay showed increased incidences of metastatic events in Dunn/VCP-1 inoculated mice compared to those in Dunn/Dunn-c inoculated mice (Table I). Metastatic events were observed only in one mouse of the five mice inoculated with Dunn cells, and none of the five with Dunn-c, whereas metastasis was seen in all of the five mice inoculated with Dunn/VCP-1 ($P<0.01$). However, there were no differences in the size of the main tumors. These results indicate that Dunn/VCPs have greater metastatic potential *in vivo* compared to the original Dunn cells.

***VCP* expression in human osteosarcoma cell lines** *VCP* expression was analyzed by using three human osteosarcoma cell lines; HOS, Saos-2, and MG-63. HOS and Saos-2 cells showed similar *VCP* expression even in the confluent condition, while confluent MG-63 cells showed a reduced *VCP* expression level compared to that of sub-confluent cells (Fig. 8).

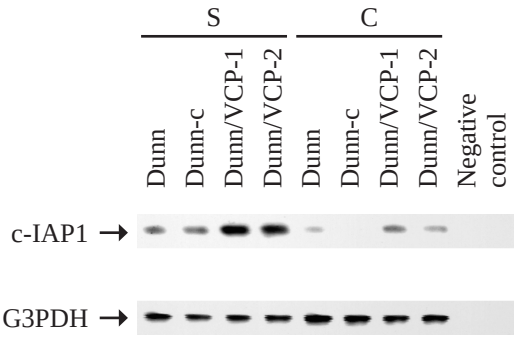


Fig. 6. RT-PCR analysis of *c-IAP1*, a gene induced by NFκB. After having been treated with TNFα for 30 min, the cells were analyzed for *c-IAP1* expression by RT-PCR. Dunn/Dunn-c cells showed reduced expression of *c-IAP1* in the confluent state, whereas Dunn/VCPs cells showed constant *c-IAP1* expression.

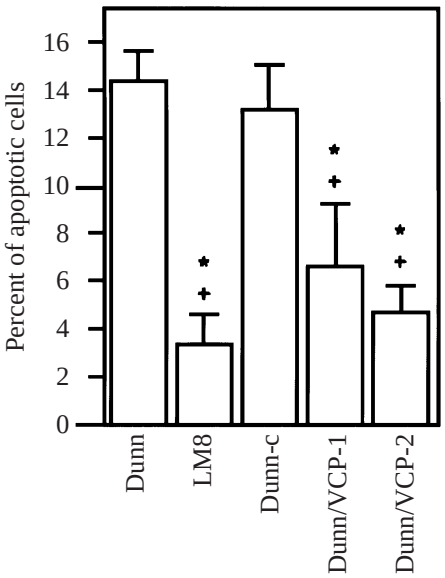


Fig. 7. TNFα-induced apoptosis assay. The cells were cultured to confluence. After TNFα treatment for 30 min at a concentration of 5 ng/ml, the cells were cultured in DMEM with 0.5% FBS for 12 h. The ratio of apoptotic cells was measured by trypan blue exclusion test. Values shown are means±SD for three experiments. * $P<0.01$ compared with Dunn cells; + $P<0.01$ compared with Dunn-c cells.

Table I. Lung Metastasis after Subcutaneous Inoculation

| Cell lines | Incidence of primary tumor | Metastasis | | |
|---|---|---|---|---|
| | | Incidence | No. of foci | Area of foci /lung (‰) |
| Dunn | 5/5 | 1/5 | 0.2±0.45[a)] | 0.68±1.53[b)] |
| Dunn-c | 5/5 | 0/5 | 0 | 0 |
| Dunn/VCP-1 | 5/5 | 5/5[*] | 4.0±2.34[**] | 4.30±2.62[**] |

Dunn, Dunn-c, Dunn/VCP-1 cells ($1\times10^7$ cells/mouse) were inoculated s.c. into the back space of male mouse aged 5 weeks ($n=5$).

Lung metastasis was determined 5 weeks after tumor inoculation as described in "Materials and Methods."

* $P<0.01$ compared to Dunn and Dunn-c by $\chi^2$ test.

** $P<0.01$ compared to Dunn and Dunn-c by Mann-Whitney's *U* test.

*a*, *b*) Mean±SD.

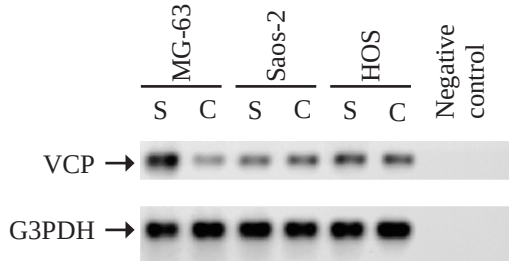


Fig. 8. *VCP* expression in human osteosarcoma cell lines. RT-PCR analysis of *VCP* in three human osteosarcoma cell lines; HOS and Saos-2 cells showed similar *VCP* expression in the confluent and sub-confluent states, whereas confluent MG-63 cells showed a reduced *VCP* expression compared to that of sub-confluent cells.

## DISCUSSION

By using SSH, we found that *VCP* gene expression was maintained in LM8 cells in the confluent condition, as well as in the sub-confluent condition. A stable VCP transfectant of the Dunn cells (Dunn/VCP-1) showed increased metastatic incidence compared to the original Dunn cells in *in vivo* metastasis assay. We also observed constant NFκB signal activation in Dunn/VCPs, which has an anti-apoptotic influence.[18)]

Apoptosis is a key event in several steps during metastasis.[28)] The steps involved in metastasis include 1) neo-vascularization at the primary site, 2) local invasion and intravasation, 3) transport and arrest at the target organ, 4) extravasation and migration, and 5) outgrowth at the metastatic sites.[6)] The role of apoptosis has been discussed in the first step, in which reduced oxygenation and overt necrosis are commonly observed.[6)] In addition, during the process of circulation and arrest at the secondary organ, massive loss of tumor cells has been demonstrated.[6)] Experimentally, less than 0.1% of cells injected into the circulation successfully form detectable lesions.[29)] Circulating cells are able to arrest in a wide variety of organs, but metastasis occurs only in a limited number of organs.[30, 31)] Most of the injected cells are capable of arrest and extravasation, but a major loss of metastatic cells occurs at the time of initial replication.[32)] Cells of high and low metastatic potential, together with non-malignant cells can similarly extravasate.[33, 34)] Instead, the survival and growth rates of the cells after the migration step are different according to the malignancy of the cells.[33, 34)] Furthermore, molecular analysis revealed that dissemination of tumor cells from the primary site is clinically a frequent event.[35, 36)]

NFκB activation is required to protect cells from the apoptotic cascade induced by TNF and other stimuli.[19, 20)] NFκB induces anti-apoptotic genes such as TNF receptor-associated factors (TRAFs), IAPs, and the Bcl-2 homolog A1/Bfl-1.[37–39)] In addition to the apoptotic-suppressing function, NFκB has been shown to regulate many genes involved in oncogenesis and metastasis, cell growth-promoting genes such as cyclin D1, cell adhesion molecules such as ICAM-1, cell surface proteases such as MMP-9, and extracellular matrix proteins such as tenascin-C.[39)] NFκB signaling starts with phosphorylation of IκBs, and subsequently ubiquitination of IκBs enables the freed NFκB to translocate into the nucleus, where it promotes expression of the target genes.[18)] In the present study, reduced NFκB signal, together with increased p-IκBα protein in the cytoplasm, was observed upon TNFα stimulation of confluent Dunn/Dunn-c cells compared to those in sub-confluent cells, which indicates that the NFκB signaling was disturbed by p-IκBα. Reduced expression of VCP was suggested to be associated with the impairment of the degradation process of p-IκBα. On the contrary, in Dunn/VCPs cells constitutively active for VCP, no difference in NFκB activation or cytoplasmic p-IκBα protein level was observed between confluent and sub-confluent cells. Dai *et al.* showed that the level of VCP correlates with the proteolytic activity of IκBα by *in vitro* assay.[10)] Stable transfectants of mutant IκBα show reduced NFκB activation in a dose-dependent manner.[21)] Taken together with these previous observations, our results indicate that VCP modulates NFκB activation by influencing the degradation process of cytoplasmic p-IκBα. NFκB activation and anti-apoptotic function of VCP was further confirmed by *c-IAP1* RT-PCR analysis and apoptosis assay following TNFα stimulation.

In the present study, confluency-dependent down-regulation of *VCP* in Dunn cells was observed. Confluency-dependent proliferation arrest of cultured cells, i.e., contact-inhibition, is a widely accepted concept, although little is known about the underlying molecular mechanism.[40)] Based on mRNA *in situ* hybridization and immunohistochemical analysis, Muller *et al.* suggested distinct cell-to-cell heterogeneity and tissue-specific patterns of VCP expression.[41)] The nucleotide sequence of the 5′-flanking region of *VCP* contains consensus binding sites for several transcriptional activators, suggesting complex regulation of *VCP* expression.[40)] Enhanced expression of *VCP* in a metastatic variant of a murine melanoma cell line has been reported.[42)] These findings suggest that *VCP* expression is involved in metastatic potential of many tumor cell types. In the human osteosarcoma cell lines analyzed, two types in *VCP* expression pattern were observed, i.e., constant *VCP* expression in the sub-confluent or confluent condition, or decreased *VCP* expression in the confluent condition.

In conclusion, the findings of constant VCP expression in LM8 cells with higher metastatic potential and an increased metastatic potential in constitutively active VCP transfectant Dunn cells suggested that VCP expression plays an important role in the metastatic process. Anti-apoptotic potential in these cells owing to constant NFκB activation via efficient cytoplasmic p-IκBα-degrading activity may regulate the increased metastatic potential of these cells.

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