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Antitumor Effect of β2-Microglobulin in Leukemic Cell-bearing Mice via Apoptosis-inducing Activity: Activation of Caspase-3 and Nuclear Factor-κB[1]

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ABSTRACT

We have reported previously that β2-microglobulin (β2m) induces apoptosis in leukemic cells *in vitro*, and that an interaction between β2m and HLA class I antigen induces apoptosis. Here we examined whether β2m can induce apoptosis in leukemic cells *in vivo* and whether it has an antitumor effect in tumor-bearing mice. Daily administration of 50 or 250 μg of β2m induced apoptosis and an antitumor effect on K562 leukemia cell-bearing mice in the same manner as tumor necrosis factor-α. In tumor tissues in β2m-treated mice, both caspase-3 and nuclear factor-κB (NF-κB) were stained more strongly than in control mice by anti-caspase-3 and anti-NF-κB p65/Rel A polyclonal antibodies. We also observed the *in vivo* immunological effects of β2m on lymphoid and hematopoietic organs, such as thymus, bone marrow, Peyer's patches, liver, and spleen in normal mice. Using antibodies against caspase-3 and NF-κB, immunohistochemical staining showed that no specific tissues were damaged or stained in normal mice. We conclude that β2m stimulates caspase-3 and NF-κB pathways to induce apoptosis, making it a useful approach to a new therapy for leukemia.

INTRODUCTION

Apoptosis is a much-studied phenomenon in cancer therapy research (1). We have purified an apoptosis-inducing factor to homogeneity from a medium conditioned by phorbol-12,13-dibutyrate-treated HL-60 cells (2). NH_2-Terminal sequence analysis revealed that apoptosis-inducing factor is identical to β2m.[3] Activated vascular endothelial cells participate in apoptosis of leukemic cells by endothelial interleukin 8 (3). We also have purified another apoptosis-inducing factor from a medium conditioned by phorbol-12,13-dibutyrate-treated HL-60 cells (4). Apoptosis-inducing activity of β2m has been described *in vitro*, and a difference and association between β2m and HLA class I antigen have been discussed (4). We reported a new function of β2m *in vitro* in leukemic as well as lymphoma cells. The inhibitory effect of β2m on cell proliferation was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and its apoptotic activity was detected by TUNEL assay. Here we demonstrate that the antitumor effect of β2m on leukemic cell-bearing mice is regulated via caspase and NF-κB activities.

MATERIALS AND METHODS

Reagents. Recombinant human TNF-α was purchased from R&D Systems, Inc. (Minneapolis, MN). Native and recombinant human β2m was given by Eiken (Nogi, Tochigi, Japan). Both forms of β2m were homogeneous in SDS-PAGE analysis. Polyclonal antiserum for caspase-3 (IgG, clone H-277; Santa Cruz Biotechnology, Santa Cruz, CA), NF-κB p65 (Rel A; Rockland, Gilbertsville, PA), and Vectastain Universal Elite ABC kit were purchased from Funakoshi (Tokyo, Japan). Proteasome inhibitor LLnL was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in DMSO. Antihuman IκBα (rabbit) antibody was purchased from Rockland.

Cell Lines and Cell Culture. The human chronic myelogenous leukemia cell line K562 (5) was obtained from American Type Culture Collection (Rockville, MD) and was maintained in GIT medium (Wako, Tokyo, Japan) as reported in our previous studies (2–4). The human myelogenous leukemic cell line HL-60 was also obtained from American Type Culture Collection and was maintained in 10% heat-inactivated FCS-supplemented RPMI 1640 (Sigma Chemical Co.).

TUNEL Assay. Resected specimens were incubated with a digoxigenin-dUTP terminal deoxynucleotidyl transferase mixture and subsequently were stained with peroxidase-conjugated antibody to digoxigenin (Apop Tag PLUS; Oncor, MD; Ref. 6), counterstained with 1% methyl green in sodium acetate (pH 4.0), and mounted. Specimens were examined and photographed under a microscope. The percentage of apoptotic cells was determined by counting >200 cells. Statistical analysis was performed by Student's *t* test, as reported in our previous study (6).

Cell Cycle Analysis. K562 cells (5×10^5) were incubated with propidium iodide and were analyzed using a CellFIT program by FACScan (Becton Dickinson, Mountain View, CA), as reported in our previous study (7). We investigated the cell cycle at 12, 24, and 48 h after incubation with or without β2m. Moreover, β2m-induced apoptosis-resistant K562 cells, which were selected using the colony formation assay at the concentration of 1×10^5/ml, were stimulated with β2m (10 μg/ml) for 72 h in the presence or absence of LLnL (6.25 μmol/l; 1 h of pretreatment; Ref. 8).

***In Vivo* Experiments.** Male BALB/c *nu/nu* mice were purchased from Japan Charles River (Kanagawa, Japan) and were age-matched (5 weeks of age) at the onset of each experiment. Mice were inoculated with 2×10^5 viable K562 cells by s.c. injection in the midline ventral position in a total volume of 0.1 ml of PBS. Test mice bearing s.c. established K562 tumors (confirmed 4 days after inoculation) were treated daily (for 14 days) with β2m in a total volume of 0.1 ml saline. As controls, saline and TNF-α were injected. Tumor size was calculated using the formula described by Kyriazis *et al.* (8) as follows: tumor volume = width^2 × length × 0.4.

Tumors were resected *in toto*, fixed in 10% neutral formalin solution (Sigma Chemical Co.), embedded in paraffin, sectioned to a thickness of 4 μm, and stained with H&E or by the TUNEL method. The injected dose of β2m was 50 or 250 μg/mouse/day. The dose of TNF-α was 200 units/mouse. Statistical analysis was performed by Student's *t* test, as reported in our previous study (6).

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[3] The abbreviations used are: β2m, β2-microglobulin; HLA, human leukocyte antigen; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; LLnL, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal.

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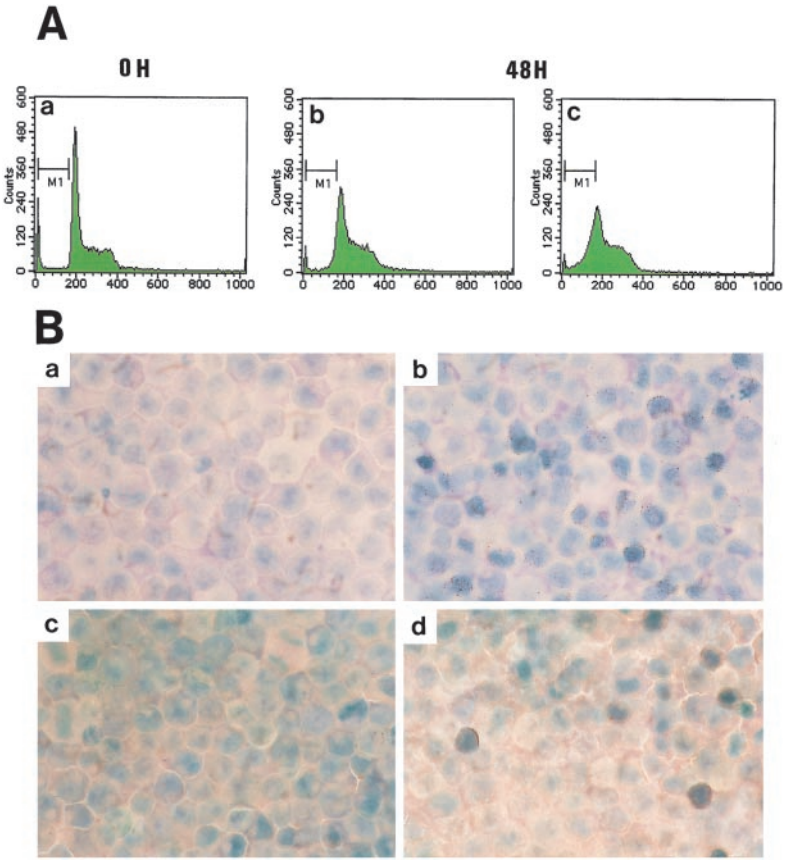

Fig. 1. *A*, cell cycle analysis was performed using a CellFIT program from FACScan (Becton Dickinson). K562 leukemic cells were with or without 10 μg/ml β2m for several hours. Each of the treated cells at 5×10^5 cells/ml was incubated with propidium iodide and analyzed. *a*, K562 cells at the starting point; *b*, K562 cells cultured without β2m for 48 h; *c*, β2m-treated K562 cells for 48 h. *B*, immunohistochemical staining of K562 cells was performed using anti-caspase-3 (*a*, control; *b*, β2m-treated) and anti-NF-κB (Rel; *c*, control; *d*, β2m-treated) polyclonal antibodies *in vitro* cultured for 48 h with β2m at the concentration of 10 μg/ml.

Immunohistochemistry of the Resected Tissues from Tumor-bearing Mice. Resected tissues from tumor-bearing mice were paraffin-embedded at the time of observation and were stored at 4°C. After thawing the paraffin, a first antibody or antiserum was added on the slide glass. After 0.5-h incubation, first antibody or antiserum was washed three times by PBS. The second biotinylated antibody was added and washed three times by PBS as directed by the manufacturer's manual.

RESULTS

Apoptosis in K562 Cells Was Induced through the Expression of Both Caspase-3 and NF-κB by β2m. We demonstrated at which phase of the cell β2m could act in K562 cells. After a 48-h incubation with β2m, sub-G_1 phase (M1) of the cells showed 23.8%, but control culture was 6.5% (Fig. 1*A*). Moreover, expression of both caspase-3 in cytoplasm and NF-κB in both cytoplasm and nuclei was detected in K562 cells incubated with β2m (Fig. 1*B*).

β2m Suppresses Tumor Growth and Induces Apoptosis of K562 Cells in Vivo. We investigated whether β2m could suppress cell growth or induce apoptosis in the case of s.c. implanted K562 cells. Ten μg/ml of β2m were injected daily from day 4 to day 11 into s.c. K562 cell tumors established in nude mice, and its antitumor and apoptosis-inducing effects were examined. The mice showed an obvious response to intratumor administration of β2m, with evident apoptosis. The β2m-treated tumor shrank to 77.3% of the size observed in the control (saline; Fig. 2). TNF-α treatment decreased the tumor size to 79.8% of that observed in the case of the control (Fig. 2).

Antitumor Effect of β2m Is Attributable to Induction of Apoptosis. To investigate whether the antitumor effect of β2m is attributable to induction of apoptosis, a histopathological examination was performed by H&E staining and TUNEL staining (Fig. 3). Histolog-

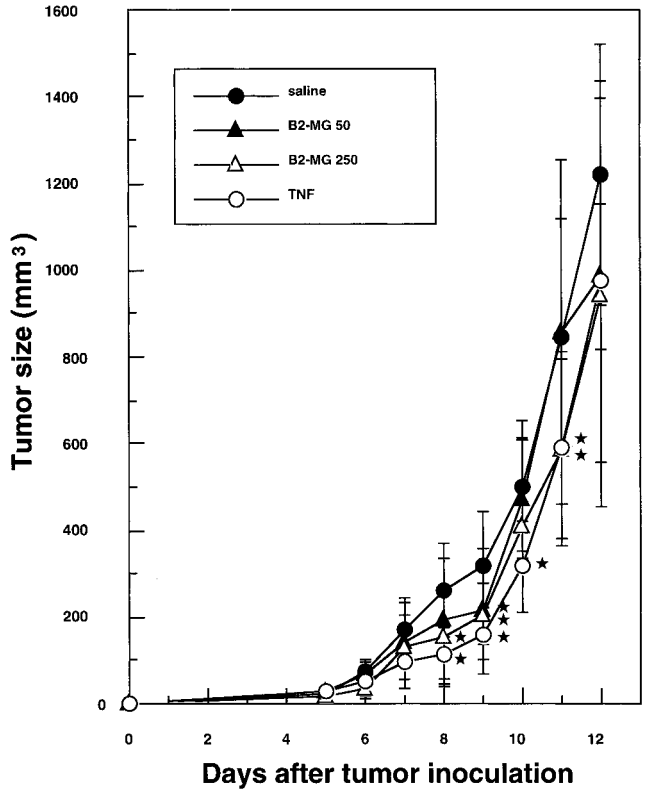

Fig. 2. Inhibition of cell growth by treatment with β2m in nude mice was demonstrated. Inhibition of the growth of K562 cells by β2m was shown. The β2m suppressed the growth of s.c. K562 cell tumors. The subepidermis of nude mice (10 examined/group) was inoculated with K562 cells, and either 50 or 250 μg of β2m, saline, or TNF-α was injected daily, as described in "Materials and Methods." As controls, saline and TNF-α were used. Data shown are from 10 nude mice. Data points are the means of three independent experiments; *bars*, SD. Statistical analysis was performed by Student's *t* test. ★, $P < 0.05$.

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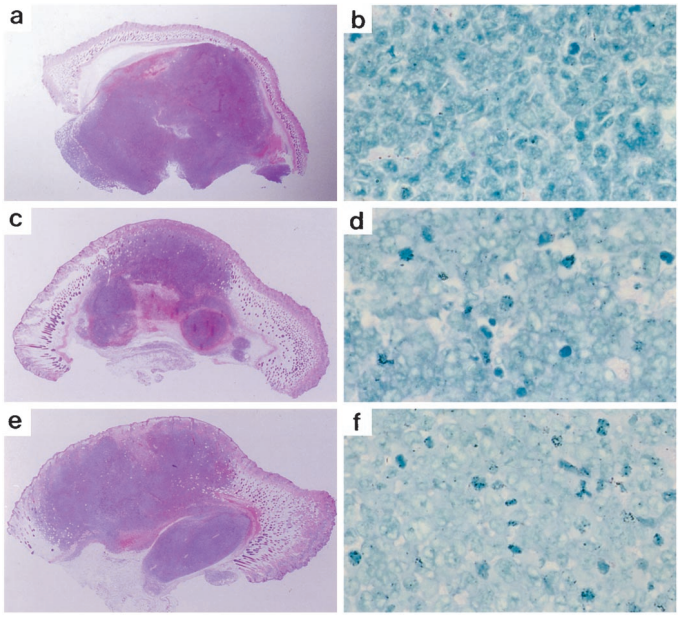

Fig. 3. Microscopic morphology of s.c. K562 tumors was shown. BALB/c *nu/nu* mice were injected s.c. with K562 cells and were subsequently injected with saline. *Left panels*, H&E staining. Apoptotic cells were detected by *in situ* staining with Apop Tag PLUS (Oncor), which gives a dark contrast, insoluble precipitate indicative of genomic fragmentation. *Right panels*, TUNEL staining. ×500. The percentage of apoptotic cells was determined microscopically by counting >200 cells on slides after *in situ* staining. *a* and *b*, saline; *c* and *d*, 250 μg of β2m; *e* and *f*, TNF-α.

ically, s.c. K562 tumors that responded to β2m (10 mice/group were examined) generally displayed homogeneous central necrosis (Fig. 3*b*). Within the viable tumor tissue, many tumor cells became smaller than control cells and showed either condensation or fragmentation of nuclei. Control K562 tumors (saline-treated groups) displayed little or no tumor necrosis and showed no change in cell size or nuclei (Fig. 3*a*). Tumor sections were stained by the TUNEL method, which is specific for apoptotic cells. The TUNEL assay showed that apoptosis was induced in 38.3 ± 0.9% of the K562 tumor cells in mice treated with β2m (Fig. 3*d*). However, control tumor cells showed little apoptosis (6.3 ± 0.9%; Fig. 3*b*).

Caspase-3 and NF-κB Expression in the Tumor Site by β2m-induced Apoptosis. Using antibodies specific for caspase-3, CD20, or NF-κB, protein expression of tumor sites and immunological tissues from normal mice injected with β2m was examined. In β2m-treated mice, tumor cells were positively stained with both anti-caspase-3 polyclonal antibody and anti-NF-κB polyclonal antibody (Fig. 4). No significant differences were observed in the immunological tissues of the normal mice, such as thymus, bone marrow, Peyer's patches, liver, and spleen (data not shown). NF-κB activation was accompanied by degradation of its cytosolic inhibitor IκB-α, which could be blocked by the proteasome inhibitor LLnL, as detected in Western blot (9). We confirmed that degradation of IκB-α in K562 cells can be blocked by 6.25 μM LLnL using Western blot (Fig. 5). We cultured K562 cells using colony formation assay and picked up 20 colonies. Furthermore, we selected one β2m-induced apoptosis-resistant clone among these colonies and examined the effect of LLnL on that clone. Apoptosis was more induced by the pretreatment of LLnL (15.94 ± 1.76%) than the pretreatment of 0.3% DMSO (7.12 ± 0.92%; $P < 0.05$).

DISCUSSION

In our previous studies, we purified two apoptosis-inducing factors derived from differentiated HL-60 cells (2, 4). These apoptosis-inducing factors were found to be identical to endothelial interleukin 8 and β2m. β2m is also an active protein in apoptosis. We measured the serum level of β2m in acute myelogenous leukemia and acute lymphocytic leukemia at the time of admission and after bone marrow transplantation. In some cases of acute myelogenous leukemia, serum levels of β2m were higher than after transplantation (data not shown). These data suggested that serum levels of β2m are partially related to the activity of leukemia. β2m interacts with HLA class I heavy chains and transduces an apoptotic signal into the caspase pathway (data not shown), which is distinct from the Fas and TNF pathway. *In vitro* studies showed the activation of caspase-3 pathway (Fig. 1), and *in vivo* studies showed that the NF-κB pathway was also stimulated by the administration of β2m (Fig. 4). In our *in vivo* experiments, β2m

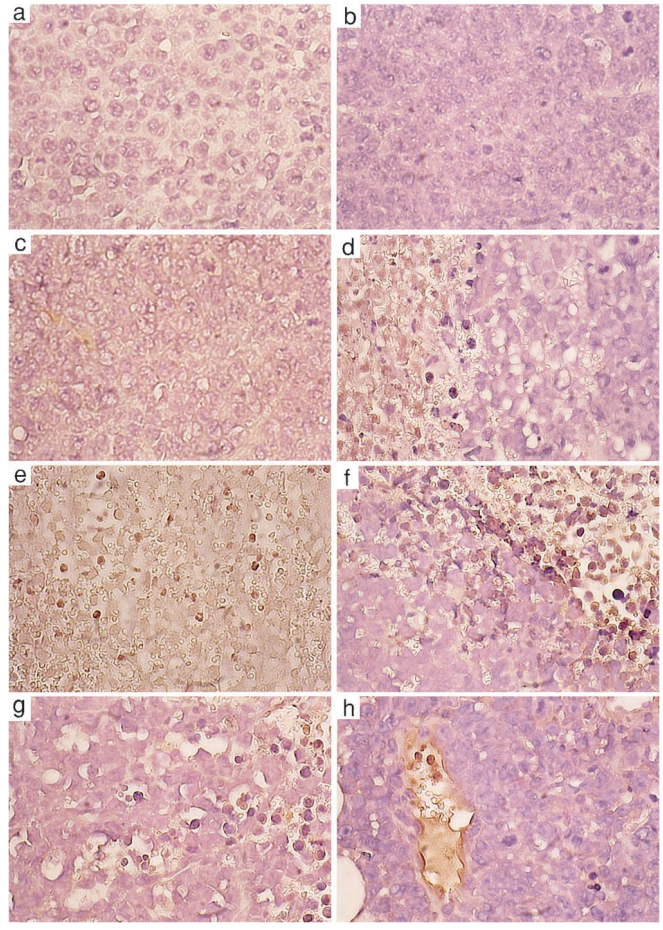

Fig. 4. Immunohistochemical staining of K562 cells from the tumor tissues of mice was performed using anti-caspase-3 (*left panels*) and anti-NF-κB (Rel; *right panels*) polyclonal antibodies. *a* and *b*, saline; *c* and *d*, 50 μg of β2m; *e* and *f*, 250 μg of β2m; *g* and *h*, TNF-α. ×500.

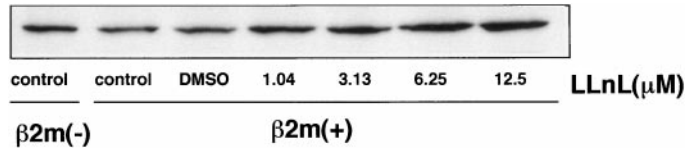

Fig. 5. Degradation of IκB-α in K562 cells can be blocked by 6.25 μM LLnL using Western blot. K562 cells were incubated with 20 μg/ml of recombinant human β2m for 24 h in the presence or absence of the preincubation with proteasome inhibitor LLnL at several concentrations for 1 h. Twenty-five μg of protein extracts were separated by 12% SDS-PAGE, transferred to polyvinylidine difluoride membranes, and subsequently hybridized with an anti-IκBα antibody. *control*, incubated with PBS.

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inhibited the growth of K562 cell tumors in the same manner as TNF-α.

The *β2m* gene knock-out mice showed increased susceptibility both to viral infection and to tumor formation. β2m-deficient mice showed defective antibody responses because of increased IgG catabolism (10). In β2m-deficient mice, perforin-dependent cytolytic responses have been found to be preserved (11, 12). Data suggesting the importance of β2m in tumor immunity and progression in melanoma and natural killer cell tolerance have been reported (13). In β2m-deficient mice, natural killer cells have shown increased sensitivity to MHC class I heavy chain-mediated inhibition (14). β2m-deficient mice are reported to be susceptible to lymphocytic choriomeningitis virus because of dysfunction of CTLs (15). Others have reported that β2m deficiency ameliorates a TGF-β1-deficient state (16). IFN-γ stimulated gene expression of β2m in cells of hepatic, monocytic, and T-lymphocytic origins (17). MHC class I assembly with β2m has been shown to be important in regulating tumor and viral immunity (18).

Caspase-3 is the primary regulator of DNA fragmentation in apoptosis (19). We have reported that the inhibition of caspase-3 or caspase-1 suppressed apoptosis that was induced by β2m in K562 cells, and that β2m-induced apoptosis was dependent on caspase cascade (4). NF-κB is also involved in interleukin-induced activation of T lymphocytes and endothelial cells (20, 21). The transcriptional factor NF-κB plays an important role in inflammatory and immune responses, but the question was raised as to whether its effect is antiapoptotic or proapoptotic, which is controversial (22, 23). NF-κB is composed of a heterodimer of p65 and p50 subunits. Its inhibitor proteins, the IκBs, are composed of two closely related kinases. These kinases phosphorylate the NF-κB p65 subunit in the transactivation domain (24). On the basis of these findings, we chose a p65 subunit to take a look at the activation of NF-κB in our system. In TNF-α-related apoptosis, both NF-κB and c-Jun NH_2-terminal kinase activation are involved (25). NF-κB is also activated by chemotherapy and by irradiation (26). Inhibition or activation of NF-κB upon tumor resistance to apoptosis induced by β2m should be addressed. Our immunohistochemical experiments showed that NFκB p65 proteins are overexpressed in cytoplasm and nuclei of cells treated with β2m, and moreover, inhibition of NFκB activation by the proteasome inhibitor LLnL increased apoptosis in β2m-induced apoptosis-resistant K562 cells. These results suggest that NFκB may act as an antiapoptotic agent in β2m-induced apoptosis. Although little is known concerning which is its ligand on tumor cells, we expect it may be a novel M_r 150,000 β2m-binding protein complex (4).

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