marrow, namely in the field of haematopoiesis in *de novo* AML and MDS.

Angiopoietin-2 can act as an anti-angiogenic factor by antagonising Ang-1 or, conversely, act under the influence of VEGF in a pro-angiogenic manner (Tait & Jones, 2004). The expression pattern of Ang-2 in MDS, MDS → OL, *de novo* AML and controls was quite similar to that of Ang-1. Their expression highly correlated with each other and the angiopoietin receptor TIE2, suggesting the presence of an autocrine circuit influencing angiogenesis. Furthermore, the correlation between the TIE2 and MVD confirmed the important role of angiopoietins in angiogenesis. Angiopoietin expressions also correlated with all the other angiogenic factors investigated, except TGFβ, indicating a close relationship amongst angiogenic factors, where expression of one influences another. Ang-1, Ang-2 and TIE2 were highly correlated with bFGF, a factor known to play a prominent role in angiogenesis (Bertolini *et al*, 2000) and one of the most commonly produced factors in tumours (Padro *et al*, 2000).

Interestingly, the expression of TGFβ was in contrast to the trend displayed by the other factors in this study, having increased expression in MDS → OL compared with MDS ($P < 0{\cdot}01$). TGFβ exerts bifunctional effects on endothelial cells *in vitro*; it can both stimulate and inhibit the proliferation of endothelial cells (Bertolino *et al*, 2006). Low doses of TGFβ stimulate endothelial proliferation, while high doses of TGFβ inhibit it. Recent studies have shown that TGFβ can regulate vascular homeostasis by balancing the signalling between two distinct TGFβ type I receptors [the endothelial-restricted activin receptor-like kinase (ALK) 1 and the broadly expressed ALK5 receptors] (Bertolino *et al*, 2006). The activation/regulation of these receptors has been shown to induce opposite effects on endothelial cell behaviour and angiogenesis. Angiogenesis can be roughly divided into an activation phase and a resolution phase. During the resolution phase, smooth muscle cells will be recruited to cover the new vascular tube and to inhibit the proliferation and migration of the endothelial cells. TGFβ would act as an inhibitory factor of endothelial proliferation directly or indirectly through the suppression of pro-angiogenic factors. Thus, the reduction of angiogenic activity/MVD in the bone marrow of MDS → OL might be attributable to the high expression of TGFβ.

In the present study, seven angiogenic factors and two receptors were studied and shown to have cooperative dynamics of expression for regulating angiogenesis in the bone marrow. However, numerous other angiogenic factors are known to be significant. When therapeutic implications are considered, the multiple proteins involved and their influence upon each other would complicate the effects of anti-angiogenic therapy. In addition, angiogenic factors often have multiple roles and are not specific surrogate markers of angiogenesis. VEGF, Ang-1 and Ang-2 act specifically upon endothelium, however, bFGF, HGF and TNFα are pleiotropic, targeting numerous cell types. As well as angiogenesis, TNFα contributes to apoptosis (Kitagawa *et al*, 1997; Sawanobori *et al*, 2003; Stifter *et al*, 2005) and bFGF is mitogenic for fibroblasts. Although angiogenesis is part of the pathogenic process in malignancy, it is also required physiologically (Bertolini *et al*, 2000). Therefore, although knowledge in this field is growing, anti-angiogenic therapy is not an easy cure, but does offer promise, as anti-angiogenic drugs target both the microenvironment and malignant cells directly (Aguayo *et al*, 2003), blocking angiogenic factors secreted by leukaemic and other cells, and breaking the autocrine and paracrine circuits which facilitate malignant growth. On the other hand, the promotion of angiogenesis in MDS → OL might even facilitate the delivery of anti-leukaemic drugs to the bone marrow environment. Further studies should determine whether anti-angiogenic therapy has differing efficacy between *de novo* AML and AML secondary to MDS, to clarify the role and importance of angiogenesis in these haematological malignancies.

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Rapid induction of IAP family proteins and Smac/DIABLO expression after proapoptotic stimulation with doxorubicin in RPMI 8226 multiple myeloma cells

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Abstract

We studied the expression dynamics of inhibitor of apoptosis protein (IAP) family members and Smac/DIABLO after *treatment* with doxorubicin in human multiple myeloma cell line RPMI 8226 and its doxorubicin-resistant variant DRR. Proapoptotic stimulation with doxorubicin rapidly induced the overexpression of mRNA as well as protein for IAPs in RPMI 8226 cells followed by a gradual decrease of their expression. Smac/DIABLO, which is known to neutralize IAPs, showed increased expression at the mRNA level after treatment; however, Western blot analysis revealed a slight decrease of the amount of protein. Immunoprecipitation analysis revealed the association of Smac/DIABLO with cIAP1 or XIAP after treatment with doxorubicin. In contrast to the RPMI 8226 cells, DRR cells did not undergo apoptosis in response to doxorubicin treatment. The DRR cells had higher levels of IAPs expression at the mRNA level and did not show a remarkable peak or decrease in the expression of mRNAs for cIAP1, cIAP2, XIAP, and survivin after treatment with doxorubicin. Furthermore, the expression of Smac/DIABLO mRNA was not up-regulated after treatment. These findings indicate that the suppression of IAPs expression by Smac/DIABLO shortly after proapoptotic stimulation might play a role in the mechanisms of apoptotic induction, and that the maintenance of high IAPs expression and low Smac/DIABLO expression after treatment might lead to the doxorubicin-resistance of multiple myeloma cells.

Keywords: Apoptosis; Drug resistance; IAP; Smac/DIABLO; Multiple myeloma

Introduction

Inhibitor of apoptosis proteins (IAPs) were first identified in baculoviruses. All IAPs, including those from viruses as well as their cellular homologues in invertebrates and vertebrates, contain 1 to 3 baculovirus IAP repeat (BIR) motifs (Deveraux and Reed, 1999; Miller, 1999). In humans, eight kinds of IAPs, survivin, cIAP1, cIAP2, XIAP, NAIP, livin, apollon and ILP-2, have been identified (Abe et al., 2005; Yamamoto et al., 2004). The BIR domains of IAPs allow them to bind to and inhibit the proteases, caspases, that modulate the apoptotic destruction of cells. Although the exact biochemical mechanism by which these proteins suppress apoptosis is under debate, survivin, for example, is known to directly bind to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades (Shin et al., 2001; Tamm et al., 1998). Survivin is widely expressed in fetal tissues, but its expression becomes restricted during development, and it is negligibly expressed in the majority of terminally differentiated adult tissues (Adida et al., 1998; Ambrosini et al., 1997). However, analysis of the differences in gene expression between normal cells and tumor cells has revealed that survivin is one of the genes that is most consistently overexpressed in tumor cells relative to normal tissues (Velculescu et al., 1999). In fact, survivin is prominently expressed in transformed cell lines and in many human cancers

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including hematopoietic cell tumors (Altieri and Marchisio, 1999).

The expression dynamics as well as the functional protein amount dynamics of IAP family proteins after stimulation for apoptosis-induction would have critical significance in regulating the apoptotic pathways of cells. Regarding the degradation of IAP family proteins, the ubiquitylation process has been a focus of attention recently. Another zinc-binding motif of IAPs, the RING domain, binds E2 ubiquitin-conjugated enzymes (UBCs). This enables RING-domain-containing proteins to recruit an E2 and catalyse the transfer of ubiquitin from the E2 to a substrate (Vaux and Slike, 2005). Such ubiquitylation might target IAPs or other IAP-interacting proteins for degradation, or might specifically change their activity. Thus, the degradation of not only IAPs themselves but also the associated proteins would be followed by complicated outcomes that should be controlled by much more complicated mechanisms.

Smac/DIABLO is also an important molecule that regulates the function of IAPs. The Smac/DIABLO protein resides in the mitochondria of healthy cells, and is released upon apoptotic stress with similar kinetics to cytochrome *c* (Du et al., 2000; Verhagen et al., 2000). Although the mechanism of Smac/DIABLO release has not been entirely resolved, this protein has been demonstrated to bind all of the IAPs tested to date (Liston et al., 2003). Smac/DIABLO can bind to the BIR domain of IAPs, thereby interfering with either caspase-3/-7 or caspase-9 inhibition.

Several chemotherapeutic drugs are known to down-regulate IAP family protein and mRNA expression and to cause caspase activation and apoptosis in human cancer cells (Tyagi et al., 2003; Wittmann et al., 2003). However, many types of cancer cells do resist chemotherapeutic induction of apoptosis in clinical situations as well as under various in vitro conditions. Thus, the effects of apoptosis-inducing drugs on the actual induction of apoptosis and the expression dynamics of various apoptosis-associated molecules in the apoptosic signaling pathways are complicated and still controversial. In the present study, we determined the expression dynamics of IAPs and Smac/DIABLO from the very early period after treatment with doxorubicin. The expression of IAPs exhibited up-regulation just after treatment with doxorubicin, followed by a gradual decrease in association with over-expression of Smac/DIABLO. Mechanisms regulating the up- and down-regulation of the expression of IAPs should provide clues to explaining the chemotherapy-resistant nature of cancer cells and to developing novel strategies to down-regulate anti-apoptotic molecules in human cancers. The implications of these findings regarding the drug resistance of cancer cells and their clinical significance are discussed.

Materials and methods

Cell lines

The establishment and characterization of the human multiple myeloma (MM) cell line RPMI 8226 was previously described (Dalton et al., 1986). The cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and routinely maintained in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Daiichi Seiyaku, Tokyo, Japan), 1% (v/v) penicillin at 100 units/ml (Invitrogen, Carlsbad, CA), and 1% (v/v) streptomycin at 100 units/ml (Invitrogen). We also generated the doxorubicin resistant variant of RPMI 8226 cell line, designated DRR, according to a previously described method (Dalton et al., 1986).

Induction of apoptosis by chemical agents

Doxorubicin hydrochloride (Wako, Tokyo, Japan) was used for inducing apoptosis in RPMI 8226 as well as DRR culture cells. Cells were treated with doxorubicin at the concentrations of 2, 5, and 10 μM in the culture medium described above.

Identification of apoptotic cells

To identify apoptotic cells by terminal deoxytransferase (TdT)-mediated dUTP nick end labeling (TUNEL), an *in situ* cell death detection kit, fluorescein (Boeringer Mannheim, Mannheim, Germany) was used as described previously (Kitagawa et al., 1998). Briefly, cells were collected before treatment and 0, 5, 1, 2, 3, and 6 h after treatment with doxorubicin, fixed with 4% paraformaldehyde for 20 min, washed with PBS and treated with 0.1% sodium citrate–0.1% Triton X-100 (Sigma) for 2 min. After washing with PBS, cells were mixed with FITC-dUTP and TdT at 37 °C for 60 min. Then, the TUNEL-positive cells were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Preparation of RNA and quantitative assay for mRNA expression of IAP family proteins and Smac/DIABLO using TaqMan RT-PCR

RNA was extracted from RPMI 8226 culture cells, non-treated (NT) cells and doxorubicin-treated (2, 5, and 10 μM) cells at 0.5, 1, 2, 3, and 6 h after treatment using Trizol (Invitrogen) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and the TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides used as specific primers and TaqMan probes for the IAP family proteins, Smac/DIABLO and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes were as follows. The sequence for the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that for the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; for the TaqMan probe it was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, the sequence for the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and that for the reverse primer it was 5′-CAAGCCACCATCACAACAAAA-3′; for the TaqMan probe it was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′ For cIAP2 mRNA, the sequences of the forward and reverse primer were 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and 5′-TCTCCTGGGCTGTCTGATGTG-3′; respectively, and the sequence for the TaqMan probe was 5′-CCCTCATC-TACTTGAACAGCTGCTAT-3′. The forward and reverse sequences for NAIP mRNA were 5′-GCTTCACAGCGCATCGAA-3′ and 5′-GCTGGGCGGATGCT-TTC-3′; respectively, while the sequence for the TaqMan probe was 5′-CCATTTA-AACCACAGCAGAGGCTTTAT-3′. The sequence of the forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and that for the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the sequence for the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. The sequence of the forward primer for Livin mRNA was 5′-TCTTCCACACAGGCCATCAG-3′ and that for the reverse primer was 5′-GTCCCCGCGCTTCCA-3′; the sequence for the TaqMan probe was 5′-ACAAGGTGAGGTGCTTCTTCTGCTAT-3′. The sequence of the forward primer for apollon mRNA was 5′-GCCGA-AGGATAGCGATCAG-3′ and that for the reverse primer was 5′-GCCCGGAA-GACGAAGAAA-3′; the sequence for the TaqMan probe was 5′-GTTGCGGCTCAACCTCCACCTATC-3′. The sequence of the forward primer for ILP-2 mRNA was 5′-CAGACTTCATTGCCGAGAGAAATCA-3′ and that for the reverse primer was 5′-CAGATTTTACAAAGCTTCTCCTCTTG-3′; the sequence for the TaqMan probe was 5′-CCCTGAAGAGCCGCTAAGGCGTCT-3′. The sequence of the forward primer for Smac/DIABLO mRNA was 5′-

GCTGGAAACCACTTGGATGAC-3′ and that for the reverse primer was 5′-TGCATATCAAACTGGCGCA-3′; the sequence for the TaqMan probe was 5′-CAGTTGGTCTTTCAGAGATGGCAGCAGA-3′. Finally, the forward primer sequence for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and that for the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; the TaqMan probe sequence was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 2 min at 50 °C (Stage 1, reverse transcription), 10 min at 95 °C (Stage 2, RT inactivation and AmpliTaq Gold activation) and then 45 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C (Stage 3, PCR). Data on the quantity of RNA (ng) for the IAPs and Smac/DIABLO were normalized using the data for GAPDH in each sample and quantitated according to a method described elsewhere (Yamamoto et al., 2004).

Western blot analysis for IAP family proteins and Smac/DIABLO and immunoprecipitaion

RPMI 8226 cells from each experimental group were suspended in RPMI 1640 medium containing 10% fetal bovine serum at a concentration of 6×10^6 cells/tube and pelleted. Cell lysates were prepared by incubating the pellets on ice for 15 min in 1 ml of a lysis buffer containing 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.02% NaN_3, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1% aprotinin 100 μM leupeptin, and 100 μM tosyl-L-phenylalanyl chloromethyl ketone (TPCK) (Sigma). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The whole cell lysate (50 μg) was subjected to 12.5% SDS-PAGE. Gels were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schull, Dassel, Germany). The membranes were blocked in 10% skim milk in PBS, incubated with a rabbit polyclonal anti-cIAP1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Smac/DIABLO (Santa Cruz Biotechnology) or a mouse monoclonal antibody to XIAP (R&D Systems, Minneapolis, MN), and after being washed were incubated with a horseradish peroxidase-conjugated anti-goat or anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark). To confirm the equivalent loading of protein in each lane, membranes were also incubated in polyclonal rabbit anti-actin antisera (Sigma Chemicals). Bands in the washed membranes were detected with an enhanced chemiluminescence (ECL) system (Amersham Life Science, Buckinghamshire, England) as described previously (Kitagawa et al., 1996, 2002).

For immunoprecipitation experiments, cell lysates which contained 100 μg of protein were incubated with antibody against Smac/DIABLO and protein A-Sepharose beads (Amersham Life Science, Buckinghamshire, England). The resulting immunoprecipitates (50–100 μg of protein) were analyzed for cIAP1 or XIAP as described above.

The densities of bands were measured by densitometric analysis with an ImageQuant scanning imager (Molecular Dynamics, Sunnyvale, CA). The relative intensities of the bands were calculated by comparing the density of the sample with that of the control.

Results

Induction of apoptosis in RPMI 8226 cells and DRR cells by doxorubicin

To detect the actual induction of apoptosis in RPMI 8226 cells by doxorubicin at the concentration of 2, 5 and 10 μM, TUNEL-positive cell ratios were determined at various times after treatment (non-treated (NT), 0.5, 1, 2, 3, and 6 h). As shown in Fig. 1A, the ratio was increased in a dose-dependent manner at each time point. The ratio showed a gradual increase when cells were treated with 5 and 10 μM of doxorubicin, while the ratio was rather stable until 3 h after treatment with 2 μM doxorubicin. In contrast, DRR cells showed only a slight increase of TUNEL-positive cell ratio even after treatment with 10 μM doxorubicin (Fig. 1B).

Expression of mRNA for IAP family proteins determined by real-time quantitative PCR in RPMI 8226 cells and DRR cells after treatment with doxorubicin

To quantitate the mRNA expression levels of IAP family proteins in RPMI 8226 cell line cells and doxorubicin-resistant DRR cells, real-time quantitative RT-PCR was performed using samples treated with 2, 5, and 10 μM of doxorubicin or non-treated cells (NT), 1, 2, 3, and 6 h after treatment. Three samples at each point were examined for IAPs expression. For simplicity, error bars were not shown in the figures. As shown in Fig. 2A, most of the IAPs exhibited a peak of expression at 1 or 2 h after treatment with 2 or 5 μM doxorubicin in RPMI 8226 cells. The expression gradually decreased thereafter by 6 h. The levels of the induction of overexpression of cIAP1, cIAP2, survivin and apollon were higher,

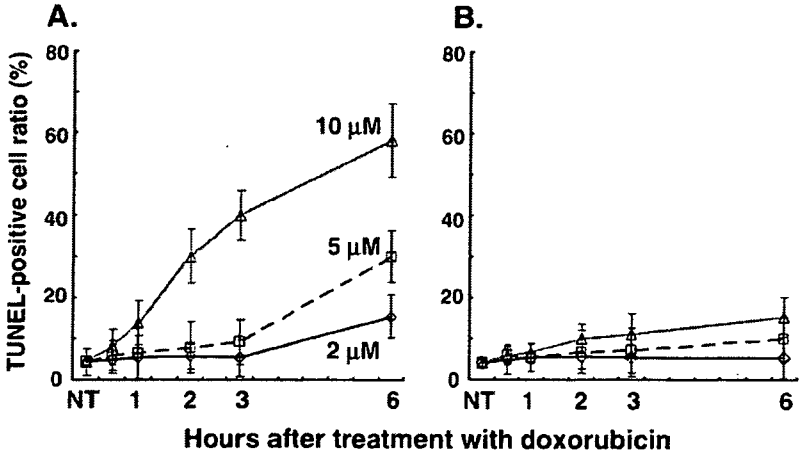

Fig. 1. Apoptotic cell ratio after treatment with doxorubicin determined by TUNEL method in doxorubicin-sensitive RPMI 8226 cells (A) and -resistant DRR cells (B). The solid line (◇—◇) indicates the ratio cells were treated with 2 μM doxorubicin, the dashed line, 5 μM doxorubicin (□--□) and the dotted line, 10 μM doxorubicin (△.....△). Error bars indicate standard deviation of the data in three samples at each point. Note that the apoptosis was induced in a dose-dependent and basically in a time-dependent manner in RPMI 8226 cells. By contrast, the apoptotic cell ratio was much lower in DRR cells.

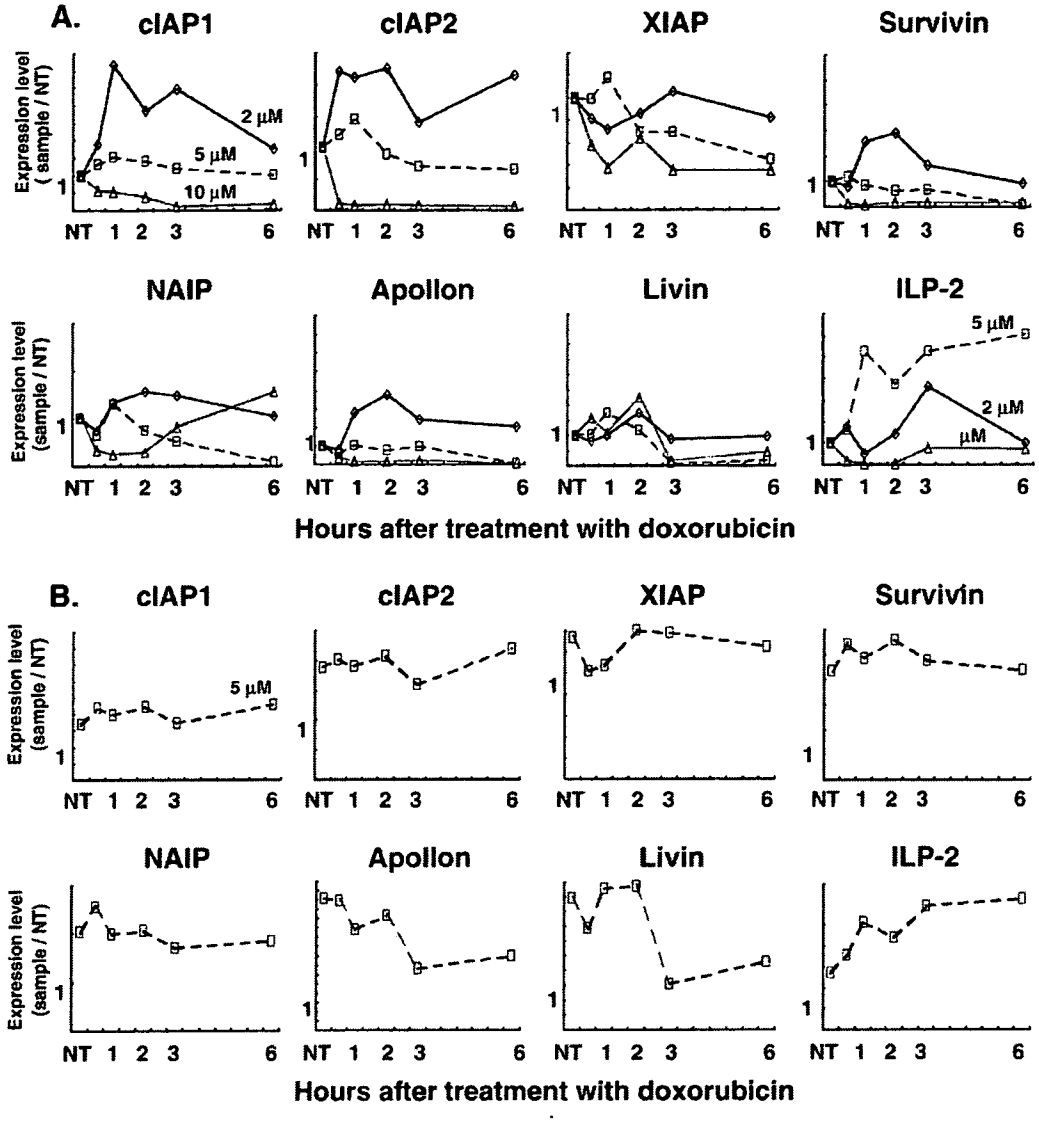

Fig. 2. Expression dynamics of mRNA of IAP family proteins in RPMI 8226 (A) and DRR cells (B) determined by the quantitative RT-PCR analysis. The expression levels of IAPs were determined in non-treated (NT) samples, and samples at, 0.5, 1, 2, 3, and 6 h after treatment with doxorubicin. The values are indicated as the ratio [IAP expression of RPMI 8226 cells or DRR cells after treatment (samples)/IAP expression of non-treated (NT) RPMI 8226 cells]. The solid line (◇—◇) indicates the value when cells were treated with 2 μM doxorubicin, the dashed line, 5 μM doxorubicin (□--□) and the dotted line, 10 μM doxorubicin (△.....△). The data were obtained from three samples at each point but for simplicity, error bars were not shown in the figures. Note that most IAPs showed increased expression forming a peak at 1 to 3 h after treatment with doxorubicin in RPMI 8226 cells. The expression gradually decreased thereafter. The DRR cells showed higher expression of IAPs than RPMI 8226 cells before treatment (NT) and the high levels of expression continued thereafter.

namely, the transient peaks were more prominent, in RPMI 8226 cells treated with 2 μM doxorubicin than in those treated with 5 μM. By contrast, RPMI 8226 cells treated with 10 μM doxorubicin did not show prominent peaks for the expression of IAPs but instead showed a gradual decrease from 1 h after treatment.

The levels of mRNA expression for IAPs were higher in DRR cells than RPMI 8226 cells before treatment with doxorubicin (non-treated samples: NT). The levels of expression in DRR cells were about 3 to 4 fold higher than those in RPMI 8226 cells. In contrast to the expression dynamics of IAPs in RPMI 8226 cells after treatment with doxorubicin, DRR cells exhibited minimal changes in expression of cIAP1, cIAP2, XIAP, survivin, NAIP and ILP-2 and continues to express high levels of the mRNAs for these IAPs at 6 h after treatment with doxorubicin (5 μM) (Fig. 2B). However, the expression of NAIP showed a small peak of expression at 0.5 h and the expression of apollon and livin was reduced by 3 and 6 h after treatment with doxorubicin. Although the expression levels of ILP-2 were similar in RPMI 8226 and DRR cells (5 μM doxorubicin), other IAPs exhibited much higher expression in DRR cells than in RPMI 8226 cells throughout the observation period.

Expression of mRNA for Smac/DIABLO determined by real-time quantitative PCR after treatment with doxorubicin

Next, to quantitate the mRNA expression of Smac/DIABLO in RPMI 8226 cells, real-time quantitative RT-PCR was performed

using the same samples as described above. As shown in Fig. 3A, Smac/DIABLO exhibited transient overexpression at 1 to 2 h after treatment with 2 or 5 μM doxorubicin in RPMI 8226 cells. Similar to the changes in IAPs, the expression levels of Smac/DIABLO were higher in cells treated with 2 μM doxorubicin than in those treated with 5 μM doxorubicin. The expression gradually decreased thereafter with 5 μM treatment, but was maintained at a high level until 6 h with 2 μM treatment. By contrast, treatment with 10 μM doxorubicin did not induce any changes of expression of mRNA for Smac/DIABLO in RPMI 8226 cells.

In DRR cells, the expression of Smac/DIBLO was almost twice as high as that in RPMI 8226 cells (non-treated samples: NT). In contrast to the remarkable up-regulation of the mRNA expression of Smac/DIABLO in RPMI 8226 cells, the DRR cells did not show a remarkable change of the expression of Smac/DIABLO after treatment even with 2 μM doxorubicin (Fig. 3B).

Expression of IAP family proteins and Smac/DIABLO determined by Western blotting after treatment with doxorubicin

To determine the dynamics of protein expression of IAPs and Smac/DIABLO after treatment with doxorubicin, Western blot analyses were performed using cell lysate from RPMI 8226 cell samples treated with 2, 5, and 10 μM of doxorubicin and NT, 0.5, 1, 2, 3, and 6 h after treatment. As shown in Fig. 4, cIAP1 and XIAP exhibited the elevated expression at 1 to 3 h after treatment with 2 μM doxorubicin. However, treatment with 5 μM doxorubicin induced overexpression at 0.5 to 2 h, and treatment with 10 μM doxorubicin induced overexpression at 0.5 h after treatment. The expression gradually decreased thereafter. By contrast, the expression of Smac/DIABLO showed a gradual decrease from 0.5 h after treatment with 2, 5, and 10 μM doxorubicin.

Interactions of IAPs with Smac/DIABLO in response to doxorubicin-treatment

To test whether IAPs actually interact with Smac/DIABLO in response to doxorubicin-treatment, lysates from RPMI 8226 cells treated with doxorubicin were immunoprecipitated with anti-Smac/DIABLO antibody and then the precipitates were immunoblotted with antibody against cIAP1 or XIAP. As shown in Fig. 5, co-precipitation of Smac/DIABLO and cIAP1 as well as Smac/DIABLO and XIAP was observed in the samples 0.5 to 2 h after treatment with doxorubicin. Taken together with the data from Figs. 3 and 4, these findings show that the expression of Smac/DIABLO was induced at the mRNA level after treatment with doxorubicin, and then the produced protein was recruited and bound to IAPs to form a IAP-Smac/DIABLO complex resulting in the rapid disappearance at the protein level.

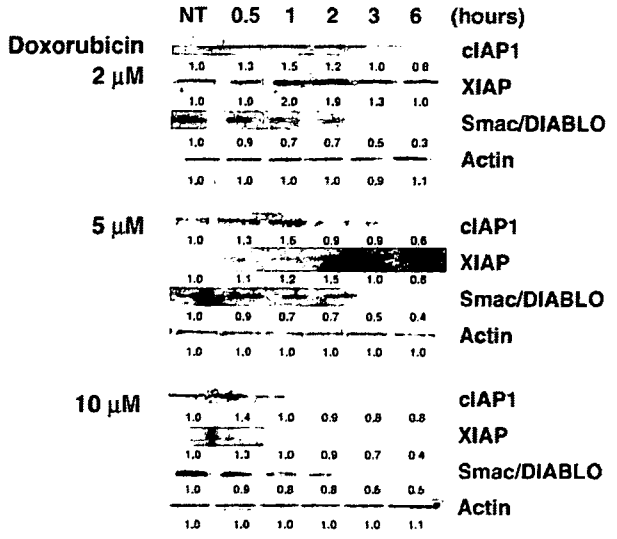

Fig. 4. Immunoblotting for cIAP1, XIAP, Smac/DIABLO in RPMI 8226 cells. Cell lysate (50 μg) from non-treated (NT) samples, and samples at, 0.5, 1, 2, 3, and 6 h after treatment with doxorubicin was used for this assay. Actin protein levels of each sample are shown to confirm that the amounts of samples loaded were almost equal. The relative intensities of bands were measured by densitometry (NT in each protein as the control, 1.0) and indicated under the photos of the gels. Note the transient increase of cIAP1 and XIAP expression in doxorubicin-treated RPMI 8226 cells in contrast to the gradual decrease of Smac/DIABLO expression after treatment with doxorubicin.

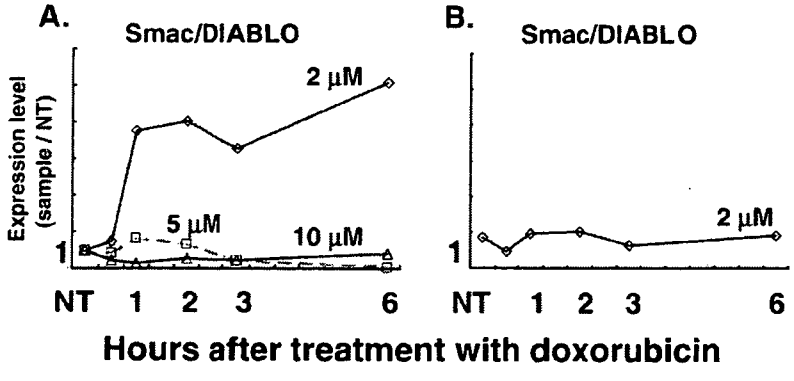

Fig. 3. Expression dynamics of mRNA of Smac/DIABLO in RPMI 8226 (A) and DRR cells (B) determined by the quantitative RT-PCR analysis. The expression levels of Smac/DIABLO were determined in non-treated (NT) samples, and samples at, 0.5, 1, 2, 3, and 6 h after treatment with doxorubicin. The values are indicated as the ratio [IAP expression of RPMI 8226 cells or DRR cells after treatment (samples)/IAP expression of non-treated (NT) RPMI 8226 cells]. The solid line (◇—◇) indicates the value when cells were treated with 2 μM doxorubicin, the dashed line, 5 μM doxorubicin (□--□) and the dotted line, 10 μM doxorubicin (△.....△). The data were obtained from three samples at each point but for simplicity, error bars were not shown in the figures. Note the increased expression of Smac/DIABLO in RPMI-8226 cells after treatment with 2 μM doxorubicin, while the expression did not show a remarkable increase in DRR cells.

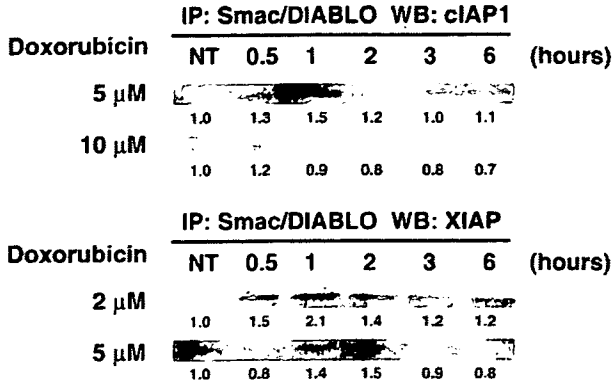

Fig. 5. Co-immunoprecipitation analysis for Smac/DIABLO and IAPs (cIAP1 or XIAP) in RPMI 8226 cells NT, 0.5, 1, 2, 3 and 6 h after treatment with doxorubicin (5 and 10 μM for cIAP1 and 2 and 5 μM for XIAP). Up-regulated association of Smac/DIABLO and cIAP1 was demonstrated 0.5 to 1 h after treatment with 2 or 5 μM doxorubicin and a significant association of Smac/DIABLO and XIAP was demonstrated 1 to 2 h after treatment with 2 or 5 μM doxorubicin. In RPMI cells treated with 10 μM doxorubicin, Smac/DIABLO and IAPs did not show the remarkable association.

This could explain why the protein analysis of Smac/DIABLO showed a gradual decrease after treatment with doxorubicin. These findings would also suggest that the decrease in the amount of IAP proteins might be related to the interaction between IAPs and Smac/DIABLO.

Discussion

In the present study we examined the possible role of IAPs and Smac/DIABLO in the regulation of apoptosis in RPMI 8226 multiple myeloma cells and the possible involvement of these proteins in the action of the chemotherapeutic agent doxorubicin and chemoresistance. Especially concerning the expression dynamics during the early period after doxorubicin treatment, we demonstrated a transient overexpression of IAPs. And the suppression of IAPs by Smac/DIABLO shortly after proapoptotic stimulation might play a role in the mechanisms of apoptotic induction. Previous studies have shown the down-regulation of IAPs expression after different types of proapoptotic stimulation such as treatment with doxorubicin, cisplatin, UV-irradiation, or TNF-α (Crnkovic-Mertens et al., 2003; Li et al., 2001; Notarbartolo et al., 2002, 2005; Yonesaka et al., 2006). However, the previous studies examined the later stage of expression dynamics in which the observation was started from 6–12 h after treatment. Our results demonstrated the transient up-regulation of IAPs at a very early stage (1 to 2 h) after proapoptotic simulation. This indicates the significance of IAPs dynamics at the very early period for understanding the biological mechanisms regulating IAPs expression as well as the interactions of IAPs with other molecules such as Smac/DIABLO. We have completed the same experiments using human T cell line, Jurkat cell line (data not shown). The results were almost the same with the present study. Thus, we prefer to speculate that the dynamics of IAPs and Smac/DIABLO after doxorubicin treatment was not specific for myeloma cells but for more general cells.

In the doxorubicin-resistant cell line DRR, we demonstrated the lower expression of Smac/DIABLO mRNA, resulting in the continuous overexpression of IAPs at the protein level, although the precise evaluation for the activation of Smac/DIABLO should be determined comparing the protein level both in the mitochondrial and cytosolic fractions. The expression dynamics of these molecules in DRR cells were thus characterized by a lack of down-regulation of IAPs and a lack of up-regulation of Smac/DIABLO after treatment with doxorubicin. Using the HL60 leukemia cell line and its multidrug resistant variant HL60R, HL60R cells were shown to overexpress the mRNAs of some IAPs as compared with HL60 (Notarbartolo et al., 2002). Doxorubicin or serum withdrawal strongly down-regulate survivin and XIAP mRNAs in HL60, while the same mRNAs are much less affected in HL60R cells. These results support the possibility that IAPs may play a role in the resistance to apoptosis of HL60R cells and further suggest that suppressor/neutralizers of IAPs such as Smac/DIABLO might have a significant role in controlling the drug-resistance of these cells. In the present study, we demonstrated the direct association of Smac/DIABLO with IAPs 1 to 2 h after treatment with doxorubicin. Smac/DIABLO is known to neutralize IAPs and thus, facilitates the proapoptotic process after apoptotic stimuli (Galluzzi et al., 2006), although the relationships between IAPs expression and Smac/DIABLO release are far from being completely understood (Liu et al., 2004; Duckett, 2005).

It would also be important to clarify the mechanisms responsible for the transient up-regulation of mRNA/protein expression for IAPs in RPMI 8226 cells after proapoptotic stimuli. Insulin-like growth factor-1 (IGF-1) and interleukin-6 (IL-6) promote the proliferation of multiple myeloma cells. IGF-1 stimulates the sustained activation of NF-κB and Akt and up-regulates a series of intracellular anti-apoptotic proteins, including FLIP, survivin, cIAP-2 and XIAP. In contrast, IL-6 does not cause sustained NF-κB activation, induces less pronounced Akt activation, and increases the expression of only survivin (Mitsiades et al., 2002a). We previously demonstrated that TNF-α is present locally in the bone marrow microenvironment and is associated with the regulation of cellular proliferation/apoptosis in hematological diseases (Kitagawa et al., 1997). TNF-α induces NF-κB nuclear translocation, cIAP-1 and cIAP-2 up-regulation, and proliferation in multiple myeloma cells (Mitsiades et al., 2002b). Thus, the expression of IAP is controlled by complex cellular signals. Further study will be necessary to clarify the mechanism of IAP induction in multiple myeloma cells in response to proapoptotic stimuli, including chemotherapy.

Our research interests deal with possible strategies to overcome the resistance to drugs and apoptosis, possibly related to IAPs expression, which characterize tumors with poor prognosis (Notarbartolo et al., 2005). Using bone marrow samples from patients with multiple myeloma, we have demonstrated that IAPs expression correlates with poor outcome in association with chemotherapy-induced overexpression of multidrug resistance genes (Nakagawa et al., 2006). Thus, the functional inhibition of specific IAPs may provide a rational basis for the development of novel therapeutic strategies. Using small

interfering (si)RNAs, which could efficiently block endogenous IAPs gene expression, HeLa cells were analyzed to test whether blockade of livin would actually be effective for inducing apoptosis in tumor cells. Silencing of livin was associated with caspase-3 activation and a strongly increased apoptotic rate in response to different proapoptotic stimuli, such as doxorubicin, UV-irradiation or TNF-α (Crnkovic-Mertens et al., 2003). Similarly, siRNA targeting survivin sensitized lung cancer cells with mutant p53 to doxorubicin (Yonesaka et al., 2006).

Here we demonstrated that proapoptotic stimulation induced the transient up-regulation of IAPs expression. This fact also indicated that cellular machineries such as the Smac/DIABLO system should work for the rapid down-regulation of the IAPs expression. Further studies should clarify the mechanisms responsible for IAPs as well as Smac/DIABLO regulation and also the regulation of down-stream molecules such as caspases in multiple myeloma cells and provide a tool for blocking the rapid induction of IAPs expression after proapoptotic stimulation with chemical agents. The highly preserved expression of IAPs and lower Smac/DIABLO expression might cooperate or interact to produce the doxorubicin-resistant conditions such as those in DRR cells. These results suggest that the DRR cells might lack the mechanisms for down-regulating IAPs and up-regulating Smac/DIABLO. Thus, to treat chemotherapy-resistant multiple myeloma cells like DRR, a novel chemotherapeutic strategy should be considered for targeting IAPs and enhancing the Smac/DIABLO system using IAP antagonists mimicking Smac/DIABLO, small-molecule BIR inhibitors, antisense oligonucleotides targeting IAPs (Mizukawa et al., 2006; Schimmer and Dalili, 2005; Wright and Duckett, 2005) or RNA interference of IAPs (Kashkar et al., 2006) in combination with proapoptotic chemotherapy.

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IMMUNOLOGY ORIGINAL ARTICLE

Interleukin (IL)-4 promotes T helper type 2-biased natural killer T (NKT) cell expansion, which is regulated by NKT cell-derived interferon-γ and IL-4

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Summary

CD1d-restricted natural killer T (NKT) cells can rapidly produce T helper type 1 (Th1) and Th2 cytokines and also play regulatory or pathological roles in immune responses. NKT cells are able to expand when cultured with α-galactosylceramide (α-GalCer) and interleukin (IL)-2 in a CD1d-restricted manner. However, the expansion ratio of human NKT cells is variable from sample to sample. In this study, we sought to determine what factor or factors are responsible for efficient *in vitro* expansion of NKT cells from various inbred mouse strains. Although the proportion of NKT cells in the spleen was nearly identical in each mouse strain, the growth rates of NKT cells cultured *in vitro* with α-GalCer and IL-2 were highly variable. NKT cells from the B6C3F1 and BDF1 mouse strains expanded more than 20-fold after 4 days in culture. In contrast, NKT cells from the strain C3H/HeN did not proliferate at all. We found that cell expansion efficiency correlated with the level of IL-4 detectable in the supernatant after culture. Furthermore, we found that exogenous IL-4 augmented NKT cell proliferation early in the culture period, whereas interferon (IFN)-γ tended to inhibit NKT cell proliferation. Thus, the ratio of production of IL-4 and IFN-γ was important for NKT cell expansion but the absolute levels of these cytokines did not affect expansion. This finding suggests that effective expansion of NKT cells requires Th2-biased culture conditions.

Keywords: natural killer T cell; interleukin-4; interferon-γ; glycolipid

Introduction

Mouse natural killer T (NKT) cells were initially identified as a T-cell subset that expresses NK cell receptors such as NK1·1, CD94 and Ly49.[1,2] The majority of NKT cells have the invariant T-cell receptor (TCR) α-chain rearrangement Vα14-Jα18 and recognize antigens presented by CD1d, a non-classical major histocompatibility complex (MHC) class I molecule.[3,4] NKT cells are continuously sensitized by endogenous antigens so that they display an effector-memory phenotype (such as $CD62L^{low}$ $CD44^{high}$)[5–7] and rapidly produce large amounts of T helper type 1 (Th1) and Th2 cytokines when stimulated with lipid antigens such as α-galactosylceramide (α-GalCer) in a CD1d-dependent manner.[2,8] NKT cells are regarded as immunoregulatory because of their cytokine profile. Moreover, NKT cells are thought to play an important role in response to infectious agents and in pathological responses such as allergies or autoimmune disease. NKT cells are cytotoxic to various tumour cell lines via Fas-ligand-, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)- and/or perforin-dependent pathways,[9–12] and play a role in tumour surveillance.[13] NKT cells activated by interleukin (IL)-12 or α-GalCer sequentially activate natural killer (NK) cells by producing interferon (IFN)-γ and induce antitumour immune responses. This in turn inhibits tumour metastasis and can suppress solid tumour growth. In some studies, it has been suggested that this ability helps to induce tumour antigen-specific CD8 T cells, thereby making an additional contribution to the immune response to cancer.[14]

In humans, counterparts of mouse NKT cells have also been found to be responsive to α-GalCer, which induces them to secrete IL-4 and IFN-γ. In addition, they have been shown to be cytotoxic to tumour cells via two different mechanisms, a CD1d-dependent and a CD1d-independent mechanism.[15] Human NKT cells have the

potential to induce antitumour responses *in vivo*. However, in patients with malignancies,[16,17] NKT cells are reduced in number and activity, and *in vivo* activation by α-GalCer leads to transient activation and long-term unresponsiveness of NKT cells.[18,19] For that reason, adaptive transfer of *in vitro* expanded and/or activated NKT cells is expected to induce effective antitumour responses.

To date, several combinations of cytokines with α-GalCer have been reported to expand NKT cells isolated from peripheral mononuclear cells. However, NKT cells present a diverse range of expansion ratios even among healthy individuals.[20,21] Although a previous study suggested that differences in NKT cell proliferation are associated with the age of the donor,[22] there is still much that remains to be determined concerning additional factors that influence NKT cell proliferation.

In this study, we used inbred mouse strains as an experimental system in which to reveal factors that affect variation in proliferation rates among individuals. Previously, we found that *in vitro* expanded NKT cells from C57BL/6 mice retained an effector-memory-like phenotype and retained the ability to produce cytokines.[23] In addition, we found that there was a marked difference in the NKT cell expansion ratio among various mouse strains and that the differences were closely related to the bias in production of Th1 or Th2 cytokines by NKT cells. Finally, we report that a relatively low rate of proliferation can be enhanced by the addition of IL-4, which creates Th2-biased culture conditions.

Materials and methods

Mice

Female C57BL/6N, BALB/cA, C3H/HeN, DBA/2N (C57BL/6 × DBA/2)F_1 (BDF1), (C57BL/6 × C3H/HeN)F_1 (B6C3F1), and SJL/J mice were purchased from Charles River Japan (Kanagawa, Japan). All mice, which were maintained in our animal facilities, were 8–11 weeks of age at the time of the experiment. All animal protocols for this study were reviewed and approved by the committee for ethics of animal experimentation at the National Cancer Center of Japan prior to the beginning of the study.

Monoclonal antibodies and reagents

Anti-IL-4 (clone 11B11) and anti-IFN-γ (clone R4-6A2) monoclonal antigen-neutralizing antibodies (mAbs) were obtained from the supernatant of a hybridoma culture maintained in serum-free medium in a CELLine CL-1000 flask (BD Biosciences, San Jose, CA) and purified by Protein G Sepharose (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden) affinity column chromatography. Anti-CD16/32 (clone 2·4G2) was obtained from a hybridoma supernatant. Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone 145-2C11), allophycocyanin (APC)-conjugated anti-IL-4 (11B11), anti-IFN-γ (XMG1·2), and a rat immunoglobulin G1 (IgG1) isotype control (clone R3-34) and Golgi Stop™ were obtained from BD Biosciences. α-Galactosylceramide (α-GalCer) was kindly provided by the Pharmaceutical Research Laboratory, KIRIN Brewery Co., Ltd (Gunma, Japan). The phycoerythrin (PE)-conjugated CD1d/α-GalCer tetramer was prepared using a baculovirus expression system as previously described.[24] Human recombinant IL-2 (rIL-2) was kindly provided by Takeda Chemical Industries Ltd (Osaka, Japan). Mouse rIL-4 was obtained from PeproTech EC Ltd (London, UK).

Flow cytometry

NKT cells were detected by multicolour flow cytometry as previously described.[23] Briefly, cells were preincubated with anti-CD16/32 mAb to block non-specific FcRγ binding and then stained with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Dead cells were excluded by propidium iodide staining and electronic gating. For detection of intracellular cytokines, cells were stimulated for 3 hr with phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) and ionomycin (1 μg/ml), with the last 1 hr of stimulation in the presence of Golgi block, in a 37°, 5% CO_2 incubator, and then washed and incubated with anti-CD16/32 mAb, followed by incubation with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Cells were then permeabilized using Cytofix/Cytoperm (BD Biosciences) and IL-4 or IFN-γ was detected using APC-conjugated mAbs. Cells were analysed by flow cytometry (FACSCalibur; BD Biosciences).

NKT cell proliferation assay

Preparation of splenic mononuclear cells and *in vitro* expansion of NKT cells were performed as previously described.[23] Briefly, spleens of each mouse strain were macerated aseptically and pushed through a nylon mesh to obtain single-cell suspensions, and erythrocytes were lysed in ammonium chloride buffer. Mononuclear cells (1×10^6 cells/ml) were cultured with α-GalCer (50 ng/ml) and rIL-2 (100 IU/ml) in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8% fetal calf serum (JRH Biosciences, Lenexa, KS), 2-mercaptoethanol (5×10^{-5} M) 100 U/ml penicillin and 100 μg/ml streptomycin for 4 days in a 37°, 5% CO_2 incubator. After 4 days in culture, the absolute number of living cells was counted using a microscope after staining of cells with 0·2% trypan blue, and the relative percentages of NKT cells were determined by flow cytometry.

Cytokine production

The cell culture supernatant was collected after 24 hr or 4 days in culture and stored at −20°. The concentrations

of IL-4 and IFN-γ were determined by enzyme-linked immunosorbent assay (ELISA) (OptEIA ELISA set; BD Biosciences).

Results

α-GalCer-induced expansion of NKT cells from various mouse strains

Mouse NKT cells show a similar variation in expansion ratios to that observed for human NKT cells. We found that the expansion ratios were different for different mouse strains (Fig. 1). Before culture, spleen cell suspensions contained a small percentage (0·8–1·5%) and a small number (7–18 × 10^3 cells/ml) of NKT cells in each mouse strain. As shown in Fig. 1, culture of spleen cells with α-GalCer and IL-2 induced expansion of NKT cells, except for C3H/HeN mice. After 4 days of culture, NKT cells constituted 6·4–40·7% of cells in the culture and had expanded 7–25-fold in BALB/c, C57BL/6, DBA/2, B6C3F1 and BDF1 mice. The CD1d-restricted TCR α-chain Vα14 dominantly associates with the high-affinity TCR β-chain Vβ8·2, or the lower affinity chain Vβ8·3, Vβ7 or Vβ2, and a genetic defect in Vβ8 is reportedly the cause of the low responsiveness of NKT cells. We next asked if the TCR-β status of NKT cells had an effect on expansion. However, we found no significant differences among the six strains that were tested, and selective proliferation did not occur (data not shown).

NKT cell proliferation ratio correlates with amount of IL-4 in supernatant from a 4-day culture

Previously, a high concentration of IL-4 and IFN-γ in supernatant from a 4-day culture was observed.[23] Firstly, we measure amounts of IL-4 and IFN-γ in the culture supernatant.

An increase in the number of NKT cells was positively correlated with the production of IL-4 in the 4-day culture (Fig. 2a). However, high levels of IFN-γ were observed in all of the mouse strains, independent of an increase in either NKT cell number or IL-4 production. Almost all CD8 T cells acquired the ability to produce IFN-γ when activated indirectly via NKT cells by α-GalCer (data not shown), so it appears that, in C3H/HeN mice, NKT cells do not proliferate. Instead, it seems reasonable that a large amount of IFN-γ might be produced by the activated NK cells and CD8 T cells.[25,26]

A previous study reported cytokine secretion of NKT cells prior to their proliferation.[2,27] Thus, we harvested culture supernatants at 24 hr, before NKT cell expansion,[27] to determine the status of cytokine production at this early stage, which is the stage at which NKT cells initially respond to culture and initiate production of IL-4. This initial response positively correlated with NKT cell expansion to some degree, although the response was weaker than that observed for cells in culture for 4 days. It is notable that IL-4 production by C3H/HeN was more robust than that observed for C57BL/6, and IFN-γ

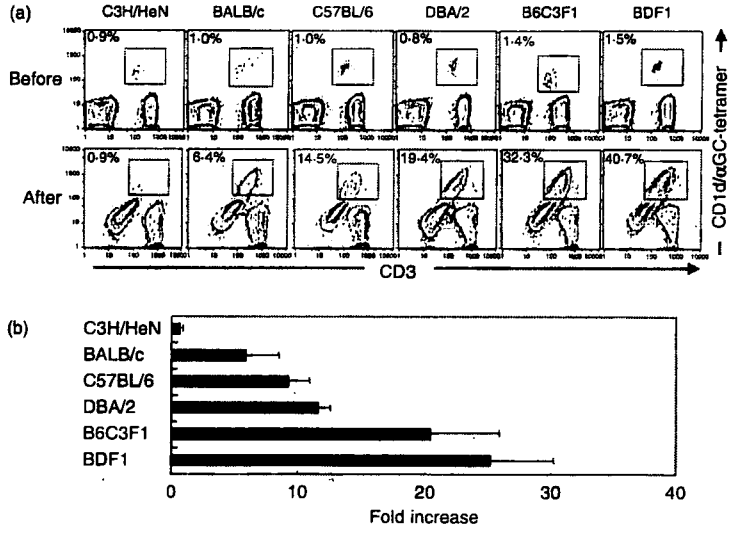

Figure 1. Expansion of natural killer T (NKT) cells *in vitro*. (a) Mouse spleen cells (1 × 10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days. Cells were stained with anti-CD3 monoclonal antibody (mAb) and CD1d/α-GalCer tetramer and analysed by flow cytometry. The percentage of NKT cells was determined for both fresh (upper row) and cultured (lower row) cells. Representative results from replicate experiments are shown. (b) The fold increase in NKT cells after culture was calculated based on living cell counts and the percentage of NKT cells in the total cell population. Data are shown as mean ± standard error of the mean ($n = 9$ for C3H/HeN, BALB/c and C57BL/6; $n = 4$ for DBA/2, B6C3F1 and BDF1).

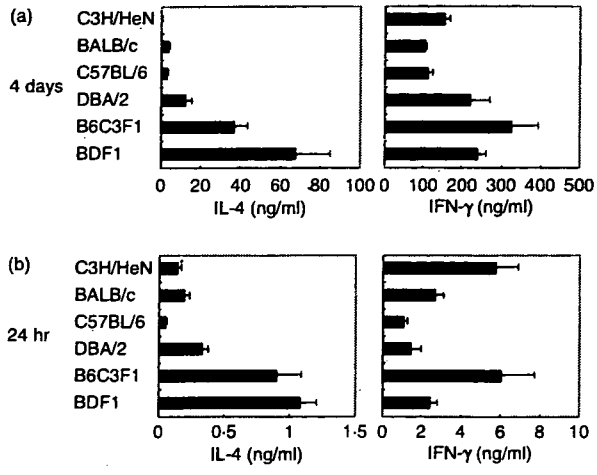

Figure 2. Production of interleukin (IL)-4 and interferon (IFN)-γ in expansion cell culture supernatants. Mouse spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days. Supernatants were collected after 24 hr (b) or 4 days (a). The levels of IFN-γ and IL-4 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Data are shown as mean ± standard error of the mean ($n = 9$ for C3H/HeN, BALB/c and C57BL/6; $n = 4$ for DBA/2, B6C3F1 and BDF1).

production of C3H/HeN mice was much higher than that of other strains (Fig. 2b). These observations lead us to speculate that IL-4 and IFN-γ produced by NKT cells work as promoting and suppressing factors, respectively, during NKT cell proliferation.

NKT cell proliferation partially depends on IL-4 and is enhanced by Th2 cytokines

We next examined the influence of IL-4 on NKT cell proliferation *in vitro*. Proliferation of these cells was accelerated by addition of IL-4 at the start of the culture period, an effect that could be partially suppressed by neutralization of IL-4 (Fig. 3). In the C3H/HeN strain, where proliferation of NKT cells was not robust, a more significant induction of proliferation by IL-4 was observed (Fig. 4). In addition, neutralization of IFN-γ using antibodies did not significantly change the proportion of NKT cells in the total cell population. However, this did appear to up-regulate the total number of living cells and lead to a concomitant increase in the total number of NKT cells (Fig. 4b). Only NKT cells can produce IL-4 when cultured with α-GalCer and IL-2,[23] so IL-4 must act as an autocrine growth factor in the expansion of NKT cells in this context.

The proportion of intracellular IFN-γ high positive NKT cells is reduced by addition of IL-4

Exogenous IL-4 promoted NKT cell expansion in C3H/HeN mice, as shown in Figs 3 and 4. We next examined

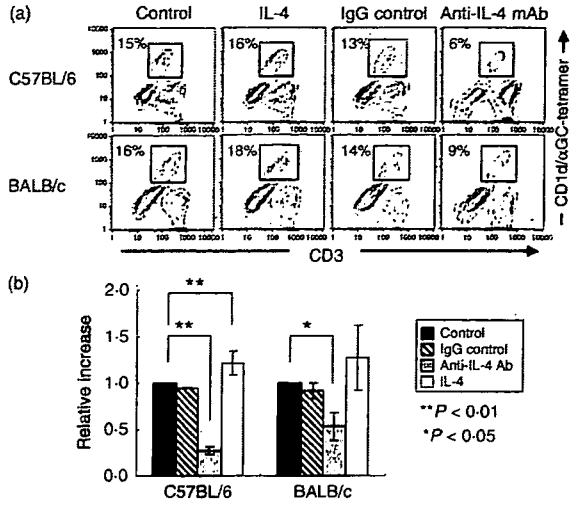

Figure 3. Expansion of natural killer T (NKT) cells in the presence or absence of interleukin (IL)-4. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days with IL-4 (10 ng/ml) or anti-IL-4 monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with control expansion culture. Data are shown as mean ± standard deviation for five independent experiments. A paired two-tailed Student's *t*-test was used for statistical analysis (*$P < 0·05$; **$P < 0·01$).

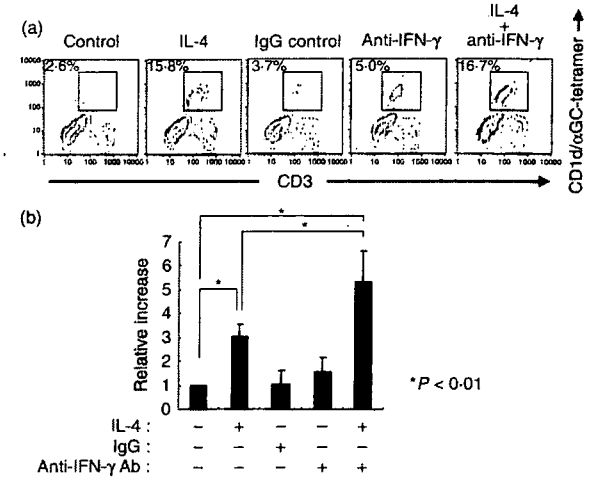

Figure 4. Expansion of natural killer T (NKT) cells from C3H/HeN strain mice in conditions that favour production of T helper type 2 (Th2)-biased cytokines. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 and with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml) for 4 days. The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as mean ± standard deviation for seven independent experiments. A paired two-tailed Student's *t*-test was used for statistical analysis (*$P < 0·01$).

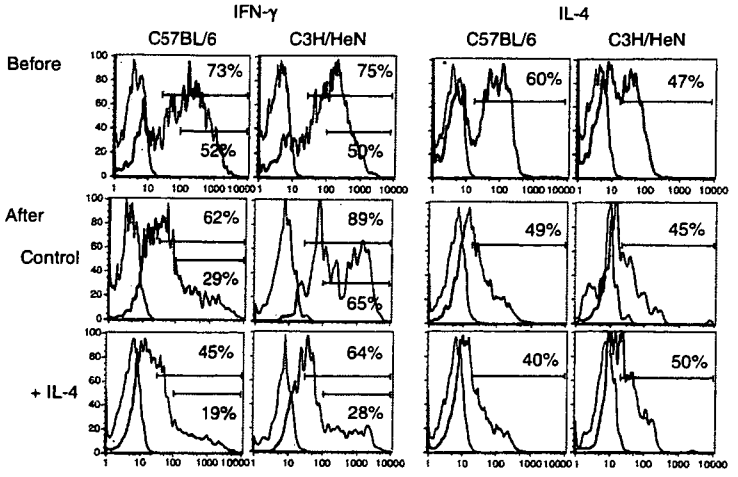

Figure 5. Cytokine production profile of natural killer T (NKT) cells treated with interleukin (IL)-4. Intracellular cytokine staining for interferon (IFN)-γ and IL-4 in NKT cells that were fresh (upper), cultured (middle), or cultured with additional IL-4 (lower) is shown. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 3 hr, stained with anti-CD3 monoclonal antibody (mAb), CD1d/α-galactosylceramide (α-GalCer) tetramer and anti-IFN-γ, anti-IL-4, or an isotype control mAb, and then detected and sorted via flow cytometry. Histogram panels for CD1d/α-GalCer-tetramer$^+$ CD3$^+$ cells are shown. Closed histograms indicate isotype controls. The percentage of total positive and high positive cells are indicated in the histograms. Data are representative of replicate experiments.

whether NKT cells cultured in Th2 conditions produced IFN-γ and IL-4. After 4 days of culture with α-GalCer and IL-2, intracellular IFN-γ- and IL-4-positive NKT cells were observed in both strains of mice. However, the proportion of intracellular IFN-γ high positive NKT cells was reduced when the cells were cultured with additional IL-4 (Fig. 5). In contrast to IFN-γ, the proportion of IL-4-positive NKT cells did not differ between cultures with and without IL-4. Therefore, NKT cells expanding as a result of induction with additional IL-4 displayed a polarized Th2 phenotype.

NKT cell expansion is accelerated by Th2-biased cytokine conditions

The SJL/J mouse strain is defective in cytokine production by NKT cells, as a consequence of a loss of high-affinity TCR to CD1d, which results from a deletion of the TCR Vβ8 subfamily genomic loci.[28,29] The proportion of NKT cells in the spleens of these mice was lower than that observed for other strains (Fig. 6a), and IFN-γ and IL-4 production after α-GalCer stimulation was also lower than that observed for other strains tested in this study (data not shown). NKT cells from SJL/J mice proliferated even in the absence of additional IL-4, as was observed for NKT cells from C57BL/6 mice. Moreover, similar to findings for NKT cells from C3H/HeN mice, the NKT cell proliferation effect could be enhanced by addition of IL-4 and further enhanced by addition of IL-4 combined with neutralization of IFN-γ (Fig. 6b).

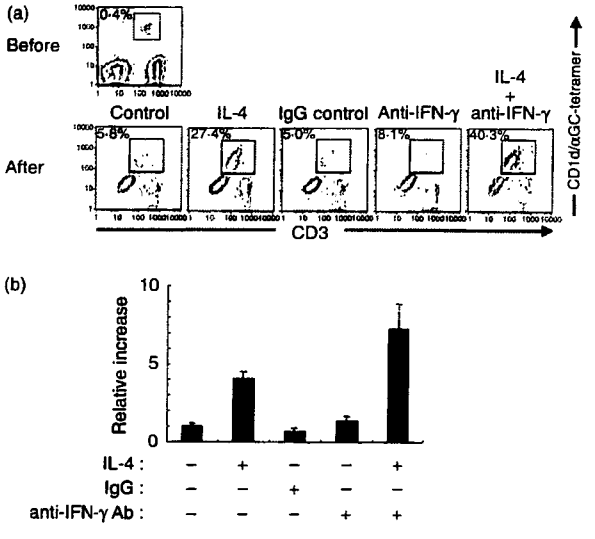

Figure 6. Expansion of natural killer T (NKT) cells from SJL/J mice *in vitro*. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as the mean of three wells ± standard deviation. Similar results were obtained in two independent experiments.

Discussion

In a previous study in which we induced expansion of NKT cells collected from human peripheral blood, we

observed wide variation in the efficiency of NKT cell expansion.[21] Similarly, when mouse NKT cells were induced to proliferate using similar methods in the present study, the ratios of expanding cell types were distinctly different in cells obtained from different mouse strains (Fig. 1). This suggests that genetic background influences or controls the difference in proliferation efficiency observed in humans and mice. However, we could not rule out the alternative possibility that the effect was a result of bipolar expansion of the cells, rather than originating from genetic variation in one or a few loci.

In this study, we have shown that the amount of IL-4 in the culture supernatant was related to the efficiency of NKT cell expansion induced by α-GalCer and IL-2. Previous studies revealed that addition of exogenous IL-2, IL-7 and IL-15 was able to augment NKT cell expansion by α-GalCer.[30–34] Similarly, in the present study we found that exogenous IL-2 augmented α-GalCer-induced NKT cell expansion in various mouse strains, with the exception of C3H/HeN mice. Moreover, addition of exogenous IL-4 promoted α-GalCer-induced NKT cell expansion in spleen cells from C3H/HeN mice. It has been shown that only NKT cells have the ability to produce IL-4 in this culture.[23] IL-4 might therefore be an autocrine or paracrine growth factor in α-GalCer-induced NKT cell expansion.

NKT cells, NK cells and some T cells when cultured with α-GalCer and IL-2 produce IFN-γ.[23] In contrast to IL-4, the amount of IFN-γ did not correlate with the efficiency of NKT cell expansion. Furthermore, we found that NKT cell proliferation in C3H/HeN mice was slightly increased by neutralization of IFN-γ in the culture. These results suggest that IFN-γ partially inhibits NKT cell expansion by α-GalCer. Interestingly, we found an inverse correlation between the IFN-γ:IL-4 ratio in the culture supernatant after 24 hr of culture and the efficiency of NKT cell proliferation (data not shown). Although higher amounts of IL-4 were detected in the culture of cells from C3H/HeN mice than in the culture of cells from C57BL/6 mice after 24 hr of culture, α-GalCer stimulated spleen cells from C3H/Ne mice produced higher amounts of IFN-γ and exhibited the highest IFN-γ:IL-4 ratio of all mouse strains tested. These results may explain the failure of NKT cell expansion in spleen cells from C3H/HeN mice.

The balance between the production of IFN-γ and the production of IL-4 by NKT cells is influenced by microenvironmental factors such as cytokines and antigen-presenting cells.[20,35–38] IL-7 and IL-12 selectively enhance IL-4 production by NKT cells.[35,36] Antigen-presenting cells such as α-GalCer-pulsed B cells selectively elicit weak IL-4 but not IFN-γ production from NKT cells.[37] There is a high IFN-γ:IL-4 ratio in cultures of spleen cells from C3H/HeN mice, which is caused by splenic NKT cells (A. Iizuka *et al.*, unpublished data) Moreover, it has been reported that the balance of IFN-γ:IL-4 production by NKT cells is developmentally controlled.[39,40] At immature stages, NKT cells predominantly produce IL-4, whereas IFN-γ secretion increases during the course of development.[39] Moreover, immature NKT cells have the ability to proliferate as compared with mature NKT cells.[39] Therefore, NKT cells in the spleen of C3H/HeN mice may be more mature than those of C57BL/6 mice, or contain only a few immature NKT cells. We assume that the failure of proliferation and the high IFN-γ:IL-4 cytokine production ratio of NKT cells in the spleen of C3H/HeN mice were attributable to their maturation stage.

Although IL-4 has opposite effects to IFN-γ and suppresses the Th1 immune response, IL-4 induces proliferation of human IL-13$^+$ NK cells[41] and CD8$^+$ T cells.[42] We found that Th2 culture conditions (in the presence of IL-4 and anti-IFN-γ mAb) facilitated NKT cell expansion induced by α-GalCer and IL-2 even in C3H/HeN and SJL/J mice. IL-4 also induces IFN-γ production by NK and NKT cells *in vivo*.[43] However, the proportion of IFN-γ-positive, but not IL-4-positive, NKT cells decreased when cells were cultured in the presence of IL-4. As in human immature IL-13$^+$ NK cells,[41] IL-4 may induce expansion of developmentally immature NKT cells which have a Th2-biased phenotype.

NKT cell maturation is controlled by the transcription factor T-bet.[44,45] Terminally differentiated NKT cells acquire a strong ability to produce IFN-γ and elicit cytotoxicity.[44] Assuming that expanded Th2-biased NKT cells after culture with α-GalCer, IL-2 and IL-4 are immature cells, it will be possible to induce terminally differentiated Th1-biased NKT cells for Th1 cell immunotherapy, such as cancer cell therapy.

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IAP Family Protein Expression Correlates With Poor Outcome of Multiple Myeloma Patients in Association With Chemotherapy-induced Overexpression of Multidrug Resistance Genes

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Multidrug-resistant (MDR) multiple myeloma (MM) patients who fail chemotherapy frequently express MDR1 protein, which serves as an efflux pump that protects neoplastic cells. The expression of lung resistance protein (LRP), which mediates intercellular and nucleocytoplasmic transport, is also correlated with chemotherapy resistance and shorter survival of MM patients. Here, we investigated the chemotherapy-induced change of MDR expression in MM patients using quantitative RT-PCR. Overall expression levels of MDR1 and LRP in MM patients were significantly higher than those in control subjects and increased after chemotherapy. More than half of the patients exhibited increased expression of MDR1 (14/26) or LRP (17/26) after chemotherapy. Also, the expression of inhibitor of apoptosis proteins (IAP) was determined in association with the prognosis of the patients. Among patients with increased MDR1-expression after chemotherapy, those with a poor outcome exhibited significant increases in survivin, cIAP1, cIAP2, and XIAP expression by chemotherapy compared with those with a good prognosis. Similarly, in the LRP expression-increased group, patients with a poor outcome showed significant increases of cIAP1 and cIAP2 expression compared with those with longer survival. In patients with reduced-MDR1 or LRP expression after chemotherapy, changes in the expression of IAPs induced by chemotherapy did not correlate with their prognosis. These findings indicate that IAP family proteins might play a role in worsening the prognosis of MM patients in association with chemotherapy-induced overexpression of MDR1 or LRP. Am. J. Hematol. 81:824–831, 2006. © 2006 Wiley-Liss, Inc.

Key words: multiple myeloma; MDR1; LRP; IAP; bone marrow

INTRODUCTION

The development of refractory disease in hematological malignancies such as multiple myeloma (MM) and acute myeloid (AML) or lymphoid leukemias (ALL) is frequently associated with the expression of one or several multidrug resistance (MDR) genes [1]. Clinical studies have established that MDR1 expression occurs in MM patients, and there is also clinical evidence of multidrug resistance [2]. In addition, the response rate to induction chemotherapy is significantly lower in patients with LRP expression than in patients without LRP expression [3]. Furthermore, MM patients with LRP expression have a shorter overall survival than those without it [4]. However, knockout mice experiments revealed that disruption of the LRP gene did not induce hypersensitivity to cytostatic agents [5]. Thus, the effects

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of LRP expression on the drug-resistance in MM patients are still controversial.

It has become possible to reverse clinical multidrug resistance by blocking P-glycoprotein-mediated drug efflux. However, potential new approaches to treat refractory diseases by using MDR modulators have not yet generated promising results. Recently, emerging knowledge about the importance of overcoming anti-apoptosis and drug resistance in treating a variety of malignancies, including MM, has raised new hope for improving the treatment outcome for patients with cancer [6]. Several targeting therapies that aim to reverse the anti-apoptotic process in MM cells have been explored in a number of experimental systems and clinical studies [6]. Thus, the aim of the present study was to investigate anti-apoptotic mechanisms that are employed in bone marrow cells from MM patients with MDR expression.

IAP family proteins, including survivin, block apoptosis induced by a variety of apoptotic triggers [7,8]. Although the exact biochemical mechanism by which these proteins suppress apoptosis is under debate, survivin is known to directly bind to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [8,9]. Survivin is widely expressed in fetal tissues, but becomes restricted during development, and is negligibly expressed in the majority of terminally differentiated adult tissues [10,11]. However, analysis of the differences in gene expression between normal cells and tumor cells has revealed that survivin is one of the genes that is most consistently overexpressed in tumor cells relative to normal tissue [12]. In fact, survivin is prominently expressed in transformed cell lines and in many human cancers including hematopoietic cell tumors [13]. It can usually be detected in the cytoplasm of tumor cells, and is therefore widely regarded as being a cytoplasmic protein [10,14,15]. However, several studies have examined the nuclear accumulation of survivin in gastric cancer cells [16] and lung cancer cells [17]. We have recently shown that ALL cells principally exhibit nuclear localization of survivin, while CLL cells possess cytoplasmic survivin [18]. Thus, the significance of nuclear-cytoplasmic localization in tumor cells is still controversial.

In the present study, we examined MM patients by focusing on the contribution of IAPs to their prognosis. First, the expression of MDR1 and LRP was compared between bone marrow samples from MM patients before and after chemotherapy using quantitative RT-PCR. The patients were divided into two groups, one consisting of those with increased MDR1 or LRP expression after treatment and the other of those with reduced expression of MDR1 or LRP after treatment. Then, the expression of IAP family proteins including survivin, cIAP1, cIAP2, NAIP, and XIAP, which suppress apoptosis by caspase and procaspase inhibition [19–22], was also determined in both groups. In MM patients with chemotherapy-induced overexpression of MDR1 or LRP expression, the increased expression of several IAPs was significantly correlated with their prognosis. The implications of these findings regarding the multidrug resistance of MM cells and their clinical significance are discussed.

MATERIALS AND METHODS

Patients

Fresh frozen bone marrow samples from control (7 cases, age, median 55, max. 74, min. 43; male: female, 1:6) and MM (26 patients; male:female, 14:12; age, median 68, max. 85, min. 36) patients who received induction chemotherapy were collected. Melphalan-based regimens utilizing melphalan/prednisone or VMCP were administered to 20 of the MM patients. Also, four MM patients were treated with VAD, and two patients received VAD followed by high-dose melphalan. The prognosis of the patients in association with their responses to induction chemotherapy was assessed by their survival times. Patients with a survival time of more than 4 years were determined to be those with a good prognosis, while patients who died within 4 years were designated as those with a poor outcome. To rule out the influence of aging on bone marrow cells, age-matched control patients were analyzed. All MM samples were collected at the time of their initial aspiration biopsy and after chemotherapy. The patients were not infected with specific viruses including HTLV-1 and had not been treated prior to the study.

The procedures followed were in accordance with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

Preparation of RNA and Quantitative Assay for mRNA Expression of MDR1, LRP, and IAP Family Proteins Using TaqMan RT-PCR

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders and MM samples before and after chemotherapy using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and the TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Se-