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Expression of IAP-Family Proteins in Adult Acute Mixed Lineage Leukemia (AMLL)

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Inhibitor of apoptosis protein (IAP)-family proteins suppress apoptotic signaling in normal/neoplastic cells in various settings. To determine the apoptosis-resistant mechanism in adult acute mixed lineage leukemia (AMLL) with biphenotypic blasts responsible for resistance against chemotherapy, the expression levels of IAP-family proteins in AMLL bone marrow cells were analyzed by quantitative RT-PCR. The overall expression levels of IAPs were higher than those in control, AML, and ALL cells. A significant difference for the expression of survivin was observed between AMLL and AML ($P < 0.05$), and differences between AMLL and ALL were significant for the expression of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$). These findings suggest that higher expression of various IAPs is associated with the chemotherapy-resistant nature of this specific type of leukemia. Am. J. Hematol. 78:173–180, 2005. © 2005 Wiley-Liss, Inc.

Key words: IAP; apoptosis; AMLL; AML; ALL; bone marrow

INTRODUCTION

The regulation of apoptotic cell death has a profound effect on the pathogenesis and progression of hematological malignancies. Acute mixed lineage leukemia (AMLL) is a relatively rare group of hematological malignancies that exhibits the expansion of biclonal or biphenotypic blasts in peripheral blood [1,2]. According to FAB criteria, AMLL may present as ALL or as one of the AML subtypes, often as M1 [2]. AMLL has a high incidence of clonal chromosomal abnormalities, the most common being the t(9;22)(q34;q11) (Ph chromosome) and structural abnormalities involving 11q23 [2]. Recently, molecular analysis revealed that the *mixed lineage leukemia* (*MLL*) gene rearrangement occurs in AMLL cases and also in a fraction of AML/ALL patients [3]. One characteristic feature of AMLL as well as *MLL* gene-rearranged leukemia is a poor patient prognosis associated with lower sensitivity to chemotherapeutic procedures [2,4]. Resistance against chemotherapy might result from the resistance to apoptosis-inducing drugs such as steroids and Ara-C [5,6]. Regarding the complicated mechanisms that regulate apoptosis in the bone marrow of acute leukemias and myelodysplastic syndromes (MDS), we previously showed that a variety of apoptosis-related molecules are active in hematopoietic cells [7–13]. However, the associated parameters and molecules involved in apoptosis in AMLL are unclear.

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IAP-family proteins, including survivin, block apoptosis induced by a variety of triggers [14,15]. Although the biochemical mechanism by which IAP-family members suppress apoptosis is under debate, survivin is known to bind directly to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [15,16]. The expression of survivin is ubiquitous in fetal tissues but is restricted during development and is negligible in the majority of terminally differentiated adult tissues [17,18]. However, an analysis of the differences in gene expression between normal and tumor cells reveals that survivin is a protein whose gene is most consistently overexpressed in tumor cells relative to normal tissue [19]. Survivin is prominently expressed in transformed cell lines and in many human cancers, including hematopoietic cell tumors [20]. It is also usually detected in the cytoplasm of tumor cells and is therefore widely regarded as a cytoplasmic protein [17,21,22]. However, several studies have shown the nuclear accumulation of survivin in gastric cancer cells [23] and lung cancer cells [24]. We recently reported that ALL cells principally exhibited the nuclear localization of survivin, while CLL cells exhibited cytoplasmic distribution [13]. Although the significance of this nuclear-cytoplasmic expression in tumor cells is still controversial, the subcellular localization of survivin should also be clarified for AMLL subjects.

We also reported that survivin exhibited higher levels of expression in acute lymphocytic leukemia (ALL) and that chronic lymphocytic leukemia (CLL) cases exhibited significant over-expression of survivin and cIAP2 [13]. In acute myelogenous leukemia (AML) cases, some of these IAP-family proteins, such as NAIP and XIAP, are expressed at significantly higher levels [25]. To focus on the contribution of IAPs to the expansion of blasts in AMLL, we examined cases of AMLL that exhibited biphenotypic proliferation of blasts. The expression levels of survivin tended to be high in AMLL samples compared with control bone marrow, AML, and ALL subjects. The expression of other IAPs, including cIAP1, cIAP2, NAIP and XIAP, which suppress apoptosis by inhibiting caspase and procaspase [26–29], was also observed in these samples. The significance of IAP-family proteins in resistance against chemotherapy in AMLL is discussed.

MATERIALS AND METHODS

Patients

Fresh-frozen and formalin-fixed paraffin-embedded bone marrow-aspirated samples from 13 individuals with no hematological disorders were used as normal controls (male/female 5:8; age, median 52 years, range: 25–84 years), 9 patients with AML (8 with M2 and 1 with M1 according to the FAB classification, male/female 5:3; age, median 41 years, range: 19–78 years), 7 patients with ALL (male/female 2:5; age, median 58 years, range: 46–87 years), and 8 patients with AMLL with biphenotypic blasts (male/female 4:4; age, median 50 years, range 17–73 years) were examined. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Flow-cytometic analysis was routinely performed for CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD16, CD19, CD20, CD33, CD34, CD41a, CD56, and HLA-DR. Among them, the data for CD19, CD13, and CD33 were tabulated to demonstrate the biphenotypic nature of blastic cells in AMLL samples (Table I). Diagnoses were based on Catovsky's standard clinical and laboratory criteria [2] including cell morphology [30,31]. All samples were collected at the time of the initial aspiration biopsy and stored at −80°C. We selected the adult M1 or M2 AML samples and adult

TABLE I. Summary of Cases With Adult AMLL

Case no.	Age (years)	Sex	Blast (%)	Cell markers (%)			Chromosome abnormality
				CD19	CD13	CD33	
1	40	F	90.2	98.7	58.3	99.4	45,XX,der(12)t(12;22)(p13;q11) −22
2[a]	57	M	4.2	31.2	50.2	55.0	46,XY
3	67	M	94.4	95.2	67.8	0.9	36,XY,−3,−3,−5,−7,−9,−13,−15,−16,−17,−20
4	61	F	95.7	95.9	6.4	56.6	46,XX
5	17	M	96.4	99.8	53.8	50.6	46,XY
6	21	F	76.0	97.3	75.2	64.0	47,XX,+8
7	43	F	92.0	97.9	67.6	83.1	46,XX,i(8)(q10)del(9)(?q), der(9)del(9)(p22)t(9;22)(q34;q11),der(22)t(9;22)
8	73	M	69.2	89.2	45.4	13.5	46,XY,del(20)(q11.2)

[a]For case 2, material for flow-cytometric analysis was not sufficient at the time of initial diagnosis, although the diagnosis was confirmed as AMLL at the time of second biopsy. For the second biopsy sample, the blast count accounted for more than 90% of the bone marrow cells and consisted of more than 90% CD19-positive cells and more than 50% CD13/CD33-positive cells.

ALL samples for the comparison with AMLL. The AML, ALL, and AMLL samples exhibited the proliferation of blastic cells accounting for more than 80% of all bone marrow cells. The patients were not infected with viruses including HTLV-1 and had not been treated with therapeutic drugs prior to the study.

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

Double Staining for Myeloid and Lymphoid Cell Markers

The phenotype of leukemic cells in AMLL was confirmed by double immunostaining using the formalin-fixed paraffin-embedded bone marrow samples. Sections were deparaffinized and incubated with monoclonal antibody against CD20 or CD79a (DAKO, Glostrup, Denmark) and polyclonal antibody against myeloperoxidase (DAKO). Next, the sections were treated with peroxidase-conjugated anti-mouse IgG followed by a DAB development system and then with alkaline phosphatase-conjugated anti-rabbit IgG (DAKO) followed by development with an alkaline phosphatase–nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphatase development system (DAKO).

Identification of Apoptotic Cells

To identify apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used as previously described [10]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO) for 15 min at room temperature. After the tissues were washed, TdT, fluorescein isothiocyanate (FITC)-dUTP and -dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37°C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed to detect FITC-dUTP labeling, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. The sections were then observed under a microscope and the proportion of TUNEL-positive cells was determined by dividing the number of positively stained cells by the total cell number (count of more than 1,000 cells).

Preparation of RNA and Quantitative Assay for IAP-Family Proteins Using TaqMan RT-PCR

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, AML, ALL and AMLL patients using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and a TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with an ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the IAP-family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes used were as follows. The sequence of the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that of the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; the TaqMan probe was 5′-CAAGGACCACCG CATCTCTACATTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer was 5′-CAAGCCACCAT CACAACAAAA-3′; the TaqMan probe was 5′-TTT ATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer was 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAA CAGCTGCTAT-3′. The forward primer for NAIP mRNA was 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer was 5′-GCTGGGCGGATGCT TTC-3′; the TaqMan probe was 5′-CCATTTAAAC CACAGCAGAGGCTTTAT-3′. The forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTT CAGCATCA-3′ and the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTT TATACTG-3′. Finally, the forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCG GAGT-3′ and the reverse primer was 5′-GAA GATGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (stage 3, PCR). The expression of survivin and other IAP-family proteins was quantitated according to a method described elsewhere [13]. Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the IAP family was normalized using the data for GAPDH in each sample, and then the ratio to the mean value of control subjects was calculated and compared.

Immunohistochemistry for Survivin and Proliferating Cells

Tissue sections (4 μm thick) of bone marrow from the control, AML, ALL, and AMLL cases were cut on slides covered with adhesive. The sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were applied to identify survivin and to characterize proliferating cells. The primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, Inc., San Antonio, TX) and monoclonal antibody Ki-67 (DAKO). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted for the primary antibody. The proportion of Ki-67-positive cells was determined in the same way as the proportion of TUNEL-positive cells.

Statistical Analysis

Statistically significant differences in the quantitative analysis were determined using the Mann–Whitney *U*-test for comparisons between the control, AML, ALL, and AMLL samples.

RESULTS

Clinicopathological Characteristics of Cases With Acute Mixed Lineage Leukemia

To determine the clinicopathological characteristics of cases with AMLL, the clinical data for cases including laboratory findings are summarized in Table I. As indicated by the flow-cytometric data, bone marrow blasts in these cases exhibited a high frequency of B-cell lineage antigen (CD19) and myeloid cell marker (CD13 and/or CD 33) expression. Thus, blasts of these cases were "biphenotypic." Chromosomal abnormalities were identified in 5 cases (cases 1, 3, 6, 7, and 8), and the Philadelphia chromosome was identified in two cases (cases 1 and 7). Although abnormalities involving chromosome 11q were identified in two cases (cases 1 and 8), the molecular rearrangement of the *mixed lineage leukemia* (*MLL*) gene located on chromosome 11q23 [32,33] was not observed at the chromosome level.

In spite of AML- and ALL-directed therapy (cytarabine, vincristine, etoposide, adriamycin, predonin, etc.), five patients failed to exhibit complete hematological remission, having blast persistence in bone marrow above 10%. Although complete remission could be induced by chemotherapy in four cases (cases 3, 6, 7, and 8), relapse with leukemic blast proliferation occurred within 6 months in two cases (cases 3 and 6, Table II). Overall, most cases exhibited a poor prognosis and the survival times after diagnosis were shorter than 14 months for 5 cases. However, one patient who received a bone marrow transplant (case 6) and the other patients who received chemotherapy (cases 7 and 8) lived.

Double Immunostaining for Myeloid and Lymphoid Cell Markers on AMLL Cells

To confirm the biphenotypic nature of blasts in the AMLL samples, double immunostaining for myeloid and lymphoid cell markers was performed. The majority of AMLL cells exhibited positive signals for B-cell markers such as CD20 or CD79a, while the myeloid cell marker (myeloperoxidase) was partially observed for many of the cases examined

TABLE II. Treatment and Outcome of Cases With Adult AMLL*

Case no.	First treatment	Response and status	Second treatment	Response status	Survival (months)
1	A-VVV	Failure	H-CPM/VP-16	Failure	3
2	H-CPM/VP-16, H-AraC+MIT	Failure	TBI + CPM	Failure	5
3	DCM, H-AraC+MIT	CR, relapse	A-VVV, H-AraC	Failure	6
4	L-AdVP, MVP	Failure	B-VVV, H-CPM/VP-16, H-AraC, L-AdVP	Failure	11
5	AdVP	Failure	A-VVV, VP-16, CAG	Failure	14
6	DC, A-VVV, H-CPM/VP-16	CR, relapse	H-AraC + MIT, BMT	CR and alive	>6
7	A-VVV	CR	H-AraC + MTX	CR and alive	>6
8	CAG	CR	DC	CR and alive	>9

*Abbreviations: A-VVV, AraC (cytarabine) + VCR (vincristine) + VLB (vinblastine) + VP-16 (etoposide); H-CPM, high-dose CPM (cyclophosphamide); H-AraC, high-dose AraC; MIT, mitoxantrone; TBI, total body irradiation; DCM, DNR (daunorubicin) + AraC + 6-MP (mercaptopurine); CR, complete remission; L-AdVP, L-Asp (L-asparaginase) + ADR (doxorubicin) + VCR + PDN (predonin) + CPM; MVP, MIT + VP-16 + PDN; B-VVV, BHAC (enocitabine) + VCR + VLB + VP-16; CAG, AraC + ACR (acurabicin) + G-CSF (lenograstim); DC, DNR + AraC; BMT, bone marrow transplantation; MTX, methotrexate.

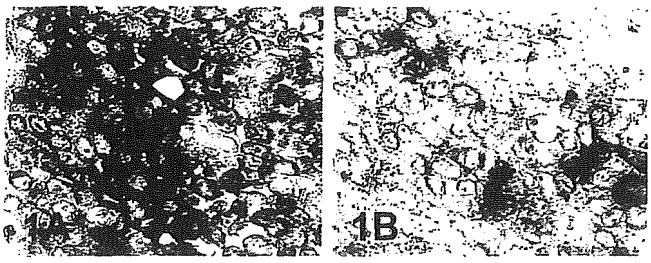

Fig. 1. Double immunostaining for a B-cell marker (CD79a) and myeloid cell marker (myeloperoxidase) in cases with AMLL (A, case 8; and B, case 6; original magnification 400×). Note that the majority of blasts stained positively for CD79a (brown) and a portion of them also stained positive for myeloperoxidase (blue) in both cases. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 1A for case 8 and Fig. 1B for case 6). These findings were consistent with the flow-cytometric analytical data shown in Table I.

Apoptotic Frequency and Proliferation Activity of Acute Mixed Lineage Leukemia

To identify the apoptotic and proliferative cells present in the bone marrow samples, the TUNEL method and immunohistochemistry for Ki-67 were performed on paraffin-embedded sections. As expected from our previous studies [7,10], the frequency of apoptosis was significantly lower in AML (median, 0.769; range, 1.06–0.219) ($P < 0.001$) and ALL bone marrow cells (median, 0.543; range, 1.18–0.072) ($P < 0.01$) than control cells (median, 2.03; range, 2.81–0.848), and the proliferative cell ratio in AML/ALL bone marrow (median, 39.7; range, 47.8–32.4/median, 45.9; range, 71.9–34.2) was significantly higher than that in control cases (median, 19.2; range, 24.3–10.0) ($P < 0.0001$ and $P < 0.001$, respectively). As shown in Table III, AMLL cells exhibited a tendency similar to AML and ALL cells in that the apoptotic ratio (median, 0.176; range, 1.69–0.021) was significantly lower than the control ($P < 0.01$) and the proliferative cell ratio (median, 26.7; range, 49.1–18.3) was significantly higher ($P < 0.01$). However, AMLL cells exhibited a relatively lower apoptotic index and also significantly lower proliferative index compared with the AML ($P < 0.05$) or ALL samples ($P < 0.05$).

Expression of IAP-Family Proteins Determined by Real-Time Quantitative PCR

To quantitate the mRNA expression levels of the IAP-family members in AMLL cells, real-time quantitative RT-PCR was performed using bone marrow samples from control, AML, ALL, and AMLL cases.

TABLE III. Apoptotic Frequency and Proliferation Activity of Bone Marrow Cells From Control and Acute Leukemia Cases*

	TUNEL$^+$ cell ratio (%)	Ki-67$^+$ cell ratio (%)
Cases	Median (max-min)	Median (max-min)
Control	2.03 (2.81–0.848)^{a,b,c}	19.2 (24.3–10.0)^{d,e,f}
AML	0.769 (1.06–0.219)^{a}	39.7 (47.8–32.4)^{d,g}
ALL	0.543 (1.18–0.072)^{b}	45.9 (71.9–34.2)^{e,h}
AMLL	0.176 (1.69–0.021)^{c}	26.7 (49.1–18.3)^{f,g,h}

*Values indicate the median, maximum, and minimum. Differences were significant between the TUNEL-positive cell ratio for control and AML ($^aP < 0.001$), control and ALL ($^bP < 0.01$), and control and AMLL ($^cP < 0.01$) as seen by the Mann–Whitney U-test. The Ki-67-positive cell ratio exhibited significant differences between control and AML ($^dP < 0.0001$), control and ALL ($^eP < 0.001$), control and AMLL ($^fP < 0.01$), AML and AMLL ($^gP < 0.05$), and ALL and AMLL ($^hP < 0.05$) as seen by the Mann–Whitney U-test.

As shown in Fig. 2, the expression of survivin ($P < 0.05$), cIAP1 ($P < 0.05$), NAIP ($P < 0.01$), and XIAP ($P < 0.01$) exhibited significant up-regulation in AMLL compared with the controls. The mRNA for survivin ($P < 0.05$) showed significantly higher levels of expression in AMLL than AML, while the expression levels of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$) in AMLL were significantly higher than those in ALL.

In summary, survivin expression in AMLL was significantly higher than the expression in control, AML, and ALL. The expression level of cIAP1 in AMLL was significantly higher than that in control, but similar with the expression in AML and ALL. Regarding cIAP2, the AMLL cases exhibited stronger expression than the control, AML, and ALL samples although the differences were not significant. NAIP expression in AMLL was significantly higher than control and ALL. The expression level of XIAP in AMLL was significantly higher than control and ALL but similar with AML. No remarkable differences were found between IAP protein expression and patients' age, sex, phenotype, or genotype for AMLL, although further analysis would be necessary because the number of cases was rather small.

These results indicate that the overall expression of IAP-family proteins in AMLL subjects tended to be higher than that for the control, AML, or ALL samples. Specifically, survivin expression in AMLL was significantly higher than that for the control, AML, and ALL samples.

Immunohistochemical Detection of Survivin in the Bone Marrow of AMLL Subjects

To investigate the distribution of survivin, immunohistochemical staining was performed on bone

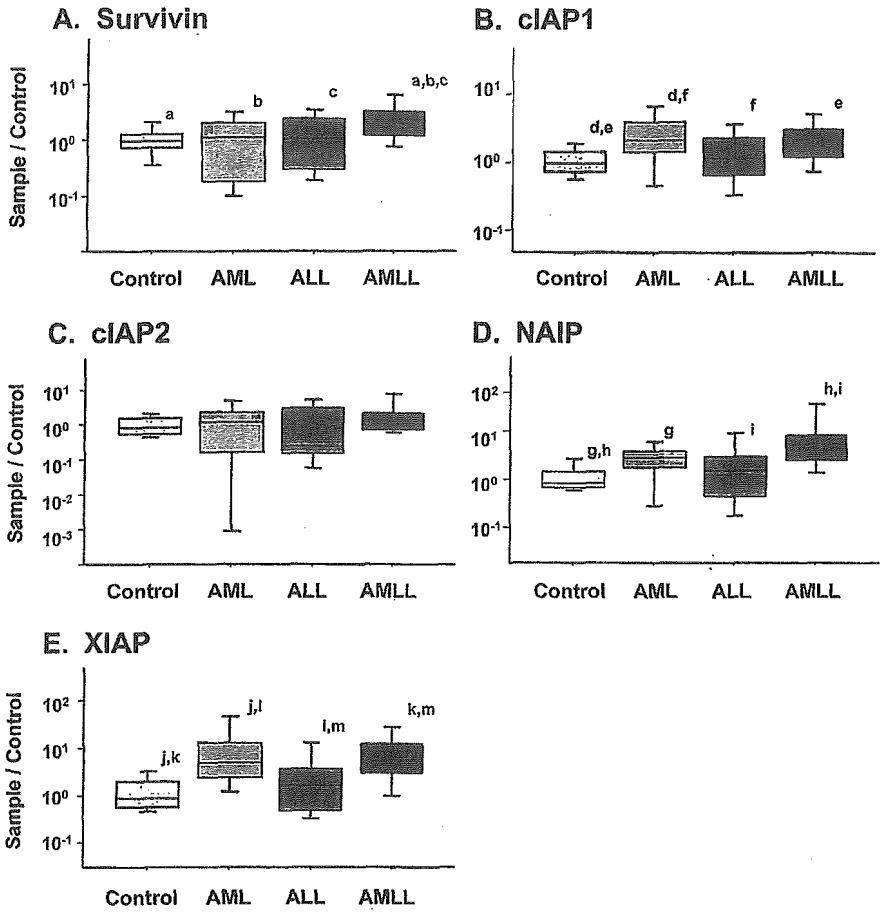

Fig. 2. Expression of IAP-family proteins in control bone marrow and acute leukemias determined by real-time quantitative RT-PCR. The relative intensity was calculated as (intensity of the reaction of IAP-family members [total Raji RNA, ng])/(intensity of the reaction of GAPDH [total Raji RNA, ng]). The intensities of the expressions from the AML, ALL, and AMLL samples are indicated as the ratios to the intensity of the control subjects. The box-bar graphs indicate the value of the control, AML, ALL, and AMLL cases: the bars indicate the 90th and 10th percentiles, and the box indicates the 75th to 25th percentiles. Differences were significant between samples as seen by the Mann–Whitney *U*-test as follows: (A) survivin—control and AMLL ($^aP < 0.01$), AML and AMLL ($^bP < 0.05$), and ALL and AMLL ($^cP < 0.05$). (B) cIAP1—control and AML ($^dP < 0.01$), control and AMLL ($^eP < 0.05$), and AML and ALL ($^fP < 0.05$). (C) cIAP2—differences were not significant. (D) NAIP—control and AML ($^gP < 0.05$), control and AMLL ($^hP < 0.01$), and ALL and AMLL ($^iP < 0.05$). (E) XIAP—control and AML ($^jP < 0.01$), control and AMLL ($^kP < 0.01$), AML and ALL ($^lP < 0.05$), and ALL and AMLL ($^mP < 0.05$).

marrow samples from AMLL subjects. As we previously showed [13], survivin was detected in only a few scattered myeloid cells in the control bone marrow samples and subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity in the control bone marrow was constant between different samples. All of the AMLL samples showed positive staining for survivin, although the staining intensity and frequency varied for each case. At the cellular level, survivin signals in AMLL cells were predominantly localized in the nucleus and also weakly in the cytoplasm (Fig. 3A). However, one case exhibited prominent cytoplasmic staining with mildly positive staining in the nucleus (Fig. 3B). The tissue sections that reacted with preimmune rabbit antibody of nonrelevant specificity showed no significant staining for any of the samples (not shown).

DISCUSSION

AMLL blasts are expected to possess more immature or intermediate characters of AML and ALL blasts because they express both myeloid and lymphoid phenotypes. Regarding the expression of survivin in myeloid neoplasms, previous studies have revealed the significant expression of survivin in AML [34,35]. Adida et al. [35] reported that survivin expression frequently occurs in AML, detecting it in 60% of a series of 125 patients analyzed, and survivin expression was found to be an unfavorable prognostic factor. In contrast, in lymphoid neoplasms, several

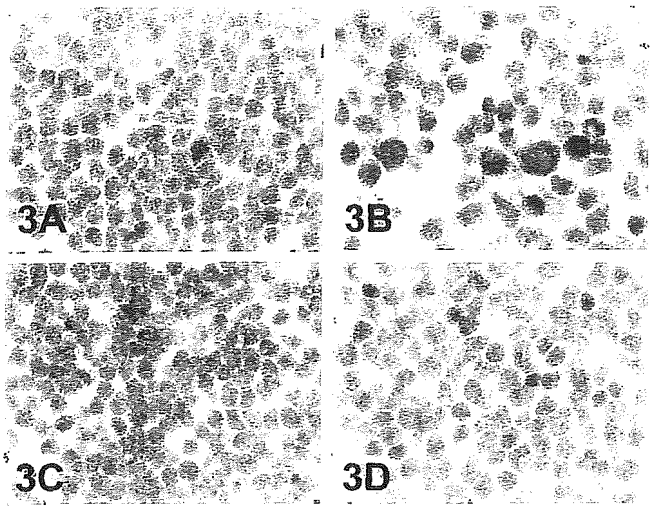

Fig. 3. Immunostaining for survivin in the bone marrow of AMLL (A, case 5; and B, case 8) in comparison with AML (C) and ALL (D) (original magnification 400×). Development was performed using the peroxidase–DAB system (brown) with hematoxylin counterstaining. Note the positive signals in the nucleus as well as the cytoplasm of AMLL cells (A) in contrast to the cytoplasmic staining (B). AML (C) and ALL (D) cases exhibited nuclear and partial cytoplasmic staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

studies investigated the dynamics of survivin expression in association with cell proliferation. The in vitro data on mononuclear cells from peripheral blood or bone marrow indicated that B-CLL cells expressed survivin in concert with CD40 and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [36]. CD40 belongs to the TNF receptor superfamily [37], and its stimulation rescues B-CLL cells from apoptosis and induces proliferation [38]. We recently found that ALL as well as CLL cells exhibited significant expression of survivin and cIAP2 [13]. Thus, both in myeloid and lymphoid neoplasms, IAPs are expressed and seemed to influence the prognosis of patients. Therefore, we can imagine that IAPs would have functions also in AMLL blasts; however, little is known about the potential roles of survivin and other IAPs in the pathogenesis of AMLL.

A major problem with leukemia treatment is drug resistance to chemotherapeutic agents, which may already be present upon diagnosis or after chemotherapy for minimal residual blasts. Resistance originates from genetic or epigenetic mutations during growth of the leukemic clone. Anti-apoptosis mechanisms, alterations of tumor suppressor genes, altered immunogenicity, and drug-resistance mechanisms act in combination [39]. AMLL exhibits strong resistance against chemotherapy, resulting in poor patient prognosis [40,41]. In the present study, expression levels of IAPs in AMLL blasts were higher than those in control samples. Furthermore, several IAPs, such as survivin, NAIP, and XIAP, exhibited stronger expression in AMLL compared with conventional acute leukemias. Thus, the IAP expression level is one criterion that can be used to explain the strong drug resistance in this category of leukemia. The IAP might function probably via the inhibition of caspase-dependent apoptotic signaling. Although we have yet to clarify the caspase-independent pathway of apoptosis in AMLL, the findings of the present study suggest that the regulation of IAPs may become a possible target of AMLL therapy in the future.

In addition to its anti-apoptotic function, survivin also helps regulate cell-cycle progression during mitosis [20]. The highly proliferative activity of AMLL bone marrow cells as well as AML/ALL cells might be associated with survivin expression. As for the expression of IAPs in AML/ALL, the present study found strong expression in some cases and control levels in others, suggesting that AML/ALL cases are heterogeneous in terms of IAP expression.

The human *MLL* gene is involved in about 50 different chromosomal translocations associated with the acute leukemia phenotype [42]. Although chromosomal rearrangement involving chromosome 11q23 was not identified, the cases in the present study were not examined for the presence of *MLL* gene rearrangement by PCR analysis at the DNA level. Further studies are necessary to clarify the interaction of the *MLL* gene and IAP-family genes in association with apoptotic signaling in AMLL blasts.

In conclusion, we showed that strong expression of IAPs, especially survivin and NAIP, occurs in AMLL. Further studies are warranted to clarify the regulatory mechanisms of IAP expression in AMLL in association with drug resistance in this leukemia.

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Expression of IAP family proteins in myelodysplastic syndromes transforming to overt leukemia

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Abstract

Bone marrow cells of patients with myelodysplastic syndromes (MDS) frequently undergo apoptosis, though the apoptotic cell ratio decreases when overt leukemia (OL) develops. Thus, we compared the expression of the inhibitor of apoptosis protein (IAP) gene family proteins in bone marrow samples from control, MDS, OL transformed from MDS (MDS → OL), and de novo acute myelogenous leukemia (AML) subjects by the quantitative real-time RT-PCR method and an immunohistochemical approach. Overexpression of mRNA for survivin, cIAP1, NAIP and XIAP was significant in MDS bone marrow cells compared with control samples. However, the expression of mRNA for survivin, cIAP1 and cIAP2 exhibited a remarkable decrease after the development of OL (MDS → OL). By immunohistochemistry, survivin was found to localize to the nucleus of myeloid cells in the majority of MDS cases. Next, the chronological changes in the expression of IAPs were determined in cases of MDS with evolution of OL. Although the expression of cIAP1 and cIAP2 revealed a sudden or gradual decrease as OL developed, survivin in many cases and XIAP in the majority of cases exhibited a peak of expression before a decline, indicating that these IAPs could be associated with the early events in the development of OL.

Keywords: Survivin; IAP; Apoptosis; MDS; Overt leukemia; Bone marrow

1. Introduction

The regulation of apoptotic cell death may have a profound effect on the pathogenesis and progression of hematological malignancies. Myelodysplastic syndromes (MDS) are a heterogenous group of hematological malignancies exhibiting peripheral blood cytopenias despite bone marrow hypercellularity [1–3]. Therefore, one characteristic feature of MDS is ineffective hematopoiesis mainly caused by frequent apoptosis of bone marrow hematopoietic cells [4,5]. Regarding the complicated regulating mechanisms regulating apoptosis in MDS bone marrow, we demonstrated previously that a variety of cytokines and their receptors, such as tumor necrosis factor (TNF)-α/TNFRI and II, Fas-ligand/Fas, play an important role in hematopoietic cells [6–10]. However, another distinctive feature of MDS is the potential to develop into overt leukemia (OL) [11]. Leukemic cells derived from MDS usually show marked proliferation in the bone marrow [12], thus these cells have gained the ability to grow rapidly and/or avoid apoptosis.

Inhibitor of apoptosis protein (IAP) family proteins, including survivin, block apoptosis induced by a variety of triggers [13,14]. Although the exact biochemical mechanism by which the IAP family suppresses apoptosis has been debated, survivin is known to bind directly to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [14,15]. The expression of survivin is ubiquitous in fetal tissues, but becomes restricted during development, and appears to be negligible in the majority of terminally differentiated adult tissues [16,17]. However, an analysis of the differences in gene expression between normal and tumor cells has revealed that survivin is one of the proteins whose gene is most consistently overexpressed in tumor cells relative to normal tissue [18]. In fact, survivin

Abbreviations: IAP, inhibitor of apoptosis proteins; MDS, myelodysplastic syndromes; OL, overt leukemia; AML, acute myelogenous leukemia

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is prominently expressed in transformed cell lines and in many human cancers including hematopoietic cell tumors [19]. Survivin is usually detected in the cytoplasm of tumor cells, and is therefore widely regarded as a cytoplasmic protein [16,20,21]. However, several studies have shown a nuclear accumulation of survivin in gastric cancer cells [22] and lung cancer cells [23]. We have reported recently that ALL cells principally exhibit a nuclear localization of survivin, while CLL cells have a cytoplasmic distribution [24]. Although the significance of this nuclear–cytoplasmic expression in tumor cells is still controversial, the subcellular localization of survivin should also be clarified in MDS, OL and de novo acute myelogenous leukemia (AML) subjects.

To focus on the contribution of IAPs to the progression from MDS to OL, we collected cases of MDS that developed into OL in the present study. The overall level of expression of survivin was relatively high in the bone marrow cells from these MDS samples compared with control bone marrow, however, the level of expression appeared lower when OL developed. The expression of other IAPs including cIAP1, cIAP2, NAIP and XIAP, all of which appeared to suppress apoptosis by inhibiting caspase and procaspase [25–28] was also demonstrated in these samples and the significance of IAP family proteins in the transformation of MDS to OL was discussed.

2. Materials and methods

2.1. Patients

Formalin-fixed paraffin-embedded bone marrow aspiration samples from 13 individuals with no hematological disorders as normal controls (male:female, 13:0; age, median 63 years, maximum 76 years, minimum 51 years), 13 patients with MDS (3 with refractory anemia (RA), 4 with RA with excess of blasts (RAEB) and 6 with RAEB in transformation (RAEB-t); male:female, 10:3; age, median 67 years, maximum 77 years, minimum 55 years) who developed overt leukemia later in the course (duration, 3 months to 3 years), and 10 patients with de novo AML (9 with M2 and 1 with M1 according to the FAB classification, male:female, 4:6; age, median 55 years, maximum 76 years, minimum 23 years) were analyzed. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Diagnoses were based on standard clinical and laboratory criteria, including cell morphology [1–3]. All samples were collected at the time of the initial aspiration biopsy and the samples from de novo AML exhibited a proliferation of blastic cells accounting for more than 80% of all bone marrow cells. The patients were not infected with specific viruses including HTLV-1 and had not been treated with therapeutic drugs prior to the study. Further, bone marrow samples were taken from MDS cases when the patients developed OL and analyzed.

In addition, fresh frozen bone marrow samples from control (5 cases, age, median 55 years, maximum 74 years, minimum 43 years; male:female, 3:2), MDS (11 cases, 3 with RA, 5 with RAEB and 3 with RAEB-t; male:female, 8:3; age, median 66 years, maximum 77 years, minimum 55 years) with transformation to OL, and de novo AML (5 cases of M2 by FAB classification, male:female, 3:2; age, median 62 years, maximum 76 years, minimum 49 years) cases were used for the PCR experiments. In MDS, we could follow the bone marrow changes before and after the transformation to OL and further, chronological changes determined at more than three points could be analyzed in six cases.

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

2.2. Identification of apoptotic cells

To identify apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used as described previously [29]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO, Glostrup, Denmark) for 15 min at room temperature. After a wash, TdT, fluorescein isothiocyanate (FITC)-dUTP and dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37 °C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed for detecting FITC-dUTP labeling, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. Sections were then observed under a microscope and the proportion of TUNEL-positive cells was determined by dividing the number of positively stained cells by the total cell number (counting more than 1000 cells).

2.3. Preparation of RNA and quantitative assay for IAP family proteins using TaqMan RT-PCR

The RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, MDS patients and de novo AML patients 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 (Perkin-Elmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (Perkin-Elmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the IAP family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (Perkin-Elmer Cetus). The primers and TaqMan probes were as follows. The sequence of the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that of the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; the

TaqMan probe was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer was 5′-CAAGCCACCATCACAACAAAA-3′; the TaqMan probe was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer was 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-3′. The forward primer for NAIP mRNA was 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer was 5′-GCTGGGCGGATGCTTTC-3′; the TaqMan probe was 5′-CCATTTAAACCACAGCAGAGGCTTTAT-3′. The forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. The forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. Conditions for the one-step RT-PCR were as follows: 30 min at 48 °C (stage 1, reverse transcription), 10 min at 95 °C (stage 2, RT inactivation and AmpliTaq Gold activation) and then 40 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C (stage 3, PCR). The expression of survivin and other IAP family proteins was quantitated according to a method described elsewhere [24]. Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the IAP family were normalized using the data for GAPDH in each sample.

2.4. Immunohistochemistry for survivin and proliferative cells

Formalin-fixed tissue sections (4 μm thick) of bone marrow from control (13 cases), MDS (13 cases) and de novo AML cases (10 cases) were cut on slides covered with adhesive. Sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were applied to identify survivin, and to characterize proliferative cells. Primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, San Antonio, TX) and monoclonal antibody Ki-67 (DAKO). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted for the primary antibody. The proportion of Ki-67-positive cells was determined in the same way as the proportion of TUNEL-positive cells.

2.5. Double staining for survivin and cell markers

The phenotype of survivin-expressing cells was identified by double immunostaining using a polyclonal antibody against survivin and a monoclonal antibody against glycophorin A (DAKO), anti-neutrophil elastase (DAKO) or anti-CD34 antibody (Nichirei, Tokyo, Japan) followed by the anti-rabbit IgG peroxidase-DAB development system and then, alkaline phosphatase-conjugated anti-mouse IgG (DAKO) followed by development with the alkaline phosphatase–nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphatase development system (DAKO).

2.6. Statistical analysis

Statistically significant differences in the quantitative analysis were determined using Mann–Whitney's *U*-test for comparisons between control, MDS, MDS → OL and de novo AML samples except for the comparison of paired samples from MDS and MDS → OL, for which Wilcoxon's test was used.

3. Results

3.1. Apoptotic and proliferative cell ratio of the bone marrow cells from control, MDS, overt leukemia derived from MDS (MDS → OL), and de novo AML subjects

To identify the apoptotic and proliferative cells in the bone marrow samples, the TUNEL method and immunohistochemistry for Ki-67 were performed on paraffin-embedded sections. As expected from our previous studies [12,29], the frequency of apoptosis was significantly higher in MDS bone marrow cells than control cells or de novo AML cells and the proliferative cell ratio in MDS bone marrow was as high as that in AML cases and significantly higher than that of control bone marrow ($P < 0.01$ by the Mann–Whitney's *U*-test). When OL developed (MDS → OL), the apoptotic cell ratio was reduced in MDS bone marrow, in spite that the proliferative cell ratio was similar to that in MDS before transformation to OL (Table 1). These findings suggested that the proliferation of blasts of MDS-derived overt leukemia in the bone marrow occurred in association with reduced apoptosis but not with the increased activity for cell proliferation.

3.2. Expression of mRNA for IAP proteins determined by real-time quantitative PCR

To quantitate the mRNA expression levels of the IAP family in MDS bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow samples from control, MDS, MDS → OL and de novo AML cases. As shown in Fig. 1, the expression of survivin ($P < 0.05$),

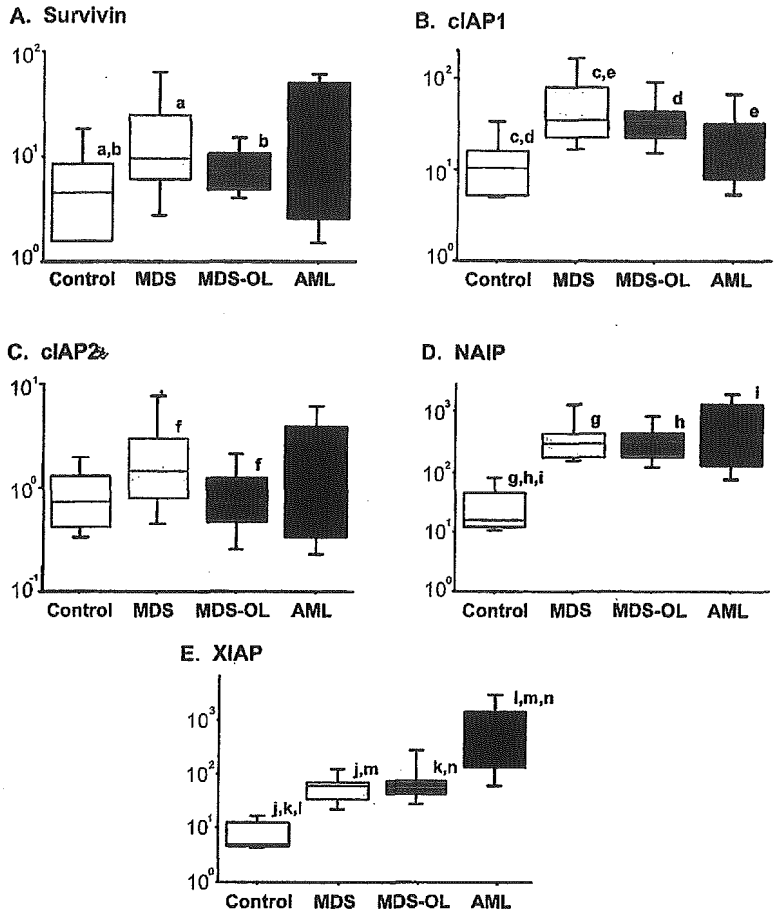

Fig. 1. Quantitative RT-PCR analysis of IAP family proteins, survivin, cIAP1, cIAP2, NAIP and XIAP in control ($n = 5$), MDS ($n = 11$), MDS → OL ($n = 11$) and de novo AML cases ($n = 5$). Relative intensity was calculated as (intensity of reaction of IAP family (total Raji RNA, ng))/(intensity of reaction of GAPDH (total Raji RNA, ng)). The box plot graphs indicate the values for MDS and AML cases. Bars indicate 90 and 10 percentile and boxes indicate 25–75 percentile. Differences were significant between the samples as follows using Mann–Whitney's U-test or Wilcoxon's test: survivin, control and MDS ($^{a}P < 0.05$), and control and MDS → OL ($^{b}P < 0.01$); cIAP1, control and MDS ($^{c}P < 0.01$), control and MDS → OL ($^{d}P < 0.01$), and MDS and de novo AML ($^{e}P < 0.01$); cIAP2, MDS and MDS → OL ($^{f}P < 0.05$); NAIP, control and MDS ($^{g}P < 0.01$), control and MDS → OL ($^{h}P < 0.001$), and control and de novo AML ($^{i}P < 0.01$); XIAP, control and MDS ($^{j}P < 0.01$), control and MDS → OL ($^{k}P < 0.01$), control and de novo AML ($^{l}P < 0.05$), MDS and de novo AML ($^{m}P < 0.01$), and MDS → OL and de novo AML ($^{n}P < 0.01$).

cIAP1 ($P < 0.01$), NAIP ($P < 0.01$) and XIAP ($P < 0.01$) exhibited a significant up-regulation in MDS compared with controls. The mRNA for survivin, cIAP1, NAIP and XIAP and cIAP2 also revealed higher level of expression in de novo AML bone marrow than control marrow, although the difference was significant only for NAIP ($P < 0.01$) and XIAP ($P < 0.05$). The expression of cIAP1 was significantly higher ($P < 0.01$) and that of XIAP significantly lower ($P < 0.01$) in MDS samples than de novo AML samples. The MDS → OL samples had lower levels of XIAP than de novo AML samples ($P < 0.01$). No significant differences were found between IAP protein expression and the patients' age, sex, or subtype of MDS or AML. Regarding the IAPs in MDS → OL bone marrow, survivin, cIAP1, and cIAP2 tended to exhibit a low level of expression compared with that in the initial MDS samples, although statistical significance was demonstrated only in the case of cIAP2 ($P < 0.05$).

These results indicated that the expression of IAP family proteins might not correlate with the frequency of apoptosis in bone marrow cells, because highly apoptotic MDS bone marrow cells exhibited higher levels of IAPs. Further, MDS → OL samples tended to have lower expression levels of IAPs and thus, the less frequent apoptosis in fully-developed OL would not to be associated with the overexpression of IAPs.

3.3. *Immunohistochemical detection of survivin in the bone marrow of control, MDS, and overt leukemia derived from MDS (MDS → OL) subjects*

To investigate the distribution of survivin, immunohistochemical staining was performed in bone marrow samples from control, MDS, and MDS → OL subjects. As we have demonstrated previously [24], survivin was detected in only a few scattered myeloid cells of the control bone marrow

Table 1
Apoptotic and proliferative cell ratio of bone marrow from control, MDS, MDS → OL and AML subjects

Cases	*n*	TUNEL$^+$ cell ratio (%)[a]	Ki-67$^+$ cell ratio (%)[a]
Control	13	2.3 (1.3–3.9)[a,b,c]	4.7 (3.2–6.7)[f,g,h]
MDS	13	15.6 (7.5–24.3)[a,d,e]	30.6 (13.9–36.4)[f,i]
MDS → OL	13	8.6 (4.4–14.8)[b,d]	29.1 (12.0–47.5)[g,j]
De novo AML	10	9.4 (4.0–20.0)[c,e]	20.4 (9.1–31.6)[h,i,j]

[a] Values indicate median (minimum–maximum). Differences were significant between the TUNEL-positive cell ratio of control and MDS ([a]$P < 0.0001$), control and MDS → OL ([b]$P < 0.0001$), and control and de novo AML ([c]$P < 0.0001$) with Mann–Whitney's *U*-test, MDS and MDS → OL ([d]$P < 0.01$) using Wilcoxon's test, and MDS and de novo AML ([e]$P < 0.05$) with Mann–Whitney's *U*-test. The Ki-67-positive cell ratio exhibited significant differences between control and MDS ([f]$P < 0.0001$), control and MDS → OL ([g]$P < 0.0001$), control and de novo AML ([h]$P < 0.0001$), MDS and de novo AML ([i]$P < 0.05$), and MDS → OL and de novo AML ([j]$P < 0.01$) with Mann–Whitney's *U*-test.

samples. The subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity in the control bone marrow was constant between different samples. All of the MDS samples revealed positive staining for survivin, although the staining intensity and frequency varied among cases. At the cellular level, survivin signals in MDS bone marrow cells were predominantly localized to the nucleus, although in some cases, a strong reaction was also observed in the cytoplasm. Survivin-positive cells basically resembled myeloid cells in morphology. Double immunostaining confirmed that the majority of survivin-positive cells also expressed neutrophil elastase, a myeloid cell marker, but not the erythroid cell marker glycophorin A (Fig. 2A and B). The CD34 antigen, stem cell marker, was expressed in some of the survivin positive cells (Fig. 2C). The differences in staining intensity and patterns for survivin were not evident between MDS samples and MDS → OL samples from the same cases. Tissue sections reacted with preimmune rabbit antibody of irrelevant specificity showed no significant staining in any of the samples (not shown).

3.4. Chronological changes of IAP expression in bone marrow samples from cases of MDS leading to overt leukemia

The apoptotic frequency of bone marrow cells exhibited a significant decrease during the transformation of MDS into OL as described earlier (Table 1). Thus, to determine whether the IAP family proteins play a role in inhibiting apoptotic signals during the development of OL, real-time quantitative PCR was performed in specimens sampled chronologically from the time of the initial diagnosis of MDS to the time of the transformation to OL. Representative patterns of IAP expression in two cases of MDS (cases 1 and 4) with progression to OL are shown in Fig. 3. The patterns of change in IAP expression could be classified into five types; (1) a gradual decrease in expression from MDS to MDS → OL (e.g., Fig. 3, cIAP2 in cases 1 and 4) (decrease: D); (2) a high expression level but a sudden fall before the development of OL (e.g., Fig. 3, cIAP1 in cases 1 and 4, XIAP in case 4) (increase followed by a decrease: ID); (3) a high level of expression that increases further (not shown in Fig. 3) (increase: I); (4) low or high level expression, then, an increase beyond the initial level followed by a drop below the control level (e.g., Fig. 3,

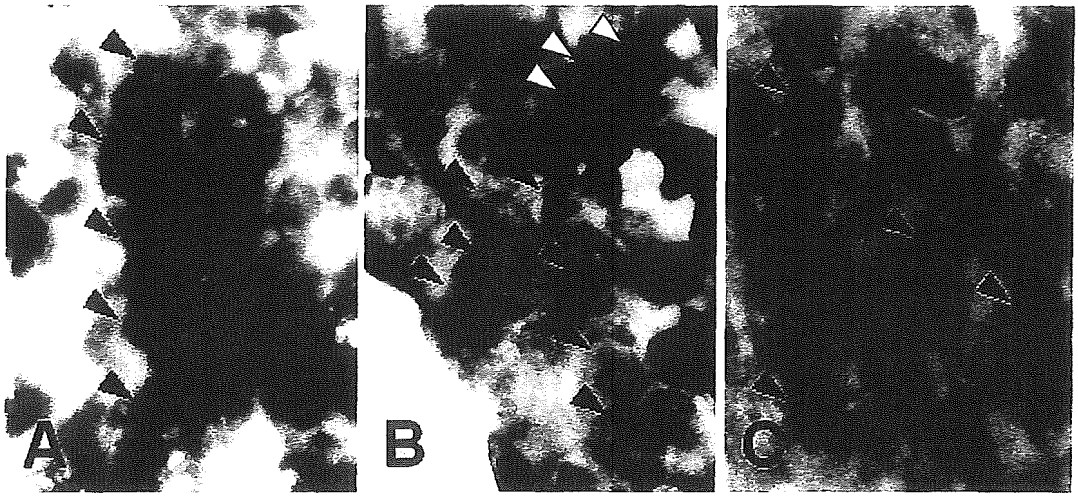

Fig. 2. Immunohistochemical detection of survivin in the bone marrow cells of MDS subjects (RAEB, 400× original magnification). Double staining for survivin (nuclear: brown) and cell markers (cytoplasmic/cell surface: blue) (A, neutrophil elastase; B, glycophorin A; C, CD34) demonstrated that survivin-positive cells were basically the myeloid series (A, neutrophil elastase-positive, arrowheads) and not the erythroid series of cells (B, black arrowheads indicate survivin-positive cells and white ones glycophorin A-positive cells). A part of survivin-positive cells were also positive for CD34 antigen (C, arrowheads). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

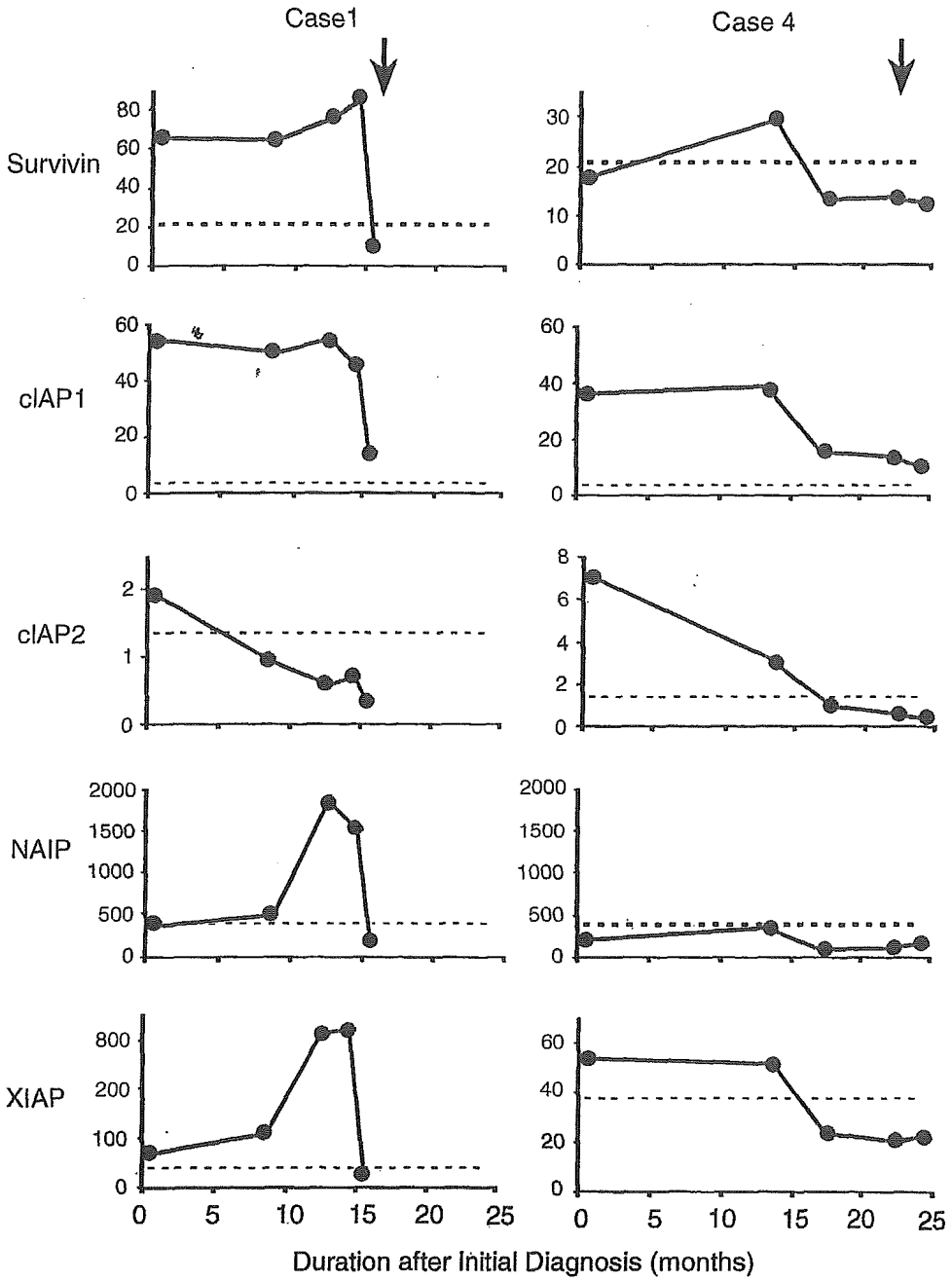

Fig. 3. Chronological changes of IAP family protein expression in MDS → OL cases (cases 1 and 4) determined by quantitative RT-PCR. Arrows indicate the time of OL evolution and dotted lines indicate median values of the control samples. Note that the dynamics of survivin and XIAP expression tended to form a peak between the time of initial diagnosis and OL development, while cIAP1 and cIAP2 expression decreased during the evolution of OL from MDS.

survivin in cases 1 and 4, NAIP in case 1) (peak formation: P); (5) stable low-level expression than an increase to the control level with no remarkable change (e.g., Fig. 3, NAIP in case 4) (not significant: N). The pattern ID and P would have the similar significance in the sense that the expression level once increased before the evolution of overt leukemia. And the changes forming peak expression (such as P and ID patterns) might be associated with the functional aspects of IAPs in the development of overt leukemia. As shown in Table 2, cIAP1 and cIAP2 expression basically revealed a sudden or gradual decrease from the initial diagnosis of MDS to the time that OL developed suggesting that these proteins are not associated with the suppression of apoptosis during the evolution of OL, although cIAP1 may have made a partial contribution in cases 5 and 6. By contrast, survivin in a half of the cases and XIAP in the majority of cases exhibited an increase in expression reaching a peak during the course (pattern 4, P). In cases with a decrease in the expression of IAP family proteins (patterns 1, D and 2, ID), the point of any peak might have been missed as

Table 2
Patterns of changes in expression of IAP family proteins during the course of evolution of OL in MDS bone marrows

	IAPs					Duration MDS → OL (months)
	Survivin	cIAP1	cIAP2	NAIP	XIAP	
Case 1	P[a]	ID	D	P	P	15
Case 2	ID	ID	D	D	P	3
Case 3	P	ID	D	ID	P	24
Case 4	P	ID	D	N	ID	24
Case 5	N	ID	ID	ID	P	35
Case 6	N	P	N	I	I	5

[a] Pattern 1, gradual decrease of expression from MDS to MDS → OL (decrease: D); pattern 2, high level of expression but a sudden decrease at the time of OL development (increase followed by a decrease: ID); pattern 3, an initially high level of expression that increases further (increase: I); pattern 4, an initially low or high level of expression, an increase beyond the initial level and then a reduction below the control level forming a peak during the course (peak formation: P); pattern 5, stable expression below the control level with no remarkable change (not significant: N).

seen in pattern 4, P. Thus, in most cases, survivin as well as XIAP could show a peak in expression during the development of OL. The expression of NAIP showed various patterns of change and did not exhibit specific changes during the transformation to OL.

In any event, MDS → OL bone marrow did not exhibit an increase in the expression of cIAP1 and cIAP2 proteins in most cases, although these proteins were highly expressed at the time of the initial diagnosis of MDS. Thus, the expression of these proteins would not be associated with the reduction in apoptosis in MDS → OL and would be secondarily regulated by other mechanisms. In contrast, survivin and XIAP might be associated with the mechanisms for avoiding apoptotic signals because they showed a peak of expression (pattern 4) or maintained a high level of expression (pattern 1 or 3) during the course of OL evolution in most cases.

4. Discussion

Regarding the expression of survivin in myeloid neoplasms, previous studies revealed overexpression in MDS samples including RA and RAEB [30] and a significant level of expression in de novo AML [31,32]. However, little is known about the potential roles of IAPs in the pathogenesis of MDS, MDS → OL and de novo AML. In lymphoid neoplasms by contrast, several studies have demonstrated the dynamics of survivin expression in association with cell proliferation. The in vitro data on mononuclear cells from peripheral blood or bone marrow indicated that B-CLL cells expressed survivin on stimulation with CD40 and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [33]. CD40 belongs to the TNF receptor superfamily [34] and its stimulation appears to rescue B-CLL cells from apoptosis and induce proliferation [35]. We have demonstrated recently that ALL as well as CLL cells exhibited significant expression of survivin and cIAP2 [24].

In MDS samples, the expression of IAPs was stronger than that in control samples. Although the number of samples would not be enough for evaluating rather a heterogeneous entity of hematological malignancy, MDS, we could find the significant elevation of expression level in MDS samples. Thus, increased apoptosis in MDS would not be attributable to a lack of anti-apoptotic regulatory mechanisms by IAP family proteins. In other words, down-regulation of IAP family expression might not be a primary event in MDS pathogenesis. To the contrary, up-regulation of IAP family proteins in MDS bone marrow appears to be an attempt at counter-balancing the primary stimuli for apoptosis. The levels of IAPs basically dropped below those of the initial MDS samples after OL developed. These findings are inconsistent with reports that MDS bone marrow cells frequently undergo apoptosis, while OL cells are less prone to apoptosis. The decline in IAP protein expression may be attributable to gene silencing, for example, by DNA hypermethylation. Although this interpretation is speculative, overexpression of IAPs in MDS would be the reversible change whether the down-regulation in MDS → OL samples might be irreversible/reversible. The mechanisms for OL cells to avoid apoptosis might include the loss of critical components of the apoptotic signaling machinery. However, a chronological analysis of IAP expression dynamics demonstrated that some IAPs exhibited a transient overexpression during the development of OL. The peak expression might be associated with the initial signaling to avoid apoptosis, although the mechanisms for down-regulation of IAP expression in MDS → OL remained unexplained. However, inhibition of apoptosis in MDS → OL should not be explained solely by the dynamics of IAP expression. Although we could not find specific cytogenetic abnormalities in MDS → OL samples, interactions with various factors including many kinds of apoptosis-regulating molecules should be clarified in the future study.

Immunohistochemical staining revealed a very high frequency of survivin expression in many of the MDS cases though only a few cells were positive in some cases. Thus, differences in the positive cell ratio would influence the differences in intensity in the overall expression of mRNA for IAPs. As suggested by the double immunostaining in the present study, the survivin positivity in myeloid cells, but not erythroid cells, might reflect the growth advantage of apoptosis-resistant neoplastic stem cells compared to normal stem cells, and also the tendency of survivin-negative erythroid cells to undergo apoptosis. It would be important to correlate the genetic events/apoptotic signals and survivin-expressing cell clones in a future study.

In addition to its anti-apoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis [19]. The highly proliferative activity of MDS bone marrow cells as well as AML cells might be associated with the expression of survivin. Adida et al. [32] reported that

survivin expression occurs frequently in de novo AML, detected in 60% of a series of 125 patients analyzed, and was an unfavorable prognostic factor. However, we could not find a significant difference in the expression of IAPs between the initial samples from MDS → OL patients who exhibited a short or long progression to OL (data not shown). As to the expression of IAPs in de novo AML, the present study demonstrated strong expression in some cases and control levels in others, suggesting that de novo AML cases are heterogeneous in terms of IAP expression. Overall, the expression of IAPs was relatively higher in de novo AML than MDS → OL samples, and a significant difference was observed in XIAP expression. Thus, the apoptosis/proliferation of bone marrow cells would be controlled via IAP family proteins differently between de novo AML and MDS → OL cases. Furthermore, it would be useful to determine XIAP expression to distinguish MDS → OL from de novo AML clinically.

Wild-type p53, but not mutant p53, represses survivin expression at both the mRNA and protein levels [36]. The modification of chromatin within the survivin promoter would explain the silencing of survivin gene transcription by p53 [37]. On the other hand, the overexpression of exogenous survivin protein rescues cells from p53-induced apoptosis in a dose-dependent manner, suggesting that loss of survivin mediates in part the p53-dependent apoptotic pathway [37]. In contrast to the high frequency of p53 mutations in many of the solid cancers, MDS (5–17%) and AML (10–20%) have been shown to demonstrate a rather low frequency of p53 mutation [38]. Therefore, p53 mutation would not appear to be the major factor controlling the overexpression of survivin in the bone marrow of patients with MDS and AML.

In conclusion, we demonstrated the transient overexpression of mRNA for several IAPs during the progression from MDS to OL, although the expression decreased below the initial level after the transformation was completed. Clinically, it would be useful to detect the increase in expression of mRNAs of IAPs for predicting the evolution of OL in MDS patients. However, the levels of mRNA could not reflect protein expression status directly. Further study is warranted to clarify the regulatory mechanisms of IAP expression in MDS → OL in association with the apoptotic signaling pathways.

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DNA-dependent protein kinase enhances DNA damage-induced apoptosis in association with Friend gp70

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Abstract

Friend leukemia virus (FLV) infection strongly enhances γ-irradiation-induced apoptosis of hematopoietic cells of C3H hosts leading to a lethal anemia. Experiments using *p53* knockout mice with the C3H background have clarified that the apoptosis is *p53*-dependent and would not be associated with changes of cell populations caused by the infection with FLV. In bone marrow cells of FLV + total body irradiation (TBI)-treated C3H mice, the p53 protein was prominently activated to overexpress *p21* and *bax* suggesting that apoptosis-enhancing mechanisms lay upstream of p53 protein in the signaling pathway. Neither of DNA-dependent protein kinase (DNA-PK)-deficient SCID mice nor ataxia telangiectasia mutated (*ATM*) gene knockout mice with the C3H background exhibited a remarkable enhancement of apoptosis or p53 activation on FLV + TBI-treatment indicating that DNA-PK and ATM were both essential. ATM appeared necessary for introducing DNA damage-induced apoptosis, while DNA-PK enhanced *p53*-dependent apoptosis under FLV-infection. Surprisingly, viral envelope protein, gp70, was co-precipitated with DNA-PK but not with ATM in FLV + TBI-treated C3H mice. These results indicated that FLV-infection enhances DNA damage-induced apoptosis via p53 activation and that DNA-PK, in association with gp70, might play critical roles in modulating the signaling pathway.

Keywords: DNA damage; Apoptosis; Friend virus; DNA-PK; ATM

1. Introduction

p53 has important roles in the cellular response to DNA damage-inducing agents such as ionizing irradiation [1–3]. Ionizing irradiation induces DNA-double strand breaks in cells, and then the stabilization and accumulation of p53 protein by phosphorylation of the N-terminal serine residues, leading to a disruption of MDM2 interaction which negatively regulates p53 [4,5]. In response to DNA damage, p53 protein is also activated by phosphorylation or acetylation allowing conformational changes convenient for the DNA binding domain to play a role. Activated p53 binds to specific DNA sequences and acts as the transcription factor whose target genes are mainly involved in cell cycle arrest and apoptosis. Although the mechanisms for the activation of p53 after DNA-double strand breaks are still unclear, the catalytic subunit of DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated (ATM) kinase are candidates for the upstream activator or the regulator of p53 [6,7]. These proteins have homology and are members of the phosphatidylinositol 3 (PI3) kinase family that can phosphorylate p53 in vitro. Wang et al. [8] have proposed that DNA-PK and ATM are similar in the selective activation of p53, but dissimilar in that DNA-PK selects for apoptosis but not cell cycle arrest, and ATM for cell cycle arrest but not apoptosis. However, others have demonstrated that not DNA-PK but

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ATM functions as the major activator of p53 in response to DNA damage in vivo [9–15]. On the other hand, the main role of DNA-PK in vivo is thought to promote the rejoining of DNA breaks by non-homologous end-joining [16,17]. Therefore, the control mechanisms of these kinases, linking DNA damage to *p53*-dependent apoptosis, are still controversial.

Viral infection has been known to have various effects on the apoptotic signaling pathways of cells, negatively and positively. For example, viral FLICE-inhibitory proteins prevent apoptosis induced by death receptors [18] and the *ets*-2 transcription factor inhibits apoptosis through a *bcl-xL*-dependent mechanism [19]. In contrast, HIV-infection causes apoptosis in $CD4^+$ T cells via various pathways [20], and Moloney murine leukemia virus is shown to enhance thymocyte apoptosis [21]. HTLV-I Tax expression promotes anti-apoptotic or apoptotic processes in T cells according to the experimental conditions [22].

Recently we found that Friend leukemia virus (FLV) infection strongly enhanced DNA damage-induced apoptosis in mice of an FLV-susceptible C3H strain [23]. Mice were infected with FLV and then treated with split low dose γ-irradiation (total body irradiation: TBI). Surprisingly, FLV + TBI-treated C3H hosts died within 10 days after TBI treatment, while mice just infected with FLV survived for more than 40 days. The hematopoietic cells, especially erythroid cells of FLV + TBI-treated C3H mice, revealed frequent apoptosis causing lethal anemia in these mice. Experiments using *p53* knockout mice with the C3H background clarified that the apoptosis was *p53*-dependent and would not be associated with changes of cell populations caused by the infection with FLV [23]. Regarding apoptosis and FLV infection, an enhancement of anti-apoptotic signaling has been observed in transformed cell lines [24,25] as well as primary erythroblasts [26]. However, effects of FLV-infection on pro-apoptotic signaling have been unknown. In the present study, we used C3H·SCID mice in which the activity of DNA-PK would be deficient [27–29] and *ATM* knockout mice with the C3H background to elucidate the mechanisms behind the enhanced apoptotic signaling in C3H cells after FLV + TBI-treatment. The results indicated that enhanced apoptosis in vivo required both DNA-PK and ATM. To further demonstrate the regulatory mechanisms of *p53*-dependent apoptotic pathways, these PI3 kinases as well as viral protein were analyzed and the relation between FLV-infection and the enhanced DNA damage-induced apoptosis was discussed. The mechanism of enhanced *p53*-dependent apoptosis in the present system might aid in generating a novel gene therapy model using *p53* by controlling the *p53*-dependent cell death.

2. Materials and methods

2.1. Mice

Eight to ten-week-old male C3H/He mice (C3H, $H\text{-}2^k$, $Fv\text{-}2^s$), C3H·SCID mice and *ATM* knockout mice with the C3H background (C3H·$ATM^{-/-}$) were bred from our colony at the Animal Production Facility of the National Institute of Radiological Sciences in Chiba. Methods for the generation of the knockout construct and $ATM^{-/-}$ mice were described elsewhere [30]. The SCID and $ATM^{-/-}$ mice with the C3H background were generated by crossing CB.17·SCID and 129/Sv $ATM^{-/-}$ mice to the C3H strain of mice, respectively, followed by backcrossing through more than 20 generations. All of the mice were reared and treated in accordance with the guidelines governing the care and use of laboratory animals at the National Institute of Radiological Sciences (approval numbers 1997-4 and 1997-17) and also the guidelines established by the Animal Experiment Committee of the Tokyo Medical and Dental University.

2.2. Viral infection and total body irradiation

An NB-tropic Friend leukemia virus (FLV) complex, originally from Dr. C. Friend, was prepared as described earlier [31,32] and injected i.p. into mice at a highly leukemogenic dose of 10^4 PFU/mouse [33]. On day 7 after inoculation with FLV, 8–10-week-old mice were treated with 3 Gy of total body irradiation (TBI). A dose of 3 Gy TBI was delivered from a GAMMA-CELL-40 at a dose rate of 1.12 Gy/min. Sham-treated mice that were not irradiated were also prepared in each experiment.

2.3. Detection of apoptotic cell

Fresh bone marrow tissue was mounted in an OCT compound (Sakura, Tokyo, Japan), frozen with liquid nitrogen and cut to make 8–10 μm-thick frozen sections. To identify apoptotic cells on frozen tissue sections 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 [34]. Briefly, frozen sections were fixed with a 4% paraformaldehyde solution for 20 min, washed with phosphate-buffered saline (PBS), incubated in 0.1% sodium citrate–0.1% Triton X-100 for 2 min, washed with PBS and then incubated with fluorescein isothiocyanate (FITC)-labeled dUTP and TdT at 37 °C for 60 min. Sections were then observed by fluorescence microscopy and the TUNEL-positive cell ratio was determined by dividing the cell number of positively stained cells by the total cell number (counting more than 1000 cells).

2.4. Antibodies

The mouse monoclonal anti-p53 antibody Pab421 (Oncogene Research Product, Cambridge, MA) was used for immunoprecipitation. Cocktails of the mouse monoclonal anti-DNA-PK antibodies 18-2, 25-4, and 42-psc (NeoMarkers, San Jose, CA) and the rabbit polyclonal

anti-ATM antibody Ab-3 (Oncogene Research Product) were used for immunoprecipitation or as primary antibodies for immunoblotting. The mouse monoclonal anti-p53 antibody Pab240 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal phospho-p53 (Ser15) antibody (Cell Signaling Technology Inc., Beverly, MA), goat polyclonal antibody for Friend MuLV (ATCC, Manassas, VA), goat polyclonal anti-Moloney MuLV gp70 antibody (Quality Biotech, Camden, NJ) which is known to cross-react with F-MuLV gp70 [34], goat polyclonal anti-Raucher MuLV gp70 antibody (ATCC) which is expected to cross-react with F-MuLV gp70 and rabbit polyclonal anti-actin antisera (Sigma Chemicals) were used as primary antibodies for immunoblotting. Horseradish peroxidase-conjugated anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark), horseradish peroxidase-conjugated anti-rabbit antisera (Dakopatts), and horseradish peroxidase-conjugated anti-goat IgG antibody (Dakopatts) were used as secondary antibodies for immunoblotting.

2.5. Immunoprecipitation and immunoblotting analysis

The bone marrow cells from each experimental group of mice were suspended in Iscove's modified Dulbecco's medium (IMDM; Sigma Chemicals, St. Louis, MO) containing 10% fetal bovine serum and pelleted. Cell lysates were prepared by incubating cell pellets on ice for 15 min in ice-cold lysis buffer containing 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.02% NaN_3, 1 mM PMSF, 0.1% aprotinin, 100 μM leupeptin, and 100 μM TPCK (Sigma Chemicals). Supernatants were separated from debris by centrifugation at 12,000 rpm for 5 min at 4 °C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates which contained 100 μg of protein were incubated with antibodies and protein A-sepharose beads (Amersham Life Science, Buckinghamshire, England). The resulting immunoprecipitates or whole cell lysates of 50–100 μg were subjected to 6–12.5% 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 primary antibodies, and after washing, were incubated with peroxidase-conjugated secondary antibodies. Bands in the washed membrane were detected with an enhanced chemiluminescence (ECL) system (Amersham Life Science) as described previously [35]. In a part of gp70 experiments, cell lysate was divided into two fractions, cytoplasmic and nuclear fraction, according to the protocol by Dignam et al. [36], and then, these fractions were used for immunoprecipitation and immunoblotting.

2.6. Reverse transcription (RT)-polymerase chain reaction (PCR)

To determine the activation of *p21* and *bax*, known as downstream molecules of p53, and to examine the expression of *DNA-PK* and *ATM* genes at the mRNA level, an RT-PCR was performed for each experimental group. The RNA was extracted from the bone marrow using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Tissue RNA (100 ng) was used as a template for the amplification. Complementary (c)DNA was synthesized using Rous-associated virus reverse transcriptase (Takara Biomedicals, Kyoto, Japan). The PCR was performed as described elsewhere [34]. Oligonucleotides as specific primers for *p21* and *bax* were synthesized by a commercial laboratory (Life Technologies Oriental, Tokyo, Japan). As a control reaction, *β-actin* was also detected in each run. The sequences of primers were as follows: *p21*: 5′ PCR primer AATCCTGGTGATGTCCGACC, 3′ PCR primer TTGCAGAAGACCAATCTGCG; *bax*: 5′ PCR primer CCAGCTCTGAACAGATCATG, 3′ PCR primer AGCTCCATATTGCTATCCAG; *DNA-PK*: 5′ PCR primer GAATTCACCACAACCCTGCT, 3′ PCR primer GCTTTCAGCAGGTTCACACA; *ATM*: 5′ PCR primer TTACGATGGCAACAGCAGAG, 3′ PCR primer TCCAGTTCTCGCTGAACCTT; *β-actin*: 5′ PCR primer TGGAATCCTGTGGCATCCATGA, 3′ PCR primer ATCTTCATGGTGCTAGGAGCCAG. The expected sizes of the PCR products were 461 bp for *p21*, 187 bp for *bax*, 188 bp for *DNA-PK*, 225 bp for *ATM*, and 175 bp for *β-actin*. *φX174/Hae*III-cut DNA was run in parallel as a molecular size marker.

2.7. Kinase assays

Kinase assays were performed according to the protocol of Shangary et al. [37] with our modification. Cell lysates were incubated with anti-DNA-PK or anti-ATM antibody for 2 h on ice and then, mixed with 25 μl of protein A-sepharose beads rocking at 4 °C for 1 h. The immunoprecipitates obtained with anti-DNA-PK or anti-ATM antibody was centrifuged, washed three times, and used for kinase assays. The immunoprecipitate was mixed with the substrate, 1 μg of p53 protein (p53 (1–393), Santa Cruz Biotechnology, Santa Cruz, CA), along with 5 μM cold ATP in kinase buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM EDTA, and 1 mM dithiothreitol). The kinase reaction was carried out at room temperature for 30 min and terminated by adding an equal volume of SDS sample buffer followed by heat inactivation. The reaction products were subjected to 10% SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was processed for immunoblotting using the phospho-p53 (Ser15) antibody as described above and then, analyzed.

2.8. Densitometric analysis

The densities of the 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.