bjh short report

Treatment of isolated central nervous system relapse in high-risk lymphoid malignancy with allogeneic bone marrow transplantation and extended intrathecal therapy

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Summary

We performed allogeneic bone marrow transplantation (BMT) with an extended period of post-transplant intrathecal (IT) chemotherapy for five patients with acute lymphoblastic leukaemia and non-Hodgkin's lymphoma who had relapsed in the central nervous system either in the very early phase or more than twice. Post-transplant IT was scheduled for a total of 12 doses over 18 months. One patient was found to have subclinical leucoencephalopathy. Disease relapse occurred in one patient and the other patients remained in complete remission for 39–196 months post-BMT. The estimated event-free survival was 80 ± 17·9% (standard error).

Keywords: central nervous system relapse, acute lymphoblastic leukaemia, allogeneic bone marrow transplantation, extended intrathecal therapy.

Due to recent advances in chemotherapy, acute lymphoblastic leukaemia (ALL) in childhood can now be successfully treated. However, about 20% of patients suffer from disease relapse, which occurs in the testicles and central nervous system (CNS) in about 2–3% of these cases (Pui *et al*, 1998; Schrappe *et al*, 2000). Patients with a first extramedullary relapse can be rescued by subsequent chemoradiotherapy, especially when the relapse occurs while off therapy (Gaynon *et al*, 1998; Ritchy *et al*, 1999). However, the disease can occasionally recur despite intensive salvage chemoradiotherapy and most patients who develop early CNS relapse during front-line chemotherapy cannot be rescued by chemoradiotherapy.

Allogeneic bone marrow transplantation (BMT) has been the treatment of choice for haematological relapse in patients with ALL (Barrett *et al*, 1994; Wheeler *et al*, 1998). However, it has been reported that the prognosis of children with a second isolated CNS relapse of ALL was very poor, even when the patients underwent BMT (Morris *et al*, 2003). Therefore, a novel treatment strategy is needed for patients with a second isolated CNS relapse of ALL.

Methods

Conditioning regimen and graft versus host disease (GvHD) prophylaxis

The conditioning regimen was based on total body irradiation in four patients. One patient received a busulphan-based regimen because the disease relapsed 1 month after cranial irradiation in the CNS. Prophylaxis for GvHD was ciclosporin A (CSA) in cases of matched siblings or short-term methotrexate (MTX) with CSA or tacrolimus in mismatched cases.

Extended intrathecal (IT) therapy

Extended IT chemotherapy post-transplant was scheduled with cytarabine (Ara-C, 30 mg/m^2) and hydrocortisone (HDC, 50 mg/m^2) every 4 weeks after BMT for 3 months, and then every 8 weeks for another 15 months for a total of 12 doses. Magnetic resonance imaging (MRI) of the brain was performed at every IT therapy. When MRI revealed abnormal findings compatible with leucoencephalopathy (Padovan *et al*,

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1998), regardless of the symptoms, further IT therapy was omitted.

Results

One patient (UPN 105) received craniospinal irradiation (CSI) during salvage chemotherapy, while the other patients did not because the duration of the second complete remission (CR) was too short to undergo scheduled CSI. A patient with Philadelphia chromosome-positive (Ph1) ALL (UPN 017) developed overt relapse 1 month after achieving the first CR, and BMT was performed 6 weeks later.

Adverse events

Veno-occlusive disease of the liver occurred in one patient (UPN 105). MRI showed leucoencephalopathy in one patient (UPN 079) without any symptoms at the sixth IT therapy, and further IT therapy was omitted. Four of the five patients developed acute GvHD. All three patients in grade III or IV were treated successfully with corticosteroids. Extensive chronic GvHD occurred in two patients (see Table I).

Disease relapse and survival

The disease relapsed 12 months after BMT in the bone marrow of one patient (UPN 178). The other patients were disease free and well for 39–196 months. The estimated event-free survival was 80·0 ± 17·9% (SE).

Discussion

Promising results have recently been reported with salvage chemoradiotherapy after the first CNS relapse in ALL (Ribeiro *et al*, 1995; Ritchy *et al*, 1999). However, the Medical Research Council reported that the prognosis of children after a second CNS relapse, including isolated and combined relapse, treated with conventional chemotherapy with or without BMT, was dismal (Morris *et al*, 2003). On the contrary, the Seattle group reported that the risk of post-transplant CNS relapse in patients with ALL who received post-transplant IT-MTX was lower than that in patients who did not receive such treatment (Thompson *et al*, 1986). In addition, they also reported that the risk of leucoencephalopathy was higher in patients who had received post-transplant IT-MTX.

Against this background, we started a novel treatment strategy for patients with ALL who experienced multiple CNS relapse and were thought to be incurable by salvage chemoradiotherapy (e.g. Ph1-ALL with CNS relapse): such patients should be treated with allogeneic BMT and post-transplant extended IT-Ara-C and -HDC.

It has been reported that IT-MTX could induce leucoencephalopathy, especially when combined with cranial irradiation (Filley & Kleinschmidt-DeMasters, 2001). Therefore, we did not use MTX as an IT therapeutic agent. Nevertheless,

Table I. Patient and donor characteristics and outcomes.

UPN	Age (years)/sex	Diagnosis	Disease status	Time from diagnosis to BMT (months)	Duration of second CR (months)	Donor	HLA disparity	Conditioning	GvHD prophylaxis	aGvHD	cGvHD	Relapse	Survival (months)	Outcome
017	3/M	Ph1-ALL	2nd CR	4	1	Sibling	matched	TBI/CA/CY	CSA	IV	None	No	196	Alive
079	3/F	Precursor-B ALL	3rd CR	20	4	Sibling	1 locus mismatched	TBI/CA/CY	CSA	I	None	No	109	Alive
105	7/F	Precursor-B ALL	4th CR	49	7	Sibling	matched	BUS/L-PAM	CSA/MTX	0	None	No	92	Alive
177	9/M	Precursor-T ALL	3rd CR	35	4	Mother	1 locus mismatched	TBI/CA/L-PAM	FK/MTX	III	Extensive	No	39	Alive
178	4/F	NHL (LBL)	4th CR	35	6	Father	2 loci mismatched	TBI/CA/CY	FK/MTX	III	Extensive	Yes (BM)	19	Dead

ALL, acute lymphoblastic leukaemia; NHL, non-Hodgkin's lymphoma; LBL, lymphoblastic lymphoma; CR, complete remission; BMT, bone marrow transplantation; HLA, human leucocyte antigen; TBI, total body irradiation; CA, cytarabine; CY, cyclophosphamide; BUS, busulphan; L-PAM, L-phenylalanine mustard; GvHD, graft *versus* host disease; CSA, ciclosporin A; MTX, methotrexate; FK, tacrolimus; aGvHD, acute GvHD; cGvHD, chronic GvHD.
HLAs were typed by serological tests only in the first three donor–recipient pairs and by DNA typing in the latter two.

subclinical leucoencephalopathy occurred in one patient in our series. IT-MTX might not always be avoided, and serial MRIs should be performed to enable early diagnosis of leucoencephalopathy, which is an irreversible complication.

The optimal schedule of post-transplant IT therapy in patients with multiple CNS-relapsed ALL is unclear. We administered post-transplant IT chemotherapy 12 times over a period of about 18 months. Due to subclinical leucoencephalopathy, one patient (UPN 079) received only six applications of IT therapy and remained disease free. Therefore, our scheduled IT therapy might continue for too long or may involve too many treatment episodes in these cases. Although a randomized study would be desirable, such a study may be difficult, as there are very few patients with multiple CNS-relapsed ALL.

It has been reported that treatment failure after the first CNS relapse was due to toxicity, bone marrow relapse and extramedullary relapse, including a second CNS relapse (Ribeiro *et al*, 1995; Ritchy *et al*, 1999). Our encouraging results might be due to sufficient systemic treatment with the conditioning regimen, along with prolonged CNS treatment by post-transplant IT chemotherapy. Of course, it might be possible to treat patients with sufficient systemic therapy and autologous BMT if we could transplant non-contaminated grafts of leukaemic cells.

Recently, the stem cell source for BMT has become quite diverse. Matched- or mismatched-related and matched-unrelated bone marrow and -unrelated cord blood donors are available. Our patients underwent allogeneic BMT from matched- or mismatched-related donors. Although there was no transplant-related mortality (TRM) in our series, GvHD and other complications, including opportunistic infection, are relatively specific in allogeneic BMT. In addition, TRM is higher in mismatched-related and matched-unrelated transplant than in matched-related donors (Nagatoshi *et al*, 2004). However, we believe that the risks of TRM are acceptable for patients with multiple CNS relapse of lymphoid malignancies, as the prognosis of patients after a second CNS relapse is very poor.

In conclusion, allogeneic BMT combined with extended IT therapy appears to be an effective treatment option for patients with lymphoid malignancy with CNS relapse; serial cranial MRI examinations should be performed to avoid clinical leucoencephalopathy.

References

Barrett, A.J., Horowitz, M.M., Pollock, B.H., Zhang, M.-J., Bortin, M.M., Buchanan, G.R., Camitta, B.M., Ochs, J., Graham-Pole, J., Rowlings, P.A., Rimm, A.A., Klein, J.P., Shuster, J.J., Sobocinski, K.A. & Gale, R.P. (1994) Bone marrow transplantation from HLA-identical siblings as compared with chemotherapy for children with acute lymphoblastic leukemia in a second remission. *New England Journal of Medicine*, **331**, 1253–1258.

Filley, C.M. & Kleinschmidt-DeMasters, B.K. (2001) Toxic leukoencephalopathy. *New England Journal of Medicine*, **345**, 425–432.

Gaynon, P.S., Qu, R.P., Chappell, R.J., Willoughby, M.L.N., Tubergen, D.G., Steinherz, P.G. & Trigg, M.E. (1998) Survival after relapse in childhood acute lymphoblastic leukemia: impact of site and time to first relapse – the Children's Cancer Group Experience. *Cancer*, **82**, 1387–1395.

Morris, E.C., Harrison, G., Bailey, C.C., Hann, I.M., Hill, F.G., Gibson, B.E.S., Richards, S. & Webb, D.K.H. (2003) Prognostic factors and outcome for children after second central nervous system relapse of acute lymphoblastic leukaemia. *British Journal of Haematology*, **120**, 787–789.

Nagatoshi, Y., Kawano, Y. & Okamura, J. (2004) Comparison of the outcomes of allogeneic bone marrow transplantation from partially mismatched related donors, matched sibling donors, and matched unrelated donors in Japanese pediatric patients: a single center result. *Pediatric Transplantation*, in press.

Padovan, C.S., Yousry, T.A., Schleuning, M., Holler, E., Kolb, H.-J. & Straube, A. (1998) Neurological and neuroradiological findings in long-term survivors of allogeneic bone marrow transplantation. *Annals of Neurology*, **43**, 627–633.

Pui, C.-H., Mahmoud, H.H., Rivera, G.K., Hancock, M.L., Sandlund, J.T., Behm, F.G., Relling, M.V., Ribeiro, R.C., Rubnitz, J.E., Kun, K.E. & Evans, W.E. (1998) Early intensification of intrathecal chemotherapy virtually eliminates central nervous system relapse in children with acute lymphoblastic leukemia. *Blood*, **92**, 411–415.

Ribeiro, R.C., Rivera, G.K., Judson, M., Mulhern, R.K., Hancock, M.L., Kun, L., Mahmoud, H., Sandlund, J.T., Crist, W.M. & Pui, C.-H. (1995) An intensive re-treatment protocol for children with an isolated CNS relapse of acute lymphoblastic leukemia. *Journal of Clinical Oncology*, **13**, 333–338.

Ritchy, A.K., Pollock, B.H., Lauer, S.J., Andejeski, Y. & Buchanan, G.R. (1999) Improved survival of children with isolated CNS relapse of acute lymphoblastic leukemia: a Pediatric Oncology Group Study. *Journal of Clinical Oncology*, **17**, 3745–3752.

Schrappe, M., Reiter, A., Ludweg, W.-D., Harbott, J., Zimmermann, M., Hiddemannn, W., Niemeyer, C., Henze, G., Feldges, A., Zintl, F., Kornhuber, B., Ritter, J., Welte, K., Gadner, H. & Riehm, H. for the German-Austrian-Swiss ALL-BFM Study Group (2000) Improved outcome in childhood ALL despite reduced use of anthracyclines and of cranial radiotherapy: results of trial ALL-BFM 90. *Blood*, **95**, 3310–3322.

Thompson, C.B., Sanders, J.E., Flournoy, N., Buckner, C.D. & Thomas, E.D. (1986) The risks of central nervous system relapse and leukoencephalopathy in patients receiving marrow transplants for acute leukemia. *Blood*, **67**, 195–199.

Wheeler, K., Richards, S., Bailey, C. & Chessells, J. for the Medical Research Council Working Party on Childhood Leukemia (1998) Comparison of bone marrow transplant and chemotherapy for relapsed childhood acute lymphoblastic leukemia: the MRC UKALL X experience. *British Journal of Haematology*, **101**, 94–103.

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Differential expression of survivin in bone marrow cells from patients with acute lymphocytic leukemia and chronic lymphocytic leukemia

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Abstract

Survivin, a member of the inhibitor of apoptosis protein (IAP) gene family, has been detected widely in fetal tissue and in a variety of human malignancies. In the current study, we investigated the expression of IAP family proteins in bone marrow samples from acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL) and control cases by quantitative real-time RT-PCR method and an immunohistochemical approach. Overexpression of survivin and cIAP2 mRNA was significant in CLL bone marrow cells ($P < 0.05$, respectively) compared with control samples. By immunohistochemistry, survivin was detected in a few scattered myeloid cells in all cases of control bone marrow. Concerning the ALL bone marrow, more than half the cases demonstrated positive expression of survivin (8 out of 13), while the majority of CLL cases (20 out of 21) exhibited intense expression of survivin. The differential subcellular localization of survivin was distinct between ALL and CLL cases. ALL cells essentially revealed nuclear localization of survivin as well as cytoplasmic signals in some cases, while CLL cells from the majority of cases predominantly showed cytoplasmic expression. Next, RT-PCR was performed for the expression of survivin and its splicing variant, survivin-2B and survivin-ΔEx3 in ALL and CLL cells, as the distribution of these variants would be regulated by nuclear/cytoplasmic transport system. In both ALL and CLL bone marrow samples, the expression of wild-type survivin was more predominant than that of survivin-2B or survivin-ΔEx3, although the expression of survivin-ΔEx3 was prominent in samples from survivin-expressing ALL cases. Thus, the splicing of survivin mRNA may be differently regulated in ALL and CLL cells, causing distinct manners of nuclear/cytoplasmic transport of survivin protein. In conclusion, our observations indicate a differential regulatory mechanism for the expression of IAP family proteins in ALL and CLL cells, although the functions of IAP families and the mechanisms of nuclear/cytoplasmic transport of survivin should be clarified in future studies.

Keywords: Survivin; IAP; Bone marrow; ALL; CLL

1. Introduction

The regulation of apoptotic cell death may have a profound effect on the pathogenesis and progression of hematological malignancies. Chronic lymphocytic leukemia (CLL) is characterized by clonal expansion of relatively mature B cells with a high percentage of cells arrested in the non-proliferative G0/G1 cell cycle phase [1,2]. The progressive rise of lymphocytes, despite the very low proportion of proliferating cells, has led to the notion that the pathogenesis of CLL is primarily related to defective apoptosis. In contrast, acute lymphocytic leukemia (ALL) cells exhibit highly proliferative character with a very low percentage of apoptotic cells [1,3,4]. Thus, ALL and CLL cells may be regulated by different types of cell-proliferation/cell-death signaling pathway. To begin to clarify the antiapoptotic pathways in lymphocytic leukemias, the expression and modulation of the family of inhibitor of apoptosis proteins (IAPs), especially survivin, were investigated and compared in control, ALL and CLL bone marrow samples.

Survivin is expressed widely in fetal tissues, but becomes restricted during development, and appears to be negligibly expressed in the majority of terminally differentiated adult tissues [5,6]. However, analysis of the differences in gene expression between normal and tumor cells has revealed that survivin is one of the genes most consistently overexpressed

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in tumor cells relative to normal tissue [7]. In fact, survivin is prominently expressed in transformed cell lines and in many of the human cancers including hematopoietic cell tumors [8].

As with other IAP family proteins, survivin blocks apoptosis induced by a variety of apoptotic triggers [9,10]. Although the exact biochemical mechanism by which survivin 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 [10,11]. Survivin is usually detected in the cytoplasm of tumor cells, and is therefore widely regarded as a cytoplasmic protein [5,12,13]. However, several studies have shown nuclear accumulation of survivin in gastric cancer cells [14] and lung cancer cells [15]. Thus, the mechanisms that control its nuclear-cytoplasmic localizations in tumor cells are still controversial.

Many cellular proteins either reside in the nucleus or shuttle between the nucleus and the cytoplasm across the nuclear envelope. In a recent study, survivin was shown to be a nuclear shuttling protein that was actively exported from the nucleus via the chromosome region maintenance 1 (CRM1)-dependent pathway [15]. CRM1 was shown to be a receptor for the nuclear export signal that bound to the nuclear export sequences of the proteins. Thus, the molecular export sequences are very important in determining the subcellular localization of proteins. Differences in the amino acid sequence of the carboxy-terminal domain of survivin determine the dramatically different localization of survivin and its splice variant, survivin-ΔEx3. Survivin-ΔEx3 lacks exon 3 but has additional sequences that could mediate its strong nuclear accumulation. Therefore, wild-type survivin localizes to the cytoplasm, while survivin-ΔEx3 accumulates in the nucleus.

Here, in the present study, overall survivin expression was significantly up-regulated in the bone marrow cells from ALL and CLL compared with the control bone marrow. However, different localization of survivin was shown by the nuclear expression in ALL and the cytoplasmic expression in CLL. Expression of other IAPs including NAIP, cIAP1, cIAP2 and XIAP, all of which appeared to suppress apoptosis by caspase and procaspase inhibition [16–19] was also determined in these samples and the significance of IAP family protein expression in lymphocytic leukemias was discussed.

2. Materials and methods

2.1. Patients

Formalin-fixed paraffin-embedded bone marrow aspiration samples from 13 patients with adult-onset ALL (7 with B-ALL and 6 with T-ALL; male:female = 5:8; age: median 48, maximum 78, minimum 19), 21 patients with B-CLL (male:female = 11:10; age: median 57, maximum 87, minimum 49) and 13 cases with no hematological disorders as age-matched normal controls (male:female = 13:0; age: median 63, maximum 76, minimum 51) were analyzed. To rule out the influence of aging effect on bone marrow cells, ALL cases with adult-onset were analyzed and cases with childhood ALL were excluded from the study. Diagnosis was based on standard clinical and laboratory criteria, including cell morphology [20–22]. All samples were collected at the time of the initial aspiration biopsy and the samples from ALL and CLL exhibited proliferation of the blastic cells accounting for more than 80% of the total 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. 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 determine apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used for the assay as described previously [23]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO, Glostrup, Denmark) for 15 m at room temperature. After washing, 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, followed by color development with DAB containing 0.3% hydrogen peroxide solution. Sections were then observed under 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 1,000 cells).

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

The RNA was extracted from the frozen bone marrow samples from seven cases with ALL (four with B-ALL and three with T-ALL), seven cases with B-CLL and eight cases with no hematological disorders using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes, 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 IAP family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized by a commercial laboratory (Perkin-Elmer Cetus). The primers and TaqMan probes were as follows. Sequences of the forward primer for survivin

mRNA were 5′-TGCCTGGCAGCCCTTTC-3′ and the reverse primer, 5′-CCTCCAAGAAGGGCCAGTTC-3′; the sequence of the TaqMan probe was 5′-CAAGGACCACCGCATCTCTACATTC-3′. For cIAP1 mRNA, sequences of the forward primer were 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer, 5′-CAAGCCACCATCACAACAAAA-3′; the TaqMan probe was 5′-TTTATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, sequences of the forward primer were 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer, 5′-TCTCCTGGGCTGTCTGATGTG-3′; the sequence of the TaqMan probe was 5′-CCCTCATCTACTTGAACAGCTGCTAT-3′. Sequences of the forward primer for NAIP mRNA were 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer, 5′-GCTGGGCGGATGCTTTC-3′; the sequence of the TaqMan probe was 5′-CCATTTAAACCACAGCAGAGGCTTTAT-3′. Sequences of the forward primer for XIAP mRNA were 5′-AGTGGTAGTCCTGTTTCAGCATCA-3′ and the reverse primer, 5′-CCGCACGGTATCTCCTTCA-3′; the sequence of the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTTTATACTG-3′. Sequence of the forward primer for GAPDH mRNA were 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′; the sequence of the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. Conditions of 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). Expression of survivin and other IAP family proteins was quantitated according to the method described elsewhere [24]. Briefly, the intensity of reaction was evaluated by the quantity of total RNA of Raji cells (ng) corresponding to the initial PCR cycle numbers to reveal the linear increase of reaction intensity (threshold cycle) in each sample on the logarithmic scale standard curve. Data of the Raji RNA quantity (ng) for IAP family were normalized by the data for GAPDH in each sample.

2.4. Immunohistochemistry for survivin, p53 and cell markers

Four micrometer-thick tissue sections of bone marrow from control, ALL and CLL 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, to characterize B cells (CD20) and T cells (CD45RO), and to identify accumulation of p53 protein. Primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, San Antonio, TX), monoclonal antibodies against CD20 (DAKO), CD45RO (DAKO) and p53 (Novocastra Laboratories Ltd., Newcastle, UK). 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 procedure was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted with the primary antibody in each staining.

Phenotype determination of survivin-expressing cells was performed by double immunostaining using polyclonal antibody against survivin and monoclonal antibody against CD20 or CD45RO followed by the 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).

2.5. RT-PCR analysis for survivin and splice variants, survivin-2B and survivin-ΔEx3

To determine the pattern for the splicing of survivin, RT-PCR analysis was performed using specific primers that could distinguish each type of splicing variant, survivn-2B and survivin-ΔEx3, by product size [25]. The PCR reaction was performed as described elsewhere [26,27]. Briefly, 100 ng of the RNA was used for RT-PCR. For complementary (c)DNA synthesis, 100 ng in 4 μl of sample RNA solution was heated at 65 °C for 5 min and cooled rapidly. After adding 20 U of ribonuclease inhibitor (Takara, Japan), 1 μl of 1.25 mM dNTP (dATP, dCTP, dGTP, dTTP, Pharmacia, Uppsala, Sweden) and 20 U of Rous-associated virus reverse transcriptase (Takara Biomedicals, Kyoto, Japan), the mixture was incubated at 40 °C for 30 min, then heated at 94 °C for 5 min and cooled rapidly. Oligonucleotides as specific primers for survivin were synthesized by a commercial laboratory (Invitrogen Life Technologies, Tokyo, Japan). The sequences of primers were as follows: forward primer, 5′-ACCGCATCTCTACATTCAAG-3′ and the reverse 5′-CTTTCTTCGCAGTTTCCTC-3′. In the control reaction β-actin was also determined using the forward primer 5′-AAGAGAGGCATCCTCACCCT-3′, and the reverse 5′-TACATGGCTGGGGTGTTGAA-3′. The PCR reaction mixture contained 10 μl of cDNA, 10 μl of 10 × PCR buffer, 11 μl of 20 mM $MgCl_2$, 16 μl of 1.25 M dNTP, 42.5 μl of DEPC-water, 100 pM forward and reverse primers, and 2.5 U of thermostable Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The amplification was achieved with a DNA thermal cycler (Perkin-Elmer Cetus). After denaturing at 94 °C for 10 min, the amplification was conducted for 45 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. This was followed by re-extension for 10 min at 72 °C. Ten microliters aliquots of the product samples were analyzed by electrophoresis on a 1.8% agarose gel and visualized by UV fluorescence after staining with ethidium bromide. The expected sizes of the PCR product were 342 bp for wild-type survivin, 411 bp for survivin-2B, 224 bp for survivin-ΔEx3 and 218 bp for β-actin. φX174/Hae III-cut DNA was run in parallel as a molecular size marker.

2.6. Statistical analysis

Statistically significant differences were determined using the Mann–Whitney's *U*-test.

3. Results

3.1. Apoptotic cell ratio of the bone marrow cells from ALL, CLL, and control cases

To identify the apoptotic cells in the bone marrow samples, the TUNEL method was performed on paraffin-embedded sections. We compared the overall TUNEL-positive cell ratio of control bone marrow with the ratio of ALL or CLL samples, although the apoptotic cells of control bone marrow were not necessarily the lymphoid cells. The apoptotic cell ratio was rather low even in the control bone marrow samples as shown in Table 1, however, the ratio was lower in ALL and CLL cases than in control cases. Differences were significant between ALL and control ($P < 0.01$ by the Mann–Whitney's *U*-test) and CLL and control cases ($P < 0.0001$). ALL cells exhibited relatively lower frequency of TUNEL-positive signals than CLL cells ($P < 0.0001$). These findings suggested that apoptosis was actually infrequent in ALL cells as well as in CLL cells.

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

To quantitate the mRNA expression levels of IAP family proteins in lymphocytic leukemia cells, real-time quantitative RT-PCR was performed using bone marrow samples from ALL, CLL and control cases. The expression of mRNA for survivin, cIAP1, cIAP2, NAIP and XIAP was found in all of the control samples although the expression levels varied. Thus, the expression intensity of IAP family proteins was demonstrated as the percentage of control in each group. Differences were significant between survivin expression of CLL and control ($P < 0.05$) and cIAP2 expression of CLL and control ($P < 0.05$) (Fig. 1). The intensities of mRNA expression of cIAP1, cIAP2, NAIP and XIAP proteins in ALL cases tended to be higher than the intensity of control cases, although the differences were not significant. This is caused by the fact that some of the ALL cases revealed very high expression, while other ALL cases had as low expression as control cases. No significant differences were found between survivin expression and patients' age, sex or phenotypic character of leukemic cells (B cell-lineage or T cell-lineage). These results indicated that the expression of survivin and cIAP2 would be significant in CLL bone marrow, whereas survivin as well as other IAP family proteins might possibly have a role only in some ALL cases.

Table 1
Apoptotic cell ratio of the bone marrow from ALL, CLL and control cases

Cases	TUNEL-positive cell ratio (%)[a]	
	Median	Maximum–minimum
ALL	0.044	0.38–0.0032[b,c]
CLL	0.13	0.98–0.011[c,d]
Control	1.08	3.65–0.58[b,d]

[a] Values indicate the median value, the maximum and the minimum values.
[b] Differences were significant between the TUNEL-positive cell ratio of ALL and control cases ($P < 0.01$) by the Mann–Whitney's *U*-test.
[c] Differences were significant between the TUNEL-positive cell ratio of ALL and CLL cases ($P < 0.0001$) by the Mann–Whitney's *U*-test.
[d] Differences were significant between the TUNEL-positive cell ratio of CLL and control cases ($P < 0.0001$) by the Mann–Whitney's *U*-test.

3.3. Immunohistochemical localization of survivin in the bone marrow of control, ALL and CLL cases

To investigate the localization of survivin, immunohistochemical staining was performed in bone marrow samples from ALL, CLL and control cases. ALL cells exhibited various degrees of survivin expression from case to case. In one case, the majority of cells stained positively, while in seven cases, staining was partial (Table 2). In five cases of ALL, survivin was not detected immunohistochemically. At the cellular level, survivin signals in ALL cells were predominantly localized to the nucleus (Fig. 2A and B), although in some cases, prominent reaction was also observed in the cytoplasm of ALL cases. Moreover, survivin was detected in most of the bone marrow samples from CLL cases by immunohistochemical staining (20 out of 21 cases). Positive staining was observed in the majority of CLL cells in half the cases (10 out of 20 survivin-positive cases), while other cases exhibited positive signals in some CLL cells (Table 2). In contrast to the subcellular localization of survivin in ALL cases, survivin in CLL cells was predominantly localized to the cytoplasm with minimal nuclear staining (Fig. 2C and D). By contrast, survivin was detected in only a few scattered myeloid cells of the control bone marrow samples (Fig. 2E). The subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity of the control bone marrow was constant between different samples. Tissue sections that were reacted with preimmune rabbit antibody with irrelevant specificity showed no significant staining in all of the samples (not shown). Double staining procedure revealed survivin-expressing cells were CD20-positive cells both in B-ALL and B-CLL (Fig. 3A and B) suggesting that these cells were actually leukemic cells.

3.4. Expression of survivin and splice variants, survivin-2B and survivin-ΔEx3 in ALL, CLL and control cases

To examine whether the differential subcellular localization of survivin between ALL and CLL cases was due to the difference in nuclear/cytoplasmic transport state, RT-PCR analysis was performed to distinguish the wild-type and

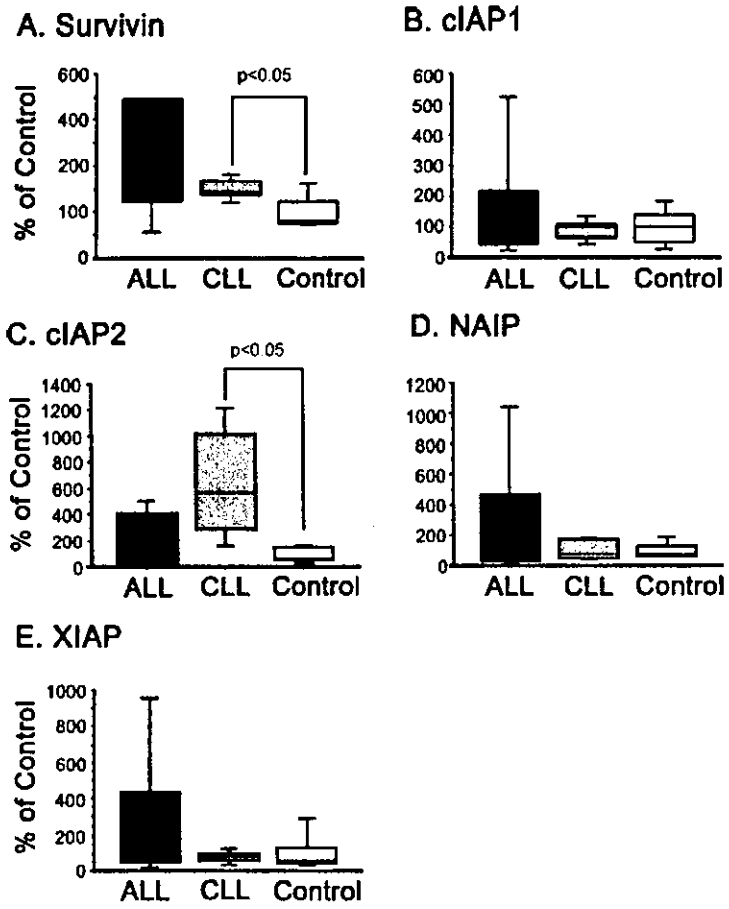


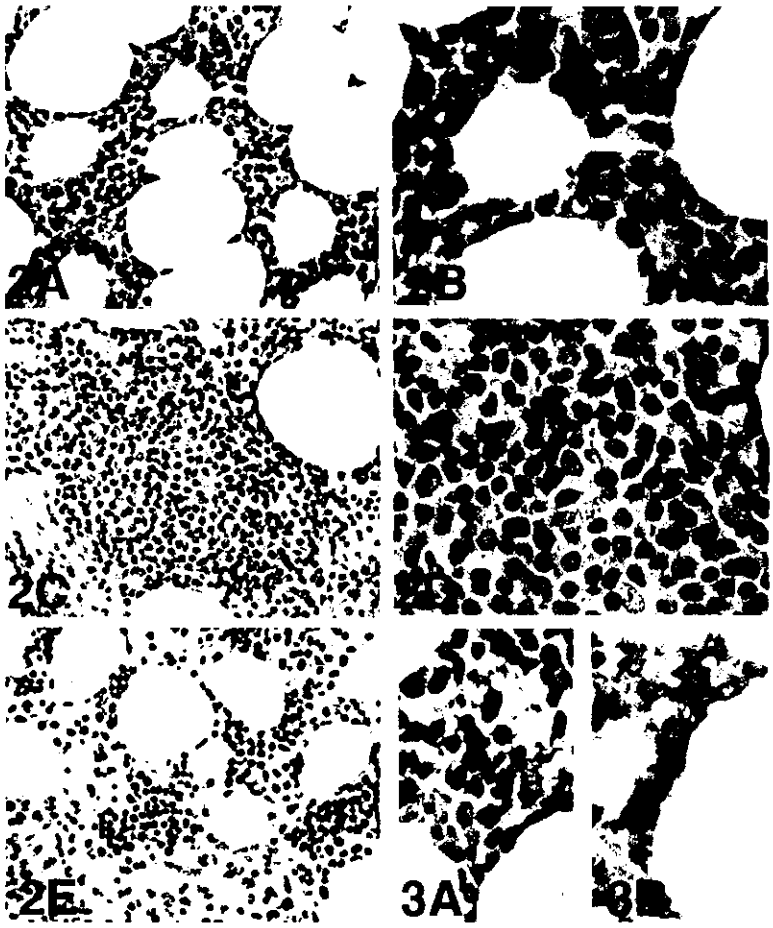
Fig. 1. ((A)–(E)) Quantitative RT-PCR analysis for IAP family proteins, survivin, cIAP1, cIAP2, NAIP and XIAP. Relative intensity was calculated as intensity of reaction of IAP family (total Raji RNA (ng))/intensity of reaction of GAPDH (total Raji RNA (ng)). The intensity of expression from ALL and CLL samples is indicated as the percentage of the intensity of control samples. The box plot graphs indicate the value of ALL, CLL and control cases. Bars indicate 90% tile and 10% tile and box indicates 75% tile to 25% tile. Differences were significant between survivin expression in CLL and control cases ($P < 0.05$) and cIAP2 expression in CLL and control cases ($P < 0.05$) by the Mann–Whitney's U-test.

splice variants of survivin. In an in vitro transfection study, the wild-type survivin and the splice variant survivin-2B have been shown to localize to the cytoplasm. In contrast, another variant, survivin-ΔEx3, has a carboxy-terminal end that is different from other types of survivin and mediates strong nuclear accumulation [15]. Thus, the difference in the splicing patterns of survivin would indicate the difference in the state of nuclear/cytoplasmic transport system. In ALL and CLL bone marrow samples, predominant expression was confined to the wild-type survivin, although a weak expression of survivin-2B and survivin-ΔEx3 was also identified (Fig. 4). The splicing for survivin-ΔEx3, appeared more frequent in ALL cases compared with CLL cases, although the expression of wild-type survivin was the strongest even in ALL cases. Therefore, splicing patterns seemed different between ALL and CLL cells. These findings suggested that

Table 2
Immunohistochemical localization of survivin in the bone marrow from ALL, CLL and control cases

Cases	Number of cases					Positive cases (%)	Subcellular localization
	Total	+++	++	+	−		
ALL	13	1	4	3	5	62	Nuclear > cytoplasmic
CLL	21	10	5	5	1	95	Cytoplasmic
Control	13	0	0	0	13	0	Nuclear/cytoplasmic, scattered myeloid cells

+++: the majority of cells exhibited intense expression; ++: more than 50% of cells revealed positive signal; +: positive staining was observed in 10–50% of cells; −: positive cells were less than 10%.



Figs. 2–3. (2) Immunohistochemical localization of survivin in the bone marrow from ALL ((A) and (B)), CLL ((C) and (D)) and control (E) cases. Development procedures were performed using the peroxidase–DAB system (brown). Note that the majority of cells were positively stained in ALL and CLL cases, while only a few myeloid cells exhibited positive signals in control bone marrow ((A), (C) and (E), original magnification 200 ·). ALL cells showed striking signals in the nucleus as well as in the cytoplasm, whereas positive signals in CLL cells were mainly cytoplasmic and not nuclear ((B) and (D), original magnification 400 ·). (3) Double immunostaining for survivin and CD20 in the bone marrow from B-ALL (A) and B-CLL cases (B) (original magnification 200 ·). For double immunostaining, development procedures were performed using the peroxidase–DAB system for survivin (brown) and the alkaline phosphatase-nitroblue tetrazolium system for CD20 (blue). In both ALL (A) and CLL (B) cases, survivin-positive cells (brown) were also positive for CD20 antigen (blue) suggesting that the leukemic cell expressed survivin.

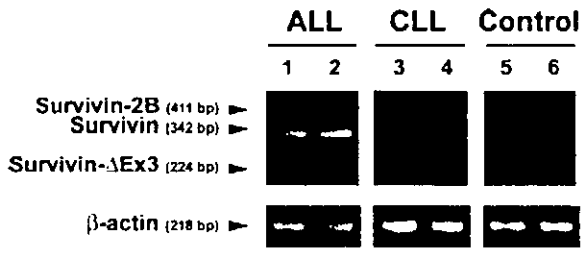


Fig. 4. RT-PCR analysis for the expression of wild-type survivin and the splice variant survivin-2B and survivin-ΔEx3 in ALL (lanes 1 and 2), CLL (lanes 3 and 4) and control bone marrow (lanes 5 and 6). In ALL and CLL samples, the predominant expression was observed in the 342 bp wild-type survivin. However, note that ALL cases have distinct 224 bp signals for survivin-ΔEx3, while CLL cases exhibit very weak 411 bp survivin-2B signals other than wild-type survivin. In some ALL cases, the expression of survivin-ΔEx3 was prominent as shown in lane 2.

the mechanism of the nuclear/cytoplasmic transport system of survivin such as the CRM1 system or the distribution of survivin-binding proteins such as caspases might be differently regulated in ALL cells compared with CLL cells.

3.5. p53 expression in the bone marrow cells of ALL and CLL cases

To determine whether p53-dependent apoptotic pathways were associated with the expression of survivin in ALL and CLL cases, immunohistochemical staining was performed for detecting p53 accumulation in bone marrow samples. As expected from the previous studies [2,4], accumulation of p53 protein was not frequent in ALL and CLL cells in the present study. A positive reaction was observed only in 8% of ALL (1 out of 13 cases) and 10% of CLL cases (2 out of 21 cases). Because the overexpression of survivin was

observed in more than half the ALL cases and the majority of CLL cases, these results suggested that survivin expression in ALL and CLL cases would not be associated with p53 mutation.

4. Discussion

Regarding the survivin expression in lymphocytic leukemia cells, previous studies have revealed an overexpression in some ALL cases [28,29] including adult T-cell leukemia [30] or a significant expression in B-CLL cases [31], while other group indicated that survivin was undetectable in the majority of B-CLL samples [32]. The in vitro data on mononuclear cells from the peripheral blood or bone marrow demonstrated that B-CLL cells expressed survivin on CD40 stimulation and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [31]. CD40 belongs to the tumor necrosis factor (TNF) receptor superfamily [33] and its stimulation appears to rescue B-CLL cells from apoptosis and induce proliferation [34]. In CLL patients, CD40L would be provided as microenvironmental stimuli by activated $CD4^+$ T cells in the bone marrow. The present data indicated that not only survivin but also cIAP2 exhibited significant overexpression in the bone marrow from CLL patients in vivo. Thus, in CLL bone marrow, multiple microenvironmental factors other than the CD40–CD40L system may also influence the expression of IAP family proteins.

In ALL samples, IAP family expression patterns other than survivin were not uniform in the present study. Some cases exhibited very strong expression, while others revealed an almost normal level of expression. As a result, although the mean intensities of cIAP1, NAIP and XIAP in ALL were higher than those of control cases, the differences were not significant between ALL and control groups. Thus, several IAP family proteins other than survivin might also play a role in some ALL cases but may not be the general factors that regulate apoptotic pathways in ALL cells.

Immunohistochemical staining revealed a very high frequency of survivin expression in CLL cells and relatively high frequency in ALL cells in the present study. Thus, immunohistochemical analysis would be useful for detecting the few remaining leukemic cells after treatment and the very early stage of leukemic relapse of ALL/CLL cases on formalin-fixed routine bone marrow aspiration samples. We confirmed that the condition in leukemic relapse did not alter the state of survivin expression in several samples from ALL and CLL cases, however, further study should be made to clarify the influence of chemotherapeutic agents on the expression patterns of survivin.

Two splice variants of survivin, survivin-ΔEx3 and survivin-2B, have been identified [25]. Study on the regulation of alternative splicing is still a new and intriguing area. Thus, how different splice forms are turned on and off is still controversial except for several instances [35]. Genetic events in ALL/CLL pathogenesis might involve and alter the splicing mechanism of survivin, although a future study should clarify the details. By transfection experiments, survivin-ΔEx3 conserves antiapoptotic properties, while survivin-2B has a markedly reduced antiapoptotic potential. In the present study, ALL cases and CLL cases exhibited enhanced expression of wild-type survivin as well as survivin-2B, while survivin-ΔEx3 was more intensely expressed in ALL cases than in CLL cases. These variants of survivin might contribute to the suppression of the apoptotic process in the bone marrow cells as expected from the present TUNEL data.

It is difficult to explain the difference in apoptotic character of ALL and CLL only by the expression of survivin and cIAP2 at this moment. However, it is possible that the ability of survivin to counteract apoptosis is modulated by its localization to the nucleus or the cytoplasm of the cell [15]. In addition to its anti-apoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis [8]. Highly proliferative activity of ALL cells but low proliferative activity of CLL cells might be associated with the differential expression pattern of survivin.

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 be a molecular explanation for the silencing of survivin gene transcription by p53 [37]. On the other hand, the over-expression 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, ALL (5–17%) and CLL (10–20%) cases have been shown to demonstrate a rather low frequency of p53 mutation [2,4]. We also observed that immunohistochemical accumulation of p53 was present only in 8% of cases with ALL and 10% of CLL cases. Therefore, p53 mutation would not appear to be the major factor controlling the overexpression of survivin in the bone marrow of ALL and CLL cases.

Acknowledgements

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References

[1] Andreeff M, Darzynkiewicz Z, Sharpless TK, Clarkson BD, Melamed MR. Discrimination of human leukemia subtypes by flow cytometric analysis of cellular DNA and RNA. Blood 1980;55:282–3.
[2] Meinhardt G, Wendtner C-M, Hallek M. Molecular pathogenesis of chronic lymphocytic leukemia: factors and signaling pathways regulating cell growth and survival. J Mol Med 1999;77:282–93.

[3] Ball LM, Pope J, Howard CV, Eccles P, van Velzen D. PCNA Ki-67 dissociation in childhood acute lymphoblastic leukaemia. An immunofluorescent laser confocal scanning microscopical study. Cell Biol Int 1994;18:869–74.
[4] Krug U, Ganser A, Koeffler HP. Tumor suppressor genes in normal and malignant hematopoiesis. Oncogene 2002;21:3475–95.
[5] Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, *survivin*, expressed in cancer and lymphoma. Nat Med 1997;3:917–21.
[6] Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC. Developmentally regulated expression of the novel cancer anti-apoptotic gene survivin in human and mouse differentiation. Am J Pathol 1998;152:43–9.
[7] Velculescu VE, Madden S, Zhang L, Lash AE, Yu J, Rago C, et al. Analysis of human transcriptomes. Nat Genet 1999;23:387–8.
[8] Altieri DC, Marchisio C. Survivin apoptosis: an interloper between cell death and cell proliferation in cancer. Lab Invest 1999;79:1327–33.
[9] LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 1998;17:3247–59.
[10] Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 1998;58:5315–20.
[11] Shin S, Sung B-J, Cho Y-S, Kim H-J, Ha N-C, Hwang J-I, et al. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 2001;40:1117–23.
[12] Gianani R, Jarboe E, Orlicky D, Frost M, Bobak J, Lehner R. Expression of survivin in normal, hyperplastic, and neoplastic colonic mucosa. Hum Pathol 2001;32:119–25.
[13] Kawasaki H, Toyoda M, Shinohara H, Okuda J, Watanabe I, Yamamoto T, et al. Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer 2001;91:2026–32.
[14] Okada E, Murai Y, Matsui K, Ishizawa S, Cheng C, Masuda M, et al. Survivin expression in tumor cell nuclei is predictive of a favorable prognosis in gastric cancer patients. Cancer Lett 2001;163:109–16.
[15] Rodriguez JA, Span SW, Ferreira CGM, Kruyt FAE, Giaccone G. CRM1-mediated nuclear export determines the cytoplasmic localization of the antiapoptotic protein survivin. Exp Cell Res 2002;275:44–53.
[16] Rothe M, Pan MG, Henzei WJ, Ayres TM, Goeddel DV. The TNFR2–TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 1995;83:1243–52.
[17] Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, et al. A conserved family of cellular genes related to the baculovirus IAP gene and encoding apoptosis inhibitors. EMBO J 1996;15:2685–94.
[18] Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996;379:349–53.
[19] Roy N, Dereraux QL, Takahashi R, Salvesen GS, Reed JC. The cIAP-1 and cIAP-2 proteins are distinct inhibitors of specific caspases. EMBO J 1997;16:6914–25.
[20] Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 1994;84:1361–92.
[21] Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink K, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee Meeting—Airlie House, Virginia, November 1997. J Clin Oncol 1999;17:3835–49.
[22] Matutes E, Owusu-Ankomah K, Morilla R, Garcia Marco J, Houlihau A, Que TH, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 1994;8:1640–5.
[23] Kitagawa M, Yamaguchi S, Takahashi M, Tanizawa T, Hirokawa K, Kamiyama R. Localization of Fas and Fas ligand in bone marrow cells demonstrating myelodysplasia. Leukemia 1998;12:486–92.
[24] Mahotka C, Kreig T, Kreig A, Wenzel M, Suschek CV, Heydthausen M, et al. Distinct in vivo expression patterns of survivin splice variants in renal cell carcinomas. Int J Cancer 2002;100:30–6.
[25] Mahotka C, Wenzel M, Springer E, Gabbert HE, Gerharz CD. Survivin-ΔEx3 and survivin-2B: two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties. Cancer Res 1999;59:6097–102.
[26] Kitagawa M, Takahashi M, Yamaguchi S, Inoue M, Ogawa S, Hirokawa K, et al. Expression of inducible nitric oxide synthase (NOS) in bone marrow cells of myelodysplastic syndromes. Leukemia 1999;13:699–703.
[27] Sawanobori M, Yamaguchi S, Hasegawa M, Inoue M, Suzuki K, Kamiyama R, et al. Expression of YNF receptors and related signaling molecules in the bone marrow form patients with myelodysplastic syndromes. Leuk Res 2003;27:583–91.
[28] Moriai R, Asanuma K, Kobayashi D, Yajima T, Yagihashi A, Yamada M, et al. Quantitative analysis of the anti-apoptotic gene survivin expression in malignant haematopoietic cells. Anticancer Res 2001;21:595–600.
[29] Paydas S, Tanriverdi K, Yavuz S, Disel U, Sahin B, Burgut R. Survivin and aven: two distinct antiapoptotic signals in acute leukemias. Ann Oncol 2003;14:1045–50.
[30] Kamihira S, Yamada Y, Hirakata Y, Tomonaga M, Sugahara K, Hayashi T, et al. Aberrant expression of caspase cascade regulatory genes in adult T-cell leukaemia: survivin is an important determinant for prognosis. Br J Haematol 2001;114:63–9.
[31] Granziero L, Ghia P, Circosta P, Gottardi D, Strola G, Geuna M, et al. Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. Blood 2001;97:2777–83.
[32] Munzert G, Kirchner D, Stobbe H, Bergmann L, Schmid RM, Döhner H. Tumor necrosis factor receptor-associated factor 1 gene overexpression in B-cell chronic lymphocytic leukemia: analysis of NF-κB/Rel-regulated inhibitors of apoptosis. Blood 2002;100:3749–56.
[33] Vogel LA, Noelle RJ. CD40 and its crucial role as a member of the TNFR family. Semin Immunol 1998;10:435–42.
[34] Fluckinger AC, Rossi JF, Bussel A, Bryon P, Banchereau J, Defance T. Responsiveness of chronic lymphocytic leukemia B cells activated via surface Igs or CD40 to B-cell tropic factors. Blood 1992;80:3173–81.
[35] Modrek B, Lee C. A genetic view of alternative splicing. Nat Genet 2002;30:13–9.
[36] Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic *survivin* gene by wild type p53. J Biol Chem 2002;277:3247–57.
[37] Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002;21:2613–22.

＜症例報告＞

血縁者間末梢血幹細胞移植後に発症した肝中心静脈閉塞症に対しprostaglandin E_1が有効であった骨髄異形成症候群

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植村　直樹
鈴木　憲史

抄　録

症例は38歳女性。骨髄異形成症候群と診断し化学療法を開始したが，徐々に病状が悪化した。そのため前処置後，同胞の姉より同種末梢血幹細胞移植を施行した。移植後，中等症の肝中心静脈閉塞症を発症したため，prostaglandin E_1 (PGE_1) を中心とする治療を開始した。その結果，骨髄の生着を確認し，総ビリルビンは減少した。黄疸の軽快と共に併発したacute respiratory distress syndrome (ARDS) も改善し，PGE_1による治療効果であると考えられた。

＜Case report＞

Myelodysplastic Syndrome (RAEB-t) with Veno-occlusive Disease after Allogenic Sibling Bone Marrow Transplantation that Ponded to Prostaglandin E_1

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Summary

The patient was a 38 year-old woman. Bone marrow examination showed 15% blasts, so myelodysplastic syndrome (RAEB-t) was diagnosed. Chemotherapy was performed, but anemia and thrombocytopenia progressed and she became dependent on blood transfusions. Since bone marrow blasts increased to 20%, after

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pretreatment with total body irradiation and cyclophosphamide, allogenic peripheral blood stem cell transplantation from her HLA-matched sister (ABO major mismatch) was performed. Immunosuppression was performed with methotrexate and cyclosporin A. On day 9 after transplantation, total bilirubin (TB) was elevated. On day 13, she had findings of acute respiratory distress syndrome (ARDS). Icterus was progressive and TB reached 11mg/dL on day 14. Abdominal ultrasound showed slight hepatosplenomegaly, but there was no tenderness of the abdomen. Her weight increased by more than 2% and moderate veno-occlusive disease (VOD) was diagnosed. ARDS became worse and she was admitted to the ICU. She was placed on a ventilator and administration of prostaglandin (PG) E_1 was started. On day 15, bone marrow engraftment was confirmed, TB decreased, and ARDS improved. On day 20, she was weaned from the ventilator and started natural breathing. Her jaundice resolved on day 26. PGE_1 appears to be effective after the onset of VOD based on the case reported here.

緒　　言

PGE_1の肝中心静脈閉塞症（veno-occlusive disease；VOD）に対する予防効果については多くの報告[1)~3)]があるが，発症後から投与して有効であった報告[4)5)]は少ない。今回，VOD発症後にPGE_1を使用したところ，副作用もなく，肝機能の改善が認められた1症例を経験したので報告する。

I　成　　績

【症例】 38歳，女性

主訴：白血球減少

既往歴：胃ポリープ，虫垂炎，乳房線維腫

家族歴・生活歴：特記すべきことなし

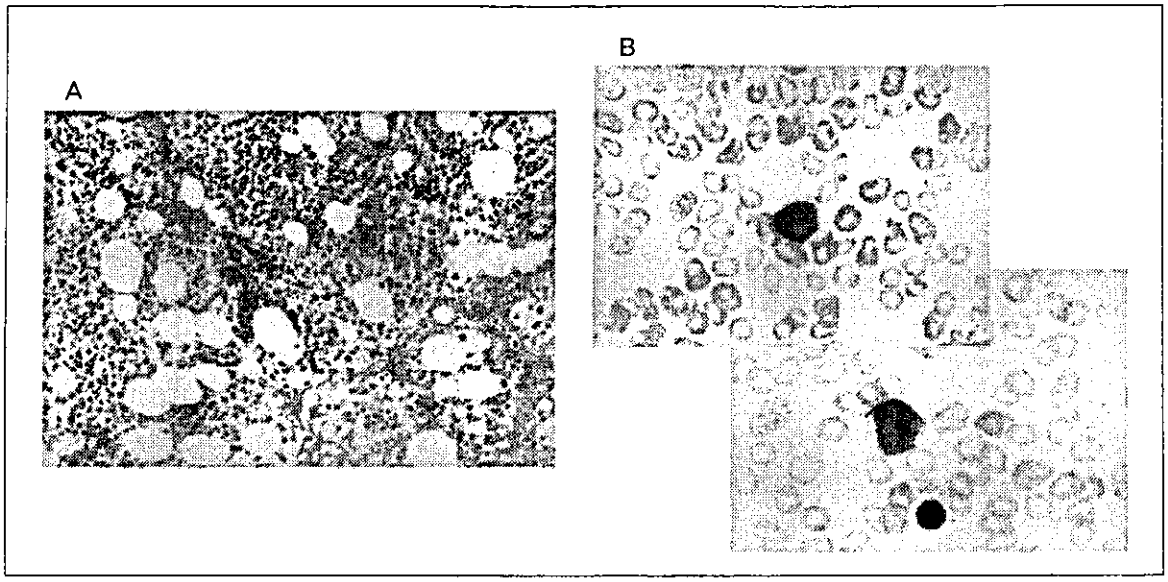
現病歴：2000年9月，検診で白血球減少を指摘された。骨髄穿刺で，MDS（RAEB-t）と診断した。DCM，A-VVV，MD-CPM，A-VVV，high dose-CPM/VP-16，high dose-AraC+MIT施行し，完全寛解に導入した。以後，DCMなどの化学療法を施行していた。2003年3月頃より，貧血，血小板減少進行，輸血依存性となった。骨髄穿刺でblastが20%に増加していたため，HLA一致の姉（ABO major mismatch）より同種末梢血幹細胞移植目的で入院した。

入院時現症：身長163cm，体重52kg，血圧104/68mmHg，脈拍50/min，呼吸音異常なし，肝脾触知せず

入院時検査所見：写真1に示すように，汎血球減少を認めた。末梢血中にblastが8%見られ，骨髄穿刺ではblastは20.2%，細胞表面マーカーはCD13，CD33，DRが陽性であった。

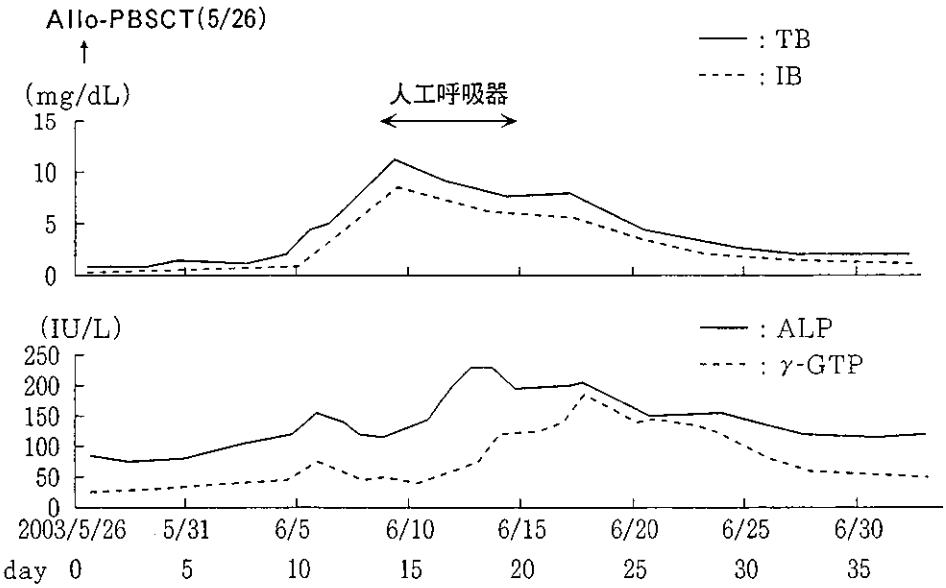
入院後経過（図1）：5月19日からtotal body irradiation (2Gy×6)+シクロホスファミド（40mg/kg×2）による前処置を開始し，5月26日（day 0）に同胞の姉より同種末梢血幹細胞移植を施行した。免疫抑制はshort term MTX+シクロスポリンAで対応した。day 9に総ビリルビン（TB）が2.1mg/dLへ上昇した。day 13には，咳嗽，呼吸困難出現，両下肺にcoarse crackle出現，胸部X-P上，心不全，両側下肺を中心としたARDSの所見を認めた。黄疸も進行し，day 14にはTBは11.0 mg/dLに上昇した（表1）。腹部エコー上，肝脾腫は軽度，IVC 18mmと拡大，腹部の圧痛は認めなかったが，2%以上の体重の増加を認めたため，Seatle groupの診断基準[6)]に従って，

写真1　骨髄/末梢血所見（2003/4/16）



A：骨髄，Hematoxylin-eosin stain（×100）　　B：末梢血，May-Giemsa stain（×100）

図1　移植後の臨床経過



MTX	22mg	15mg	15mg						
cyclospolin A	150mg		135mg		120mg		80mg		
methylpredonisolone			1000mg	500mg	250mg	125mg	80mg	40mg	20mg	10mg
heparan sulfate Na				2500IU		1250IU			
PGE_1				360μg	240μg	120μg	60μg	20μg	
ursodeoxycholic acid			600mg						

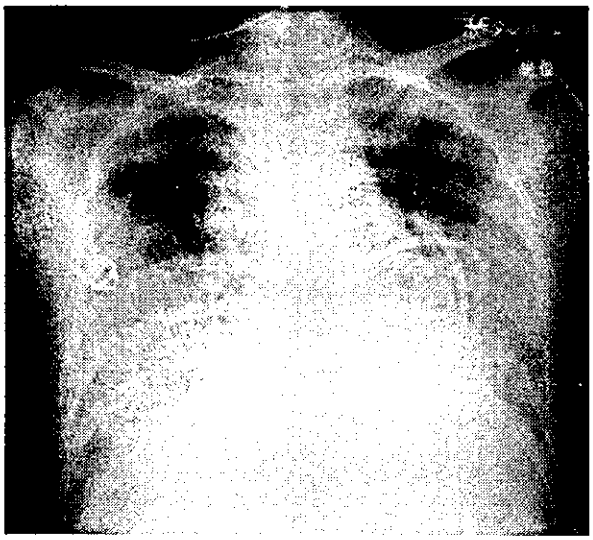
VOD（中等症）と診断した。ARDSが増悪傾向にあったためICUにて人工呼吸器管理とした〔**写真2**-(a)〕。PGE_1（注射用プロスタンディン®，0.3μg/kg/hr），ステロイドパルス（1g×3日）およびヘパラン硫酸（2500IU）の投与を開始した。day 15，骨髄穿刺施行して生着を確認した。また，TBも減少して，day 19にはARDSも改善した〔**写真2**-(b)〕。day 20に抜管して自発呼吸に切り替えた。day26には黄疸が軽快した。day 60には，TBは1.1mg/dLに低

表1 移植14日後（2003/6/9）の臨床検査データ

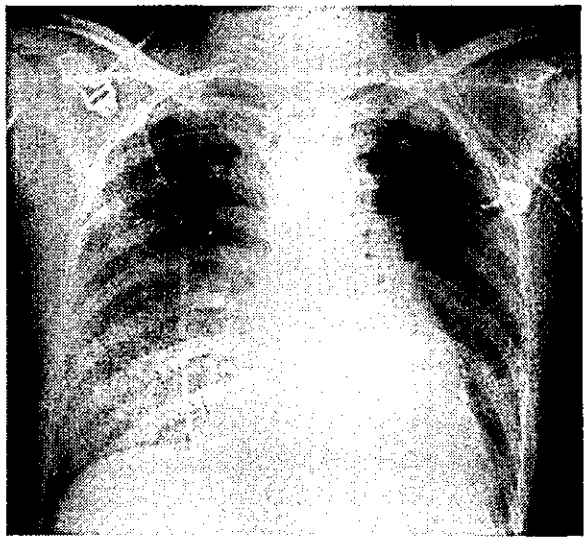
peripheral blood		blood chemistry	
WBC	7000/μL	GOT	11 IU/L
RBC	404×10⁴/μL	GPT	9 IU/L
Hb	12.4g/dL	LDH	339 IU/L
Ht	36.5%	ALP	124 IU/L
MCV	90.3fl	γ-GTP	40 IU/L
MCH	30.8pg	TB	11.0mg/dL
PLT	2.0×10⁴/μL	D.B.	8.7mg/dL
Baso	0.5%	BUN	66mg/dL
Myelo	2.5%	Cre	1.6mg/dL
Meta	1.0%	Na	146mEq/L
St	56.5%	K	3.0mEq/L
Seg	19.0%	Cl	107mEq/L
Ly	3.0%	CRP	12.5mg/dL
Mono	17.5%		

写真2　移植14，19日後の胸部X線

(a) 2003年6月9日（day 14）

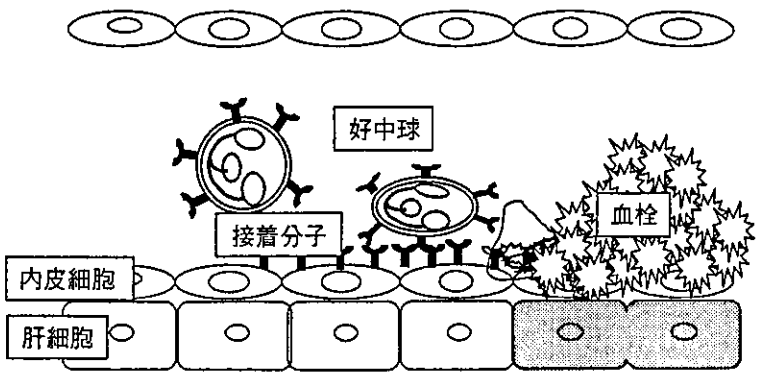


(b) 2003年6月14日（day 19）



下し，皮膚，肝臓，消化管の移植片対宿主反応病（Graft-versus-Host Disease，GVHD）も認められず，順調に回復していた。day 150にGVHDを発症したが，ステロイド，ネオーラル®の増加により軽快した。day 180に末梢血にblastを認めたため再発を疑い，day 210とday 230にドナーリンパ球輸注を施行した。その結果，病勢の進行は抑制できたが改善までには至らなかったため，臍帯血を用いた再移植を予定している。

図2　VODに対するPGE$_1$の想定される作用機序



《PGE$_1$の作用》

血管内皮細胞への白血球の集積を抑制
血小板凝集を抑制する
肝細胞内のCa上昇を抑え，肝細胞障害を抑制する
胆汁排泄を促し，ビリルビンの上昇を抑制する

Ⅱ　考　　察

VODは，骨髄移植後早期に発症する肝腫大，黄疸，水分貯留を三徴候とする症候群である。その成因は骨髄移植の前処置により，肝臓の小静脈の内皮に障害が起きて血栓が生じ，肝静脈の鬱滞や肝細胞の壊死を起こすためと考えられている。発症頻度はIBMTR，EBMTRによれば6％とされるが，今後，造血幹細胞移植の適応拡大に伴い発症の増加や重症化が予想される。

VODの発症頻度を高める危険因子として，移植前からトランスアミナーゼが高値の例，超大量前処置施行例，前処置中の感染例，再移植例，HLA不一致または非血縁者間移植施行例，移植前処置のTBI使用例などが挙げられる。本例ではTBI（12Gy）を含む前処置が，VOD発症の一因と思われた。

VODの70％は自然回復するといわれており，初期治療は水，電解質バランス管理を中心とした対症療法が一般的である。薬物はt-PA，活性型プロテインC，あるいはヘパリンなどが使用されているが，未だ治療薬あるいは予防法として確立したものはない。

PGE$_1$は血管拡張作用や抗血小板作用などを有しており，その製剤は末梢循環障害の治療などに広く使用されている。VODに関しては，Ibrahimら[4]はPGE$_1$（500μg/day×4～7日）を9例のVODに投与し，全例で効果があったと報告している。しかし，予防投与で有効であったとする報告は多いが，発症後にPGE$_1$を投与して有効であったとする報告は，我々が検索した限りでは本邦の成人で本例が5例目であった。

PGE$_1$が効果を示した機序としては，①血管内皮への白血球集積を抑制し，血管内皮細胞障害を抑制した。②肝細胞内cAMPを増加して，門脈系や肝組織血流量を改善した。③毛細胆管に作用して胆汁排泄を促進し，ビリルビン上昇を抑制した。④抗血小板作用により血栓形成を抑制したことなどが想定される（図2）。

今回は，同種末梢血幹細胞移植後に発症したVODに対してPGE$_1$を中心とした治療を行ったところ，肝機能の改善が認められ副作用もなかった。PGE$_1$はVODに対して安全に使用できる治療薬となりうる可能性があると考えられる。

文　　献

1) Gluckman, E. et al. : Use of prostaglandin E$_1$ for prevention of liver veno-occlusive disease in leukemic patients

treated by allogeneic bone marrow transplantation, British Journal of Haematology 74, 277～281, 1990.

2) Baglin, T. P. : Veno-occlusive disease of the liver complicating bone marrow transplantation, Bone Marrow Transplantation 13, 1～4, 1994.

3) 森尾聡子ほか：同種骨髄移植後の肝中心静脈閉塞症に対するプロスタグランディン E_1 の予防投与の試み，臨牀血液 35, 846～852, 1994.

4) Ibrahim, A. et al. : Hepatic veno-occlusive disease following bone marrow transplantation treated by prostaglandin E_1, Bone Marrow Transplant. 7 (Suppl. 2) 53, 1991.

5) 平安山英穂ほか：同種骨髄移植後の肝中心静脈閉塞症（VOD）に対する PGE_1 の有用性について，日本造血幹細胞学会雑誌 37, 175, 1998.

6) McDonald, G. B. et al. : Venocclusive disease of the liver after bone marrow transplantation : diagnosis, incidence, and predisposing factors, Hepatology 4, 116～122, 1984.

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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.