Table 2. (*Continued*)

Affy ID	Gene name	Symbol	Fold-change
204174_at	Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	0·7239532
223090_x_at	Transmembrane protein vezatin	VEZATIN	0·72426665
226392_at	RAS p21 protein activator 2	RASA2	0·7243885
227990_at	Step II splicing factor SLU7	SLU7	0·72443694
218823_s_at	Potassium channel tetramerisation domain containing 9	KCTD9	0·7246428
202804_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	ABCC1	0·7248081
227808_at	DnaJ (Hsp40) homolog, subfamily C, member 15	DNAJD1	0·7252901
208798_x_at	Golgi autoantigen, golgin subfamily a, 8A	GOLGIN-67	0·72628635
213292_s_at	Sorting nexin 13	SNX13	0·72653073
222996_s_at	CXXC finger 5	CXXC5	0·72690076
201237_at	Capping protein (actin filament) muscle Z-line, alpha 2	CAPZA2	0·7269243
224990_at	Hypothetical protein LOC201895	LOC201895	0·72730917
232008_s_at	Bobby sox homolog	BBX	0·7273624
225951_s_at	LOC440309	CHD2	0·727875
213005_s_at	Ankyrin repeat domain 15	ANKRD15	0·72830474
213385_at	Chimerin (chimaerin) 2	CHN2	0·72833586
212952_at	Calreticulin	CALR	0·72959316
219356_s_at	Chromatin modifying protein 5	SNF7DC2	0·7297071
215785_s_at	Cytoplasmic FMR1 interacting protein 2	CYFIP2	0·7301472
227167_s_at	Mesenchymal stem cell protein DSC96		0·73024756
204951_at	Ras homolog gene family, member H	RHOH	0·7306572
229050_s_at	Hypothetical protein MGC16037	MGC16037	0·73081815
224778_s_at	TAO kinase 1	TAOK1	0·7310451
218191_s_at	LMBR1 domain containing 1	C6orf209	0·7311856
200728_at	ARP2 actin-related protein 2 homolog	ACTR2	0·7312589
228959_at	CDNA		0·73128814
224827_at	Dendritic cell-derived ubiquitin-like protein	DC-UbP	0·73133826
218478_s_at	Zinc finger, CCHC domain containing 8	ZCCHC8	0·732107
201864_at	GDP dissociation inhibitor 1	GDI1	0·7322083
212069_s_at	KIAA0515	KIAA0515	0·73250145
202769_at	Cyclin G2	CCNG2	0·7329358
221751_at	Solute carrier family 2, member 3 pseudogene 1	PANK3	0·7336308
223054_at	DnaJ (Hsp40) homolog, subfamily B, member 11	DNAJB11	0·73389965
208765_s_at	Heterogeneous nuclear ribonucleoprotein R	HNRPR	0·7345736
212080_at	Similar to CDNA sequence BC021608	LOC143941	0·73511535
212838_at	Dynamin binding protein	DNMBP	0·73558724
243910_x_at	Cullin-associated and neddylation-dissociated 1	TIP120A	0·73592746
205034_at	Cyclin E2	CCNE2	0·7360657
205267_at	POU domain, class 2, associating factor 1	POU2AF1	0·7366251
202108_at	Peptidase D	PEPD	0·7367287
205367_at	Adaptor protein with pleckstrin homology and src homology 2 domains	APS	0·7371733
1562836_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	DDX6	0·73732203
217823_s_at	Ubiquitin-conjugating enzyme E2, J1	UBE2J1	0·7376396
204658_at	Transformer-2 alpha	TRA2A	0·7381278
225893_at	MRNA; cDNA DKFZp686D04119		0·73877865
226874_at	Kelch-like 8 (Drosophila)	KLHL8	0·73884803
217118_s_at	Chromosome 22 open reading frame 9	C22orf9	0·7391449
209463_s_at	TAF12 RNA polymerase II	TAF12	0·73976564
226134_s_at	Musashi homolog 2	MSI2	0·7397834
1553906_s_at	FYVE, RhoGEF and PH domain containing 2	FGD2	0·739792
209748_at	Spastin	SPG4	0·7398436
212995_x_at	Hypothetical protein FLJ14346	FLJ14346	0·7402725
200967_at	Peptidylprolyl isomerase B	PPIB	0·74033284
204391_x_at	Tripartite motif-containing 24	TIF1	0·74084216
221520_s_at	Cell division cycle associated 8	CDCA8	0·7408515

Table 2. (*Continued*)

Affy ID	Gene name	Symbol	Fold-change
223059_s_at	Chromosome 10 open reading frame 45	C10orf45	0·7411011
224890_s_at	Similar to CG14977-PA	LOC389541	0·7412293
206061_s_at	Dicer1, Dcr-1 homolog	DICER1	0·74136984
201885_s_at	Cytochrome b5 reductase 3	DIA1	0·74161553
212665_at	TCDD-inducible poly(ADP-ribose) polymerase	TIPARP	0·74172264
201242_s_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	0·74187577
218167_at	Archaemetzincins-2	LOC51321	0·7419247
214715_x_at	Zinc finger protein 160	ZNF160	0·7425452
200654_at	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, beta polypeptide	P4HB	0·74279094
202171_at	Zinc finger protein 161	ZNF161	0·7429878
224250_s_at	SECIS binding protein 2	SECISBP2	0·7430157
222673_x_at	Similar to hypothetical protein MGC17347	LOC159090	0·74343455
1557961_s_at	602536302F1 NIH_MGC_59		0·7434646
202660_at	Family with sequence similarity 20, member C	ITPR2	0·7436526
217901_at	Desmoglein 2	DSG2	0·7440809
222691_at	solute carrier family 35, member B3	CGI-19	0·7441896
205739_x_at	Zinc finger protein 588	ZNF588	0·74437064
224415_s_at	Histidine triad nucleotide binding protein 2	HINT2	0·7444139
209898_x_at	Intersectin 2	ITSN2	0·74446803
203660_s_at	Pericentrin (kendrin)	PCNT2	0·74484545
203947_at	Hypothetical protein LOC283267	CSTF3	0·74533767
212382_at	Transcription factor 4	TCF4	0·7455324
212560_at	Chromosome 11 open reading frame 32	SORL1	0·7456149
218095_s_at	TPA regulated locus	TPARL	0·74569106
216187_x_at	Kinesin 2	KNS2	0·74619925
224599_at	CGG triplet repeat binding protein 1	CGGBP1	0·74662995
230795_at	Histone H4/o	HIST2H4	0·74673605
201266_at	Thioredoxin reductase 1	TXNRD1	0·7467521
201195_s_at	Solute carrier family 7, member 5	SLC7A5	0·74680513
221760_at	Mannosidase, alpha, class 1A, member 1	MAN1A1	0·7468957
225285_at	Branched chain aminotransferase 1, cytosolic	BCAT1	0·747043
217776_at	Retinol dehydrogenase 11	RDH11	0·7471061
219259_at	semaphorin 4A	SEMA4A	0·74722433
223165_s_at	Inositol hexaphosphate kinase 2	IHPK2	0·74733293
201791_s_at	7-dehydrocholesterol reductase	DHCR7	0·74754477
208993_s_at	Peptidyl-prolyl isomerase G	PPIG	0·7477576
202951_at	Serine/threonine kinase 38	STK38	0·7478889
203648_at	TatD DNase domain containing 2	TATDN2	0·74821633
202716_at	Protein tyrosine phosphatase, non-receptor type 1	PTPN1	0·74856865
227601_at	KIAA1627 protein	KIAA1627	0·74859655
204028_s_at	RAB GTPase activating protein 1	RABGAP1	0·74878204
209539_at	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	ARHGEF6	0·7491753
202027_at	Chromosome 22 open reading frame 5	C22orf5	0·74919754
1555762_s_at	Megakaryoblastic leukemia 1	MKL1	0·7492855
212205_at	H2A histone family, member V	H2AFV	0·7496612
219553_at	Non-metastatic cells 7, protein expressed in	NME7	0·7496923
208723_at	Ubiquitin specific peptidase 11	USP11	0·749806
222499_at	SIL1 homolog, endoplasmic reticulum chaperone	SIL1	0·74986565

Next we examined the effect of CD40 stimulation on apoptosis induction in MLMA cells. As shown in Fig. 6(b), when CD40 was stimulated by the addition of CD40-ligand (CD40L) in the presence of interleukin-4, induction of apoptosis in MLMA cells was mediated by both BCR- and CD20-cross-linking. Furthermore, when both CD40 and BAFF were simultaneously stimulated, better inhibition of BCR-induced and CD20-induced apoptosis was observed than those mediated by each of them alone.

Discussion

In the present study, we have clearly shown that BAFF can inhibit apoptosis mediated by BCR or CD20 in MLMA cells that exhibit a mature B-cell phenotype. The BCR is thought to play a crucial role in clonal selection and clonal expansion in the process of B-cell development to expand high-affinity clones against exogenous antigens and eliminate self-acting or low-affinity clones.[31] Although its precise function is yet to be clarified, CD20 is thought to play a role in B-cell development by mediating lipid raft-related signalling.[32] Our findings indicate that BAFF contributes to the regulation of B-cell development by modulating apoptotic elimination of B cells mediated by BCR and CD20. Furthermore, our results also indicate that BAFF can enhance cell proliferation in MLMA cells.

A number of studies have attempted to elucidate the molecular basis of the function of BAFF.[24,33–39] A major focus of recent investigations has been the pro-survival signalling of BAFF-R. Activation of the alternative NF-κB pathway (processing of NF-κB2 and the nuclear translocation of p52/RelB heterodimers) is a major outcome of BAFF-R-stimulation,[2,24,36] whereas BAFF-R also weakly activates the classical NF-κB pathway mediated by NF-κB1 and low-level nuclear translocation of p50/RelA DNA-binding activity is induced. Coincident with previous reports, we also observed that BAFF induced cleavage of both NF-κB2 and NF-κB1 in MLMA cells (Fig. 4). APRIL only activates NF-κB1 via either BCMA or TACI and did not inhibit CD20- and BCR-mediated apoptosis in MLMA cells, so the anti-apoptotic effect of BAFF is thought to be mediated mainly by NF-κB2 activation.

Recent studies have shown that NF-κB directly binds to the promoter region of the Bcl-2 gene and induces transcriptional activation.[39] Since Bcl-2 has an anti-apoptotic function, the elevated level of Bcl-2 protein is thought to be important for BAFF-mediated B-cell survival. In addition, BAFF is reported to temporarily inactivate GSK-3β via AKT-mediated phosphorylation.[34] Since GSK-3β has been found to cause apoptosis by inducing the degradation of Mcl-1 (an anti-apoptotic Bcl-2 family member) and compromising mitochondrial membrane integrity,[40] the BAFF-mediated phosphorylation of GSK-3β is thought to also participate in the anti-apoptotic effect of BAFF. Consistent with previous reports, we observed that BAFF treatment also induced both an increase in Bcl-2 expression and the transient phosphorylation of GSK-3β in MLMA cells. Therefore, it is likely that the inhibitory effect of BAFF against apoptosis mediated by CD20 or BCR is also mainly the result of NF-κB2-mediated Bcl-2 expression and the transient inactivation of GSK-3β.

The pro-apoptotic BH3-only Bcl-2 family member Bim was shown to sequestrate Bcl-2 and play an essential role for BCR cross-linking-induced apoptosis.[41] Moreover, Mcl-1 was shown to inhibit Bim selectively and to be essential both early in lymphoid development and later on in the maintenance of mature B lymphocytes.[42] Therefore, future investigation of the involvement of Bim and MCL-1 in the BAFF-induced inhibition of CD20-mediated and BCR-mediated apoptosis in MLMA cells should be interesting.

Interestingly, our findings indicated that BAFF treatment also induces the expression of a series of genes related to cell survival. For example, both the microarray analysis and the flow cytometric analysis revealed increased expression of CD40 after the treatment. Since CD40 is known to mediate pro-survival signalling upon interaction with CD40L expressed on activated T cells,[43] it is suggested that BAFF-mediated up-regulation of CD40 expression also contributes to B-cell survival *in vivo*. Indeed, we observed in this study that simultaneous stimulation with CD40 and BAFF resulted in better inhibition of BCR-induced and CD20-induced apoptosis than stimulation with by each of them alone. Therefore, it may be possible that BAFF-mediated up-regulation of CD40 inhibits apoptosis induction synergistically with the effect of BAFF *in vivo*.

The microarray analysis also revealed up-regulation of several genes involved in either the inhibition of apoptosis or the proliferation of B cells (Table 1). For example, Myb has been demonstrated to directly up-regulate Bcl-2 and suppresses apoptosis.[44,45] EBI3 is a subunit of interleukin-27 that increases proliferation of B cells.[46] CFLAR is known to inhibit the activation of caspase 8.[47] Therefore, our data may indicate that mechanisms other than NF-κB2-mediated Bcl-2 up-regulation are involved in the anti-apoptotic effect of BAFF in MLMA cells. Furthermore, because direct cross-talk between the BCR-signalling and BAFF-signalling systems has been reported,[48] BAFF-mediated signals may also be able to directly influence the BCR-mediated apoptotic signalling system.

It is well documented that BAFF-mediated signalling is involved in the survival of malignant B cells.[2,49] For example, BAFF is reported to be an autocrine pro-survival and proliferation factor for B-cell chronic lymphocytic leukaemia and multiple myeloma.[2,49–51] BAFF is also thought to promote cell survival and proliferation in Hodgkin and non-Hodgkin lymphoma. Therefore, BAFF and BAFF-R might be potential molecular targets in the treatment of B-cell malignancies.[52–54] Using a combination of blocking of BAFF-signalling and activation of apoptosis induction, such as CD20 cross-linking, a novel therapeutic approach would be developed.

In conclusion, BAFF-mediated signalling inhibited CD20-mediated or BCR-mediated apoptosis in MLMA cells. Although more detailed experiments are clearly needed, MLMA cells should provide a model for investigating the molecular basis of BAFF's effect on B cells *in vitro* and will help to elucidate how B cells survive in an immune system in which BAFF-mediated signalling is involved.

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original article

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Childhood cancer in Japan: focusing on trend in mortality from 1970 to 2006

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Objective: This paper describes the mortality rates and trends from childhood cancer at the population level over a 37-year period in Japan and other developed countries.

Materials and methods: Age-standardized mortality rates were calculated by the direct method using age-specific mortality rates at 5-year age intervals and weights based on the age distribution of the standard world population. The joinpoint regression model was used to describe changes in trends.

Results: For all cancers combined, the mortality rate during 2000–2006 was 2.20 per 100 000 population for boys and 1.89 for girls. Mortality for all cancers combined decreased since 1970s in Japan. A stable trend was observed in recent 5 years for girls. For leukemia, a declining trend was observed in the whole period for girls and in 1976–2006 for boys. Mortality rates for childhood central nervous system tumors have remained stable at a low level during 1980–2006.

Conclusions: The present study provides updated figures and trends in childhood cancer mortality in Japan and other developed countries. This will help to estimate care needs and to plan intervention and the quantity of appropriate childhood cancer treatment.

Key words: cancer, childhood, epidemiology, mortality, time trends

introduction

It is estimated that ~3000 Japanese children aged from birth to 18 years will develop cancer. Although childhood cancer is rare compared with adult cancer, it is the fourth most common cause of death among children aged 0–14 years in Japan, according to the report given by the Ministry of Health, Labor and Welfare of Japan in 2005. A population-based study in Osaka prefecture in Japan indicated that death due to childhood cancer declined from 1972 to 1995, while the incidence increased in the same period [1]. In the United States, an estimated 10 400 new cases and 1545 deaths are expected to occur among children aged 0–14 years in 2007 [2]. During recent three decades, the incidence of childhood cancer increased ~0.6% annually. In contrast, mortality from childhood cancer declined by 1.3% per year during 1990–2004 [3]. A population-based study among European children since the 1970s showed that the overall incidence of childhood cancer has increased by 1.0% per year, while mortality has declined by 3.6% per year in the past three decades [4, 5].

The decrease in mortality from childhood cancer has been suggested to be due to the effects of improvements in diagnosis and therapy. For all childhood cancers combined, 5-year relative survival has improved markedly over the past three decades, from <50% before the 1970s to ~80% today [2].

There is no national childhood cancer registry system in Japan, and recent childhood cancer mortality has not been well characterized in terms of temporal and geographic trends. This paper describes the occurrence of death from childhood cancer at the population level over a 37-year period in Japan using official death certification data, which record 100% of deaths in Japan. The aim of this study was to ascertain the general mortality trend for each sex and to study the moment at which a shift in the trend occurred.

materials and methods

The number of death by cause, stratified for sex and by 5-year age group for cancer for the period 1970–2006, was derived from vital statistics compiled by the Ministry of Health, Labor and Welfare of Japan. Population figures were obtained from census data and intercensus estimates, by calendar year, age and gender. Population censuses of Japan are conducted every 5 years by the Statistics Bureau, Ministry of Internal Affair and Communications. For comparison, we also calculated the cancer mortality rate in other developed countries, including Canada (1970–2004), the United States (1970–2005), Italy (1970–2003), UK (1970–2005) and New Zealand (1970–2004). Deaths at age 0–4, 5–9 and 10–14 years were derived from the World Health Organization (WHO)

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mortality database. Estimates of the population, generally based on official censuses, were based on the same WHO database.

During the study period, three different revisions of the International Classification of Disease were used. In Japan, this included International Classification of Diseases (ICD)-8 from 1970 to 1978, ICD-9 from 1979 to 1994 and ICD-10 from 1995 onward. Since the differences were minor in various revisions, we recorded six cancer sites, including all cancer combined (ICD-8: 140–209; ICD-9: 140–208; ICD-10: C00–97), leukemia (ICD-8: 204–207; ICD-9: 204–208; ICD10: C91–C95), lymphomas (ICD-8: 200–202; ICD-9: 200–202; ICD-10: C81–85), central nervous system (CNS) tumors (ICD-9: 191–192; ICD–10: C70–C72), malignant kidney tumors (ICD-8: 189; ICD-9: 189; ICD-10: C64–C68) and malignant bone tumors (ICD-9: 170; ICD-10: C40–C41). In order to avoid possible bias due to changed ICD, the analysis of CNS tumors, malignant bone tumors and lymphomas (United States only) was restricted to data from 1980 onwards.

Age-standardized mortality rates were calculated by the direct method using age-specific mortality rates for 5-year age intervals and weights based on the age distribution of the standard world population. All rates are expressed per 100 000 children-years.

Table 1. Childhood cancer mortality rate (per 100 000) in Japan and other selected countries (boys)

Period of death	Japan	Canada	United States	Italy	UK	New Zealand
Total malignant tumors						
1970–1974	6.19	7.69	6.47	8.72	7.20	8.45
1975–1979	5.86	6.10	5.25	7.96	6.53	7.59
1980–1984	4.99	5.34	4.60	6.96	5.06	7.04
1985–1989	4.13	4.52	3.74	5.50	4.13	7.17
1990–1994	3.37	3.43	3.33	5.40	3.96	4.94
1995–1999	2.90	2.82	2.87	4.53	3.42	4.83
2000–	2.20	2.65	2.68	3.64	3.00	3.58
Leukemia						
1970–1974	3.39	3.58	2.90	3.94	3.02	3.44
1975–1979	3.10	2.81	2.23	3.50	2.79	3.07
1980–1984	2.46	2.06	1.76	2.85	2.11	2.89
1985–1989	1.91	1.79	1.41	2.20	1.52	2.76
1990–1994	1.54	1.17	1.20	1.99	1.41	1.69
1995–1999	1.21	0.90	0.97	1.64	1.18	1.99
2000–	0.84	0.85	0.85	1.25	0.91	0.78
Lymphomas						
1970–1974	0.61	0.76	–	1.14	0.73	0.77
1975–1979	0.66	0.62	–	0.86	0.65	0.75
1980–1984	0.65	0.49	0.39	0.62	0.40	0.51
1985–1989	0.55	0.32	0.31	0.51	0.29	0.58
1990–1994	0.36	0.21	0.23	0.47	0.25	0.15
1995–1999	0.18	0.16	0.16	0.41	0.20	0.23
2000–	0.14	0.12	0.12	0.27	0.20	0.12
Central nervous system tumors						
1980–1984	0.40	1.17	0.95	1.41	1.12	1.50
1985–1989	0.40	1.18	0.86	1.05	1.10	1.66
1990–1994	0.46	0.97	0.86	1.19	1.10	1.79
1995–1999	0.49	0.83	0.79	0.93	0.94	1.35
2000–	0.43	0.81	0.75	0.87	0.85	1.43
Malignant kidney tumors						
1970–1974	0.18	0.35	0.24	0.45	0.33	0.34
1975–1979	0.16	0.20	0.17	0.34	0.26	0.33
1980–1984	0.12	0.13	0.14	0.23	0.20	0.24
1985–1989	0.09	0.10	0.10	0.19	0.09	0.22
1990–1994	0.07	0.06	0.09	0.13	0.12	0.26
1995–1999	0.06	0.05	0.08	0.13	0.13	0.09
2000–	0.05	0.12	0.08	0.09	0.09	0.08
Malignant bone tumors						
1980–1984	0.15	0.18	0.16	0.33	0.26	0.12
1985–1989	0.15	0.16	0.12	0.24	0.18	0.30
1990–1994	0.14	0.11	0.11	0.19	0.14	0.04
1995–1999	0.13	0.12	0.11	0.14	0.13	0.17
2000–	0.09	0.12	0.13	0.15	0.15	0.24

The joinpoint regression model was used to describe changes in trends [6]. We allowed for up to four joinpoints for each model. The computation of mortality rates and their standard errors was implemented in SAS 9.0. Joinpoint analyses were carried out using Joinpoint software 3.3.1 from the Surveillance Research Program of the US National Cancer Institute. Time trends were assessed for all childhood cancer combined and for six major categories, including leukemia, lymphoma, malignant brain tumor, malignant kidney tumor and malignant bone tumor.

The standardized mortality ratio (SMR) by sex was calculated for 47 prefectures in Japan by taking the ratio of the observed to expected deaths. The z-value was computed for each SMR, on the basis of the assumption that observed deaths follow a Poisson distribution. The maps were developed using adjusted SMR by gender.

results

mortality

Tables 1 and 2 give age-adjusted mortality rates in Japan and five other developed countries for all malignant tumors and for

Table 2. Childhood cancer mortality rate (per 100 000) in Japan and other selected countries (girls)

Period of death	Japan	Canada	United States	Italy	UK	New Zealand
Total malignant tumors						
1970–1974	5.10	6.12	5.13	6.90	5.55	6.85
1975–1979	4.61	4.83	4.07	5.90	4.69	6.35
1980–1984	3.88	4.24	3.59	5.48	4.27	4.39
1985–1989	3.30	3.43	3.06	4.36	3.81	5.27
1990–1994	2.75	2.80	2.69	4.19	3.01	3.81
1995–1999	2.23	2.73	2.39	3.29	2.65	3.54
2000–	1.89	2.06	2.28	2.86	2.47	3.06
Leukemia						
1970–1974	2.86	2.80	2.26	3.28	2.43	3.08
1975–1979	2.50	2.34	1.70	2.53	1.82	1.86
1980–1984	1.79	1.71	1.30	2.17	1.59	1.66
1985–1989	1.50	1.37	1.09	1.51	1.26	1.84
1990–1994	1.20	0.89	0.91	1.47	0.89	1.04
1995–1999	0.88	0.87	0.78	1.07	0.91	1.34
2000–	0.68	0.46	0.69	0.82	0.76	0.90
Lymphomas						
1970–1974	0.33	0.39	–	0.54	0.31	0.27
1975–1979	0.35	0.18	–	0.39	0.27	0.41
1980–1984	0.31	0.23	0.16	0.26	0.22	0.21
1985–1989	0.28	0.22	0.13	0.28	0.14	0.25
1990–1994	0.25	0.12	0.09	0.16	0.09	0.10
1995–1999	0.10	0.09	0.08	0.17	0.09	0.20
2000–	0.06	0.39	0.06	0.18	0.09	0.05
Central nervous system tumors						
1980–1984	0.39	1.01	0.84	1.13	0.93	1.43
1985–1989	0.38	0.88	0.77	0.99	0.98	1.37
1990–1994	0.44	0.75	0.77	0.90	0.88	1.26
1995–1999	0.47	0.84	0.71	0.72	0.74	0.88
2000–	0.42	0.69	0.69	0.78	0.71	1.00
Malignant kidney tumors						
1970–1974	0.20	0.32	0.25	0.44	0.37	0.38
1975–1979	0.11	0.23	0.19	0.33	0.26	0.36
1980–1984	0.12	0.13	0.15	0.27	0.18	0.00
1985–1989	0.07	0.11	0.13	0.18	0.18	0.10
1990–1994	0.07	0.10	0.09	0.18	0.15	0.27
1995–1999	0.05	0.14	0.11	0.11	0.12	0.21
2000–	0.06	0.11	0.10	0.10	0.12	0.11
Malignant bone tumors						
1980–1984	0.17	0.20	0.16	0.26	0.29	0.13
1985–1989	0.16	0.14	0.12	0.27	0.26	0.31
1990–1994	0.12	0.13	0.13	0.23	0.14	0.05
1995–1999	0.14	0.12	0.11	0.16	0.13	0.18
2000–	0.11	0.16	0.11	0.12	0.20	0.25

the main types of childhood cancer. A total of 33 059 childhood cancer deaths were reported in Japan during 1970–2006, of which 353 cancer deaths occurred in 2006. For all cancers combined, the mortality rate during 2000–2006 was 2.20 per 100 000 population for boys and 1.89 for girls. Leukemia was the most common diagnosis. Death rates from leukemia were 0.84 for boys and 0.68 for girls. Mortality from childhood CNS tumors was 0.43 for boys and 0.42 for girls. Geographic variations were observed. The rates of childhood CNS tumor and malignant kidney tumor were lower for both genders in Japan than in other countries.

temporal changes in mortality

Trends of age-standardized mortality from childhood cancer are shown in Figures 1 and 2 and Tables 3 and 4. Mortality for all cancers combined decreased since 1970s in Japan. For boys, a declining trend of 1.58% per year ($P < 0.05$) was observed during 1970–1979, followed by an accelerated decline of 3.78% per year ($P < 0.05$) during 1979–2006. For girls, mortality was high in the 1970s and remained stable in 1996–2006 at a low level, after two significant periods of decline (1972–1995 and 1995–1999). The average annual per cent change (AAPC) in recent 10 years was −3.8% ($P < 0.05\%$) for boys and −1.9% ($P < 0.05$) for girls. In recent 5 years, declining trend only occurred in boys. The average annual per cent change during 2002–2006 was −3.8% ($P < 0.05\%$) for boys, and for girls a nonsignificant decline was observed from 2002 (AAPC = −0.6%, $P > 0.05$) for girls.

The mortality rate from leukemia in boys remained stable during 1970–1976 (APC = −1.10, $P > 0.05$) and then declined by 4.77% per year ($P < 0.05$) during 1976–2006. For girls, mortality decreased by 4.53% per year ($P < 0.05$) throughout the whole period. The average annual change in recent 10 years was −4.8% ($P < 0.05\%$) for boys and −4.5% ($P < 0.05\%$) for girls. Similar decline trends were also observed in Canada, the United States, Italy, UK (girls) and New Zealand.

In contrast with the dramatic decline in mortality for childhood leukemia, mortality rates from childhood CNS tumor in Japan remained stable at a low level for both genders during 1980–2006. The average annual change in recent 10 years was 0.5% ($P > 0.05$) for boys and 0.0% ($P > 0.05$) for girls. On the contrary, Canada, the United States, UK and New Zealand (girls) showed significant declining trends in the whole period.

With reference to the pattern of mortality for lymphomas, death rates for boys were stable during 1970–1985 and declined significant thereafter by 8.56% per year. The trend for girls leveled off during 1970–1991 and showed a declining trend of 11.85% per year during 1991–2006; however, except for New Zealand females, the death rates in other countries for both genders significantly declined throughout the whole period.

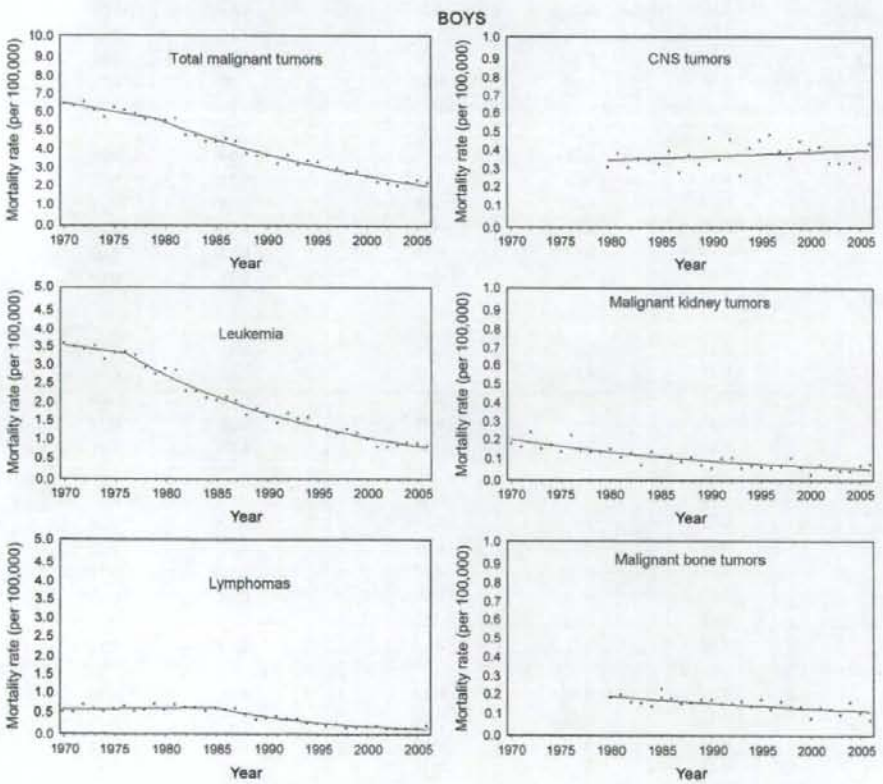

Figure 1. Mortality rates of childhood cancer deaths, boys, Japan, 1970–2006.

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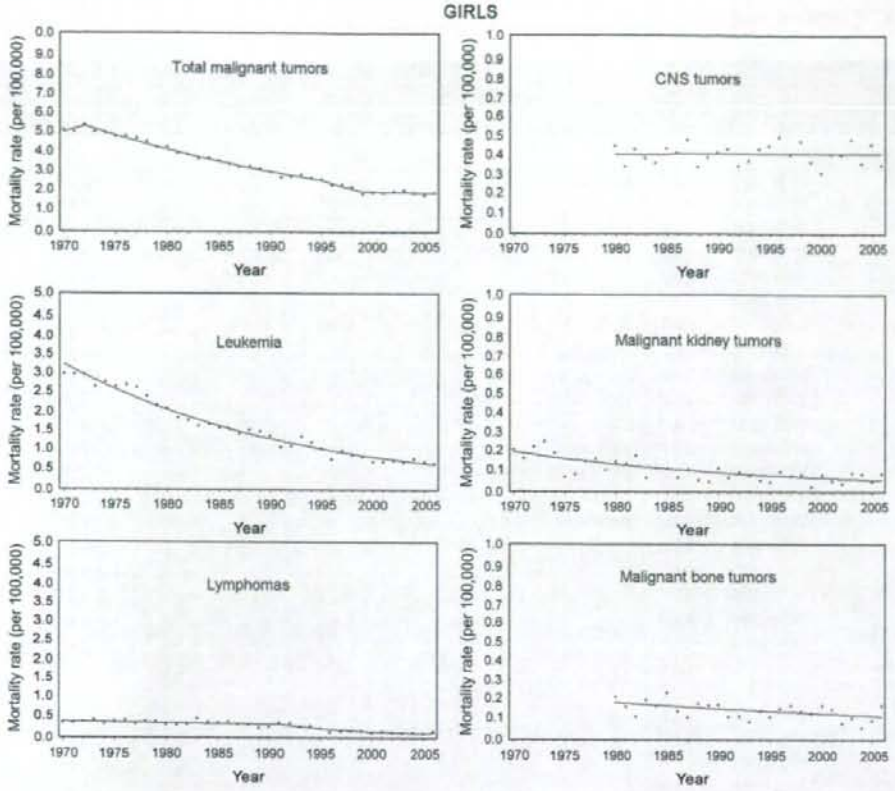

Figure 2. Mortality rates of childhood cancer deaths, girls, Japan, 1970–2006.

Regression analysis also revealed that the death rate for malignant kidney tumors declined by 4.12% per year for boys and 3.98% per year for girls during 1970–2006. Similar trends were observed for malignant bone tumor. Mortality decreased by 2.03% per year for boys and 1.79% per year for girls throughout the whole period.

Mortality rates varied from prefecture to prefecture in Japan. A map of SMR by gender is shown in Figure 3. The SMR was significantly highest among children in Kochi prefecture for boys and Tokushima and Kagoshima prefectures for girls.

discussion

In this study, we quantified the childhood cancer burden in Japan, focusing on mortality, and compared these figures with other developed countries. The results indicated that mortality from childhood cancer in Japan is substantial, while the number of deaths is small. There were 33 059 cases of childhood cancer death over the period 1970–2006 in Japan. Approximately 400 deaths each year were attributed to cancer in children aged 0–14 years. Mortality from all cancers combined in Japan is comparable to that in the European, North American and Oceanic countries included in this study for contrast.

The joinpoint regression method was used in our research to evaluate the trend in childhood cancer deaths. This method has allowed a detailed and accurate description of the pattern of childhood cancer mortality since it identifies the calendar years in which statistically significant changes in trends occurred. This offers a clearer picture of actual trends in mortality over long periods of time rather than using only one trend statistics. We also reported the average annual percentage change in this study. The AAPC can be used to characterize a short segment based on a joinpoint model fit over a much longer series. This is especially advantageous for situations when the data are sparse (e.g. a rare cancer or data from a small geographic area) [7]. Our results showed a declining cancer mortality rate for boys in the whole period and a stable trend for girls in recent 5 years. It is unlikely that the observed time trends in the mortality rate are due to variations in the completeness and accuracy of the population data because the analyzed data were provided by official sources and based on the population census. The significant time trend observed for most tumor types is congruent with improvements in diagnosis, therapy and supportive care.

The dramatic decrease in mortality observed for childhood leukemia, which accounts for ~50% of all childhood cancer

Table 3. The APC of childhood cancer mortality rates (boys)

Country	Trend 1		Trend 2		Trend 3		Trend 4		AAPC	
	Years	APC	Years	APC	Years	APC	Years	APC	Last 10 observations	Last 5 observations
Total malignant tumors										
Japan	1970–1979	−1.58*	1979–2006	−3.78*					−3.8*	−3.8*
Canada	1970–2004	−3.64*							−3.6*	−3.6*
United States	1970–1998	−3.22*	1998–2005	−0.26					−0.3	−0.9
Italy	1970–1985	−2.32*	1985–1989	−8.69	1989–1993	6.96	1993–2003	−5.89*	−5.9*	−5.9*
UK	1970–2005	−2.93*							−2.9*	−2.9*
New Zealand	1970–2004	−2.50*							−2.5*	−2.5*
Leukemia										
Japan	1970–1976	−1.10	1976–2006	−4.77*					−4.8*	−4.8*
Canada	1970–2004	−5.00*							−5.0*	−5.0*
United States	1970–1984	−4.95*	1984–2005	−3.39*					−3.4*	−3.4*
Italy	1970–2003	−3.69*							−3.7*	−3.7*
UK	1970–2005	−3.74*	2003–2005	−27.41					−9.6	−16.4
New Zealand	1970–1997	−2.12*	1997–2004	−18.03*					−14.7*	−18.0*
Lymphomas										
Japan	1970–1985	0.39	1985–2006	−8.56*					−8.6*	−8.6*
Canada	1970–2004	−6.10*							−6.1*	−6.1*
United States	1980–2005	−5.63*							−5.6*	−5.6*
Italy	1970–2003	−4.46*							−4.5*	−4.5*
UK	1970–2005	−4.56*							−4.6*	−4.6*
New Zealand	1970–2004	−2.57*							−2.6*	−2.6*
Central nervous system tumors										
Japan	1980–2006	0.48							0.5	0.5
Canada	1980–2004	−2.13*							−2.1*	−2.1*
United States	1980–2005	−1.07*							−1.1*	−1.1*
Italy	1980–2003	−2.19*							−2.2*	−2.2*
UK	1980–2005	−1.25*							−1.2*	−1.2*
New Zealand	1980–2004	−0.86							−0.9	−0.9
Malignant kidney tumors										
Japan	1970–2006	−4.12*							−4.1*	−4.1*
Canada	1970–1996	−7.91*	1996–2004	17.70*					14.5*	17.7*
United States	1970–1987	−5.46*	1987–2005	−1.73*					−1.7*	−1.7*
Italy	1970–2003	−4.91*							−4.9*	−4.9*
UK	1970–2005	−3.64*							−3.6*	−3.6*
New Zealand	1970–2004	−1.99							−2.0*	−2.0*
Malignant bone tumors										
Japan	1980–2006	−2.03*							−2.0*	−2.0*
Canada	1980–2004	−2.32*							−2.3*	−2.3*
United States	1980–1990	−4.41*	1990–2005	1.31					1.3	1.3
Italy	1980–2003	−4.43*							−4.4*	−4.4*
UK	1980–2005	−2.93*							−2.9*	−2.9*
New Zealand	1980–2004	−0.23							−0.2	−0.2

*$P < 0.05$.

APC is the annual per cent change; AAPC is average annual per cent change.

deaths, is consistent with improvements in survival, particularly for patients with acute lymphoblastic leukemia. This increase in survival is due to more effective antileukemic therapy, such as multidrug chemotherapy protocols, with a reduction in the number of relapses and resistant disease, but also due to improvements in supportive care, such as antibiotics, antifungal treatment, blood banking, transplant procedures and pediatric intensive care. In fact, the 5-year survival rate of acute lymphoblastic leukemia increased from 20% to 30% in the 1960s to 60% to 75% in the 1980s in developed countries. Current survival rates are ~80% for acute lymphoblastic leukemia (ALL) [8] and 50%–70% for acute myelogenous leukemia. In Japan, a population-based study in Osaka prefecture indicated that the 5-year survival rate of childhood leukemia increased from 32.4% in 1975–1984 to 60.4 in 1985–1994 [1]. National incidence trends could not be

Table 4. The APC of childhood cancer mortality rates (girls)

Country	Trend 1		Trend 2		Trend 3		Trend 4		AAPC	
	Years	APC	Years	APC	Years	APC	Years	APC	Last 10 observations	Last 5 observations
Total malignant tumors										
Japan	1970–1972	3.24	1972–1995	−3.21*	1995–1999	−6.46*	1999–2006	−0.57	−1.9*	−0.6
Canada	1970–2004	−3.42*							−3.4*	−3.4*
United States	1970–1977	−4.46*	1977–1995	−2.72*	1995–2005	−1.07*			−1.1*	−1.1*
Italy	1970–2003	−2.80*							−2.8*	−2.8*
UK	1970–2005	−2.73*							−2.7*	−2.7*
New Zealand	1970–2004	−2.57*							−2.6*	−2.6*
Leukemia										
Japan	1970–2006	−4.53*							−4.5*	−4.5*
Canada	1970–2004	−5.28*							−5.3*	−5.3*
United States	1970–1980	−6.09*	1980–2005	−3.14*					−3.1*	−3.1*
Italy	1970–2003	−4.33*							−4.3*	−4.3*
UK	1970–2005	−3.88*							−3.9*	−3.9*
New Zealand	1970–2004	−3.17*							−3.2*	−3.2*
Lymphomas										
Japan	1970–1991	−1.13	1991–2006	−11.85*					−11.8*	−11.8**
Canada	1970–2004	−4.55*							−4.6*	−4.6*
United States	1980–2005	−4.39*							−4.4*	−4.4*
Italy	1970–2003	−3.93*							−3.9*	−3.9*
UK	1970–2005	−4.56*							−4.6*	−4.6*
New Zealand	1970–2004	−0.35							−0.4	−0.4
Central nervous system tumors										
Japan	1980–2006	0.03							0.0	0.0
Canada	1980–2004	−1.50*							−1.5*	−1.5*
United States	1980–2005	−0.87*							−0.9*	−0.9*
Italy	1980–2003	−2.28*							−2.3*	−2.3*
UK	1980–2005	−1.68*							−1.7*	−1.7*
New Zealand	1980–2004	−2.32*							−2.3*	−2.3*
Malignant kidney tumors										
Japan	1976–2006	−3.98*							−4.0*	−4.0*
Canada	1970–2004	−2.90*							−2.9*	−2.9*
United States	1970–1991	−4.60*	1991–2005	0.16					0.2	0.2
Italy	1970–2003	−4.62*							−4.6*	−4.6*
UK	1970–2005	−3.49*							−3.5*	−3.5*
New Zealand	1970–2004	−2.91*							−2.9*	−2.9*
Malignant bone tumors										
Japan	1980–2006	−1.79*							−1.8*	−1.8*
Canada	1980–2004	−0.24							−0.2	−0.2
United States	1980–2005	−1.59*							−1.6*	−1.6*
Italy	1980–2003	−3.52*							−3.5*	−3.5*
UK	1980–2005	−2.22*							−2.2*	−2.2*
New Zealand	1980–2004	1.52							1.5	1.5

*$P < 0.05$.

APC is the annual per cent change; AAPC is average annual per cent change.

obtained in the current study. Research in Great Britain [9, 10], Italy [11] and Sweden [12] showed increased trends in childhood leukemia. A report from Britain indicated that small peaks in the incidence of ALL in 1976 and 1990 coincided with the years immediately following influenza epidemics [13]. Other explanations of the increased trend were characteristics of the environment, such as population mixing, although the etiology of cancer remains complicated and largely unknown.

The stable trend in mortality for childhood CNS tumor implied a modest increase trend in the incidence rate in Japan because of the survival improvement reported in childhood CNS tumors in developed countries in recent decades, while progress in therapy for brain tumors has not been as great as for leukemia. For CNS tumors, computed tomography, which was introduced in the 1970s, and magnetic resonance imaging, which has been used widely since the 1980s, has become

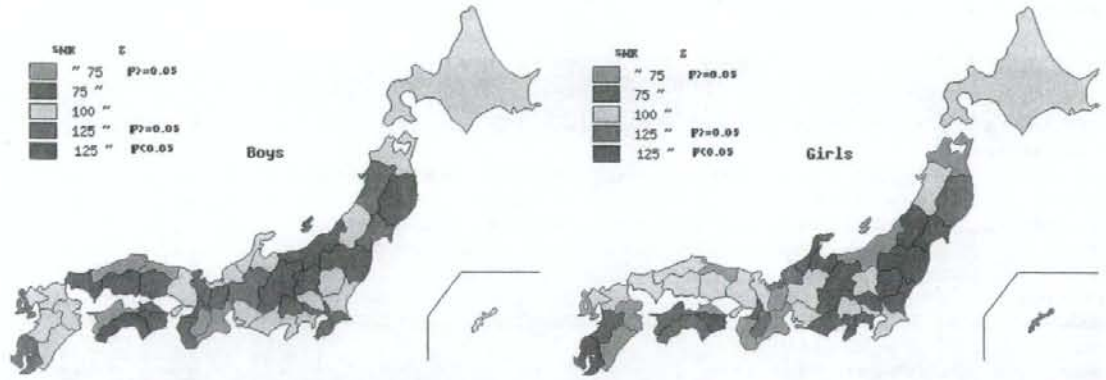

Figure 3. Standardized mortality ratios for childhood cancer in Japan, 2000–2006 by prefecture.

a standard tool for CNS tumor diagnosis and evaluation [14]. Furthermore, improvements in neurosurgical techniques have occurred during the past two decades, including stereotactic surgery, Cavitron Ultrasonic Surgical Aspirator and so on. Childhood cancer survival research from Osaka prefecture in Japan reported a slight increase in 5-year survival [1]. Incidence trends were not evaluated in this study. Data from the population-based cancer registry of Hokkaido prefecture in Japan indicated that the incidence of childhood brain tumors has been increasing, though the cause is unknown [15]. Other studies conducted in developed countries reported a significant increase in childhood CNS tumor incidence [10, 12, 16–21]. This has been explained by changes in detection and/or reports of childhood CNS tumors [22]. Because magnetic resonance imaging became ubiquitous at tertiary pediatric centers in the mid-1980s, it is likely to have increased the rate of detection; however, in the current study, the mortality rate of childhood CNS tumors in Japan was low and constant since the 1980s, and no significant increase in the number of deaths occurred in the middle of the 1980s to support the suggestion that the incidence increase was due to improved diagnostic techniques, if this increase really exists in Japan, and it seems unlikely to explain the long-term continued leveling off of mortality. The etiology of childhood CNS tumors remains largely unknown. Environmental factors are suggested to have a relationship with brain tumors. Further investigation in this field is needed to identify the incidence trends and reasonable explanations for these trends in Japan.

A previous childhood cancer mortality study in Japan presented data up to 1998. Furthermore, trend analysis was according to the correlation coefficient between the mortality rate and death year. Our analysis provides an updated mortality rate and reliable time trend analysis. In general, the mortality trends observed in other developed countries were compatible with Japan, although some differences were apparent. For example, a decrease in mortality during 0–14 years was observed in leukemia in the United States, Canada, Italy, New Zealand and Japan; however, the mortality rate from CNS tumors has decreased in the United States, Canada, UK and Italy in recent two decades. No evidence of decline appeared during 1980-2006 in Japan. For lymphoma, the decline occurred relatively late in Japan, compared with a significant decline without a leveling off period in the United States, Canada, Italy and UK. There is no simple explanation for these trend disparities. It is possible that the distribution of the histology pattern is markedly different among different countries, even in the same diagnostic group. The possible causes for these disparities in the childhood cancer death rate (e.g. late diagnosis, poor treatment quality, lack of health insurance and difficulty in accessing health care) need to be studied further.

A high mortality rate was observed in Kochi prefecture in boys and Tokushima and Kagoshima prefectures in girls. As mentioned above, the geographic disparity might be due to differences in cancer incidence and survival in different regions. Studies of the relationship between social class and childhood cancer have not been consistent. Research from Brazil suggested that higher decreases in the mortality rate were observed in more developed regions, possibly reflecting better health care [23]. We did not perform a similar ecologic study here, because of the small number of death, and we could not even calculate mortality by subtype by prefecture. Further detailed individual-level study is needed to identify a more reasonable explanation for the mortality disparities in childhood cancer.

A few points should be borne in mind when interpreting these findings. Some stable trends in the present study, such as mortality in lymphoma, and malignant bone tumors in New Zealand are more difficult to explain because of the small absolute number and substantial random variation. Other limitations included the wide time span and changes in diagnostic capabilities during the study period, and we were not able to collect any information on social status, employment of individuals and other genetic, environmental factors that would have allowed us to analysis etiological hypotheses.

Despite these limitations, when considering the absence of a national cancer registry system in Japan, estimates of incidence may have their own limitations (for example, they may be significantly influenced by errors in diagnosis and classification); evaluation of death may be an alternate effective method to identify more population-based point estimates of

mortality from childhood cancer under these circumstances. Furthermore, the results presented here are based on 100% national coverage and provide an important baseline for monitoring the further progress against childhood cancer in Japan. Analysis of trends in national mortality rates over several decades may provide additional insight into the burden and impact of childhood cancer and suggest more targeted avenues for interventions that further delineate and ultimately reduce mortality from childhood cancer.

conclusions

The present study provides updated figures and trends in childhood cancer mortality in Japan and other developed countries. This will help to estimate care needs and to plan interventions and the quantity of appropriate childhood cancer treatment. Comprehensive efforts designed to identify risk factors for childhood cancer, promote early detection and reduce morbidity and mortality are warranted.

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Human Osteoblasts Support Hematopoietic Cell Development in vitro

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Key Words
Cell adhesion molecule · Cell signaling · Cytokines · Hematopoiesis · Osteoblasts · Stem cell factor

Abstract
Background/Aim: Although osteoblasts are thought to be the major component of the hematopoietic stem cell niche in the bone marrow microenvironment, the role of osteoblasts in hematopoiesis is still unclear. The ability of human osteoblasts to support early hematopoiesis was investigated. ***Methods and Results:*** Human CD34+ bone marrow cells cultured on human osteoblasts were capable of surviving without addition of cytokines and differentiated into myeloid cells with slight proliferation. The results of immunohistochemical experiments suggested activation of FAK and AKT in hematopoietic cells attached to osteoblasts. When stem cell factor, Flt3-L, and IL-3 were added to the coculture system, each cytokine distinctively enhanced proliferation and differentiation of CD34+ bone marrow cells. ***Conclusion:*** The results suggest that human osteoblasts have the ability to support hematopoietic cell development in vitro.

Introduction

Bone marrow stromal cells, including fibroblasts, adipocytes, macrophages, endothelial cells, and osteoblasts, have been ascribed an important role in hematopoiesis. In addition to the effect of cytokines secreted by stromal cells, direct contact between hematopoietic and stromal cells or extracellular matrix synthesized by bone marrow stromal cells has been implicated in the formation of various hematopoietic cells [1, 2]. Having the ability to self-renew in this environment, hematopoietic stem cells (HSCs) proliferate and differentiate into hematopoietic cells of various lineages, e.g. lymphocytes and myeloid cells.

HSCs are thought to be located in a specific stromal niche in the bone marrow for cell maintenance, proliferation and differentiation. Recent publications have highlighted that osteoblasts are the major component of the HSC niche in the bone marrow microenvironment [3–5]. Zhang et al. [3] presented osteoblasts as key components of the niche that supports HSC development, being functionally controlled via the bone morphogenetic protein signaling pathway. They also suggested that N-cadherin and β-catenin are responsible for the interaction between osteoblasts and long-term HSCs. Calvi et al. [4] demonstrated that osteoblasts are a regulatory com-

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ponent of the HSC niche and control stem cell function through the Notch 1-Jagged 1 pathway. They further stated that parathyroid hormone affects the ability of osteoblasts to expand HSCs. Arai et al. [5] reported that the tight adhesion between HSCs and osteoblasts induced through the Tie2/Ang-1 signaling pathway plays an important role in the maintenance of HSCs in a quiescent state in the bone marrow niche. Despite these findings, however, the role of osteoblasts in hematopoiesis is still unclear.

In this study, we investigated the effect of a primary human osteoblast culture on proliferation and differentiation of human CD34+ bone marrow cells and found that human osteoblasts have the ability to maintain and support hematopoietic cell development in vitro. Primary human osteoblast cultures may provide an in vitro model for the hematopoietic microenvironment.

Materials and Methods

Recombinant human stem cell factor (SCF), human fms-like tyrosine kinase 3 ligand (Flt3-L), and human interleukin (IL)-3 were purchased from Pepro Tec (London, UK). All cytokines were dissolved in phosphate-buffered saline and diluted with culture medium to the concentration indicated. The following mouse monoclonal antibodies (mAbs) against human antigens were used: carboxyfluorescein succinimidylester (CFS)-conjugated anti-CD184 (CXCR4) from Dako Cytomation (Glostrup, Denmark), phycoerythrin- and allophycocyanin-conjugated anti-CD33, phycoerythrin-cyanine (PC) 5-conjugated anti-CD19, PC7-conjugated anti-CD34, allophycocyanin-conjugated anti-CD45, purified anti-CD29 (β_1-integrin), anti-CD49d (α_4-integrin), anti-CD106 (VCAM-1), and anti-fibronectin from Beckman/Coulter (Westbrook, Mass., USA), purified anti-CD166 (activated leukocyte cell adhesion molecule), anti-β-catenin, anti-N-cadherin, and anti-paxillin from Becton Dickinson Biosciences (San Diego, Calif., USA), anti-vinculin from Chemicon International (Temecula, Calif., USA), and anti-osteocalcin from Takara Bio (Shiga, Japan). Purified mAbs were labeled with the Zenon™ Alexa Fluor® 488 and 546 mouse IgG1 labeling kit according to the manufacturer's protocol (Invitrogen, Carlsbad, Calif., USA). The rabbit polyclonal Abs against phospho-specific AKT from Cell Signaling Technology (Beverly, Mass., USA) and phospho-specific FAK from Affinity BioReagents (Golden, Colo., USA) were also used. Unless otherwise indicated, all other chemical reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Human CD34+ bone marrow cells and a primary culture of human osteoblasts purchased from Cambrex Bio Science Walkersville (Walkersville, Md., USA) were used. The cells had been isolated from human tissue after obtaining informed consent from the donor and were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich) at 37°C under a humidified 5% CO_2 atmosphere. The primary culture of human osteoblasts was isolated from the spongy section of the bone head via an explant using the methods established by the provider. The provider confirmed that the majority of the cells were positive for alkaline phosphatase and bone mineralization (von Kossa stain), representing osteoblast phenotypes.

Cells from the primary culture of human osteoblasts were plated and grown to approximately three-fourth confluence on a 12-well tissue plate (Asahi Techno Glass, Chiba, Japan). The CD34+ cells were plated at a concentration of 4×10^4 cells/well/2 ml on the human osteoblasts in RPMI 1640 medium supplemented with 10% fetal calf serum, with and without cytokines. Each cytokine was added at a concentration of 100 ng/ml. After cultivation for the periods indicated, the cells were harvested with 0.25% trypsin plus 0.02% ethylenediamine tetraacetic acid (Immuno-Biological Laboratories, Gunma, Japan), and the number of cells per well was counted. All experiments were performed in triplicate, and cell numbers are reported as means ± SD. For the histological studies, cells were cultured on type-I collagen-coated coverslips (Asahi Techno Glass). At the end of the culture period, the cells on the coverslips were examined after either May-Grünwald-Giemsa staining or immunohistochemical staining.

RT-PCR was performed as described previously [6]. The primer sets used in this study are listed in table 1. The PCR products were separated on 1.5% agarose gel.

For immunostaining, coverslips were fixed with ice-cold acetone for 15 min, incubated with fluorescence-labeled mouse mAbs at room temperature for 30 min, and washed in phosphate-buffered saline. In case of the rabbit polyclonal Abs, secondary goat anti-rabbit IgG Ab conjugated with Alexa Fluor 546 (1:300 dilution, Invitrogen) was used for labeling. The cells were then stained with 4′,6-diamidino-2-phenylindole (200 ng/ml, Sigma-Aldrich) and Alexa Fluor 633-conjugated phalloidin (Invitrogen).

Confocal laser scanning was performed with a FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan). A water immersion objective (×40, NA1.7) was used, and simultaneous multifluorescence acquisitions were performed with the 351- and 488-, 546-, and 633-nm laser lines to excite 4′,6-diamidino-2-phenylindole, Alexa Fluor 488, 546, and 633, respectively. Fluorescence images were selected using appropriate multifluorescence dichroic mirrors and band pass filters using the sequential acquisition mode.

A multicolor immunofluorescence study was performed using a combination of carboxyfluorescein succinimidylester, phycoerythrin, PC-5, and PC-7. Cells were stained with fluorescence-labeled mAbs and analyzed by flow cytometry (EPICS-XL, Beckman/Coulter), as described previously [7]. The gates were set to exclude dead cells and osteoblasts, and 1×10^4 gated cells were analyzed. Data are displayed as histograms of log-fluorescence intensity versus log-fluorescence intensity.

Results

As described in Materials and Methods, the primary culture of human osteoblasts used in this study was qualified and confirmed by the provider, i.e. that the majority of the cells represent osteoblast phenotypes. Using im-

Table 1. Primer sequences

Primer		Sequence	Product size, bp
IL-1α	Sense	5′-TGCTGCATTACATAATCTGG-3′	738
	Anti-sense	5′-TCATGAAATCCTAGGTCTGT-3′	
IL-1β	Sense	5′-CTGGAGAGTGTGATCCCAA-3′	212
	Anti-sense	5′-GACACAAATTGCATGGTGAA-3′	
IL-3	Sense	5′-ACCTTCGAAGGCCAAACCTG-3′	271
	Anti-sense	5′-AGAGAACGAGCTGGACGTTG-3′	
IL-6	Sense	5′-GAGTACAAAAGTCCTGATCC -3′	587
	Anti-sense	5′-TATTTGAGGTAAGCCTACAC-3′	
IL-7	Sense	5′-AAAGTTTCAGAAGGCACAAC-3′	314
	Anti-sense	5′-CTAGGAAGCATTCCACTCTG-3′	
IL-9	Sense	5′-TCTCAGATGACCAATACCAC-3′	216
	Anti-sense	5′-TCATATCTTGCCTCTCATCC-3′	
G-CSF	Sense	5′-ATAGCGGCCTTTTCCTCTAC-3′	863
	Anti-sense	5′-TGATGTTCGGGAGTCAAACC-3′	
M-CSF	Sense	5′-GCAACTGCCTGTACCCCAAA-3′	614
	Anti-sense	5′-CTGAGCAGGGCAGATGGATG-3′	
GM-CSF	Sense	5′-AGCATGTGAATGCCATCCAG-3′	434
	Anti-sense	5′-TTGGTCCCTCCAAGATGACC-3′	
Flt3-L	Sense	5′-TGGATGGAGCGGCTCAAGAC-3′	136
	Anti-sense	5′-TGTTGGTCTGGACGAAGCGA-3′	
LIF	Sense	5′-GCTGTTGGTTCTGCACTGGA-3′	621
	Anti-sense	5′-ACTCCTGAGATCCCTCGGTT-3′	
OPG	Sense	5′-AGATCCTGAAGCTGCTCAGT-3′	319
	Anti-sense	5′-AAAGCCTCAAGTGCCTGAGA-3′	
SCF	Sense	5′-ACAGCTTGACTGATCTTCTG-3′	711
	Anti-sense	5′-TTGTAAGACTTGGCTGTCTC-3′	
SDF-1α	Sense	5′-ATTCAGGAGTACCTGGAGAA-3′	522
	Anti-sense	5′-CAGTGTCTGAAGAAAGGACA-3′	
VEGF	Sense	5′-CTACCTCCACCATGCCAAGT-3′	577
	Anti-sense	5′-AGATCTGGTTCCCGAAACCC-3′	
CD166	Sense	5′-GTATTCCAGAACACGATGAG-3′	272
	Anti-sense	5′-TATCTCTGGACAACTAGGAC-3′	
Fibronectin	Sense	5′-GGATGACTCGTGCTTTGACC-3′	321
	Anti-sense	5′-TGCCACTGTTCTCCTACGTG-3′	
N-cadherin	Sense	5′-GTGCTGATGTTTGTGGTATG-3′	514
	Anti-sense	5′-CTGAAGTTCAGTCATCACCT-3′	
Jagged-1	Sense	5′-CAGGACCTGGTTAACGGATT-3′	936
	Anti-sense	5′-CGTTTCTACAAGGGTTGCTC-3′	
GAPDH	Sense	5′-CCACCCATGGCAAATTCCATGGCA-3′	598
	Anti-sense	5′-TCTAGACGGCAGGTCAGGTCCACC-3′	

munohistochemical staining, the majority of cells were positive for osteocalcin (fig. 1a), an osteoblast-specific protein, indicating that the cells indeed possess the characteristics of osteoblast.

First, we investigated the expression of adhesion-related molecules in the osteoblasts by immunohistostaining. As shown in figure 1b, filamentous distribution of CD106 was detected by confocal microscopy. Fibronectin was also expressed in the form of filaments around the cells, but occasionally it was distributed in the form of coarse bundles (arrowhead). Expression of the protein N-cadherin in the form of a fine mesh was also detected by immunohistostaining (fig. 1b). We also detected mRNA expression of adhesion-related molecules CD166,

fibronectin, N-cadherin, and Jagged-1 in the osteoblasts (fig. 1c).

Next, we characterized cytokine expression of cells in the primary culture of human osteoblasts. RT-PCR revealed mRNA expression of cytokines, including IL-1β, IL-6, Flt3-L, leukemia inhibitory factor (LIF), osteoprotegrin (OPG), stromal cell-derived factor (SDF-1α), and vascular endothelial growth factor (VEGF), in osteoblasts (fig. 1c). No expression of other cytokines, i.e. IL-1α, IL-3, IL-7, IL-9, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), or SCF, was detected under the same conditions (fig. 1c).

To investigate the effect of human osteoblasts on hematopoiesis in vitro, we cultured human CD34+ bone marrow cells on cells from the primary culture of the human osteoblasts. When human CD34+ bone marrow cells were cultured alone in the absence of cytokine addition, they almost all died (fig. 2). By contrast, the human CD34+ bone marrow cells were maintained and proliferated slightly on human osteoblasts in the absence of cytokine supplementation. As shown in figure 2, part of the hematopoietic cells adhered to osteoblasts, forming a small colony, whereas another part of hematopoietic cells floated (data not shown). The culture initially contained 40,000 human CD34+ bone marrow cells, 2 and 4 weeks later 40,000–160,000 and 120,000–270,000 hematopoietic cells were collected, respectively.

We analyzed the subsets of the hematopoietic cells that had survived on the osteoblasts by flow cytometry. When examined after 2 weeks of coculture, the majority of hematopoietic cells were CD33+ myeloid cells (fig. 3). In addition, the human osteoblasts had the ability to maintain a few human CD34+ bone marrow cells (fig. 3), and a small amount of hematopoietic cells still remained to express CD34 after 2 weeks of coculture.

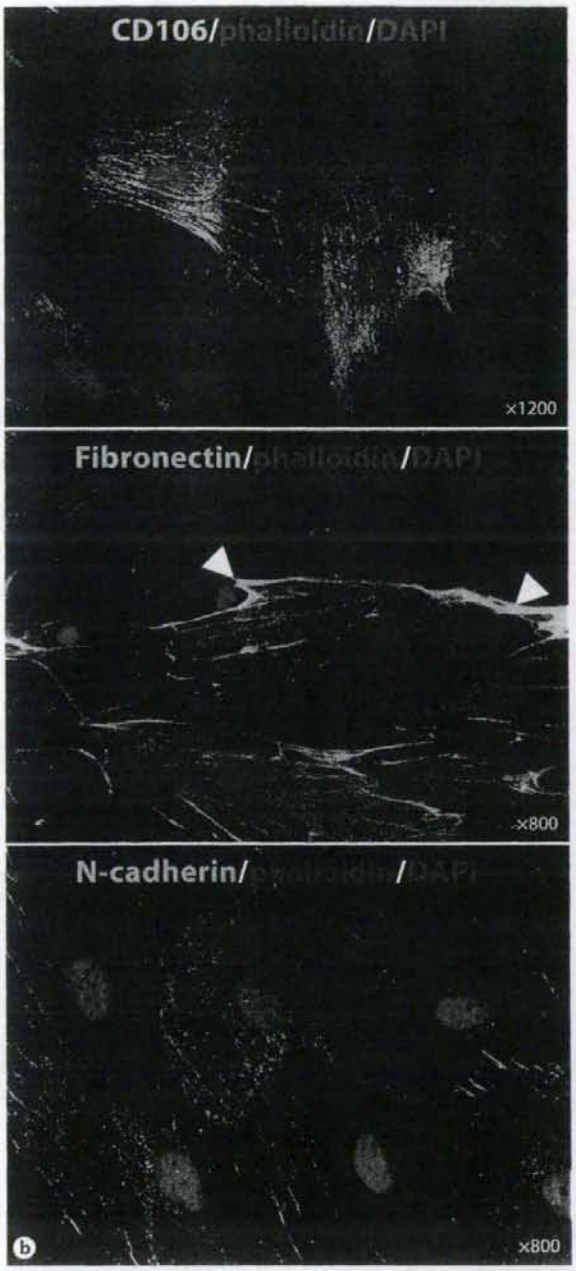
It is noteworthy that occasionally proliferation of CD19+ B-lineage cells on osteoblasts was observed, and one experiment revealed that 46.2 and 13.0% of the hematopoietic cells were B-lineage cells after 2 and 4 weeks of coculture, for example (data not shown). However, the proliferation of B-lineage cells seems to be dependent on the lot of human CD34+ bone marrow cells. Since CD34+ bone marrow cells contained a sizable population of CD19+CD34+ cells in those cases (data not shown), it is most likely that osteoblasts can maintain the B-lineage-committed cells rather than promote B-cell differentiation.

Fig. 1. Expression of osteocalcin and cell adhesion molecules in a primary culture of osteoblasts detected by immunohistochemistry. **a** Osteoblast primary culture grown on coverslips was stained with fluorescein-labeled antibodies to the osteocalcin and examined by confocal microscopy.

Next, we investigated the effect of addition of cytokines, including SCF, Flt3-L and IL-3, on the growth and differentiation of human CD34+ bone marrow cells cultured on human osteoblasts. In the absence of osteoblasts, SCF and IL-3 differently supported the proliferation of hematopoietic cells and 180,000 ± 42,426 and 1,295,000 ± 49,497 cells had grown after a 2-week cocultivation from an initial 40,000 CD34+ bone marrow cells, respectively (fig. 3b). By contrast, Flt3-L induced no significant proliferation of hematopoietic cells (fig. 3b).

In the presence of osteoblasts, however, the subsequent hematopoietic cell number was significantly increased compared with hematopoietic cells cultured with cytokine alone. When 55,000 ± 21,213 hematopoietic cells had grown from an initial 40,000 CD34+ bone marrow cells after a 2-week cocultivation on human normal osteoblasts, cytokines SCF, Flt3-L, and IL-3 markedly increased hematopoietic cell recovery to 990,000 ± 183,848, 355,000 ± 77,782, and 2,370,000 ± 367,696, respectively (fig. 3c). In each case, the majority of cells were CD33+ (fig. 3c). An especially remarkable increase in the CD34+ cell number was achieved with the combination of osteoblasts and IL-3 (fig. 3d).

We further investigated the effect of other factors which may affect either osteoblasts or CD34+ bone marrow cells, including parathyroid hormone, vitamin D, MIP-1α, BMP-2, IL-7, and LIF, whereas no significant ef-



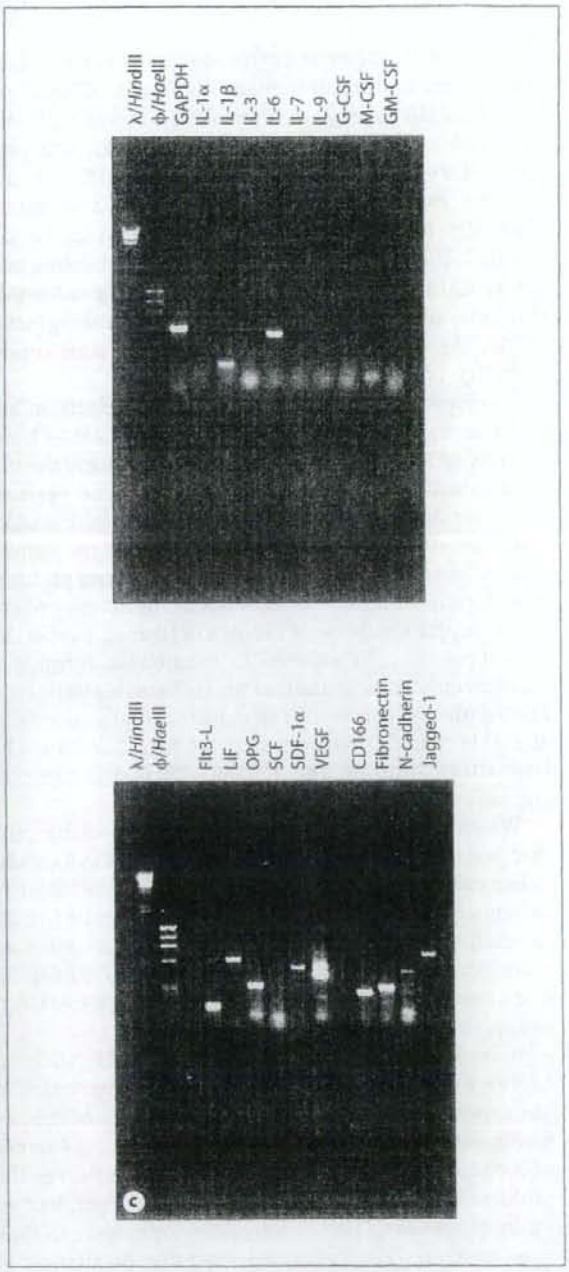


Fig. 1. b The same samples were stained with fluorescein-labeled antibodies to the cell adhesion molecules indicated and examined by confocal microscopy. **c** Expression of cytokines and cell adhesion molecules in osteoblasts detected by RT-PCR. To investigate mRNA expression of the cytokines and cell adhesion molecules indicated, total RNAs were extracted from a primary culture of human osteoblasts and RT-PCR analysis was performed. The PCR products obtained were subjected to electrophoresis with molecular weight markers ϕ/X174/*Hae*III and λ/*Hin*dIII.

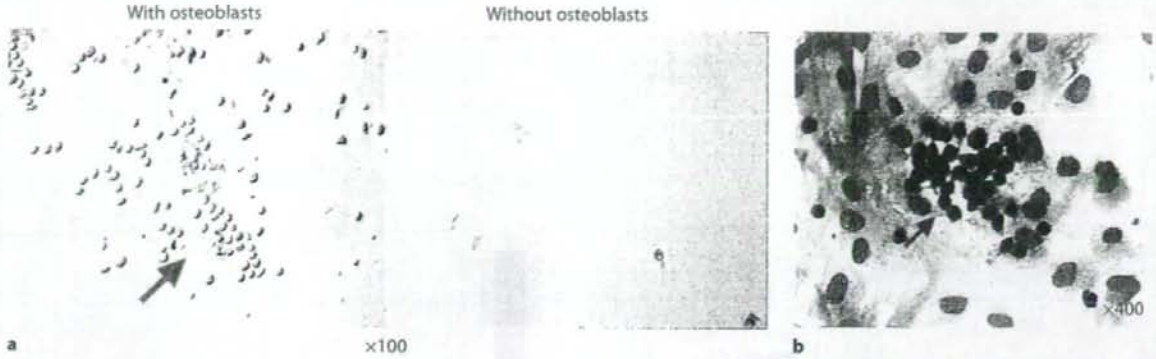


Fig. 2. Human CD34+ bone marrow cells grown on osteoblast primary cultures. **a** Human CD34+ bone marrow cells were cultured for 2 weeks on osteoblast primary cultures grown on coverslips and examined by phase-contrast microscopy (magnification ×100). **b** After May-Grünwald-Giemsa staining, cells were examined by light microscopy. The small nuclear cells gathered at the center are human CD34+ bone marrow cells (arrow), and the large nuclear cells present at the circumference are human osteoblasts (magnification ×400).

fect on the growth and differentiation of hematopoietic cells cultured on osteoblasts was observed (data not shown).

It was suggested that hematopoietic cells adhere to bone marrow stromal cells via binding between β_1- and α_4-integrin, a complex of CD29 and CD49d, expressed on hematopoietic cells and CD106 on stromal cells [8]. Consequently, the intracellular signaling required for cell survival and maintenance was initiated in hematopoietic cells [9]. As shown in figure 4, immunohistostaining experiments revealed that hematopoietic cells adhered to osteoblasts as they got twisted to the CD106 that filamentously expressed on osteoblasts. Immunohistostaining also showed that the CD166 staining pattern was similar to that of fibronectin and that some of the hematopoietic cells expressed CD166, and those cells looked like those adhering to osteoblasts that also express CD166. Both the hematopoietic cells and osteoblasts also expressed other signaling molecules, including paxillin, β-catenin, and vinculin (fig. 4). Paxillin expression in the hematopoietic cells was much greater than in osteoblasts (fig. 4).

We also investigated the phosphorylation of signaling molecules in hematopoietic cells using activation-specific antibodies. As shown in figure 5, confocal microscopic analysis showed phosphorylation of FAK and AKT, and there was clear colocalization of phosphorylated FAK and AKT with CD49d and CD29, respectively (fig. 5, bottom panels).

Thus, we tested the effect of cell adhesion mediated by CD29 and CD49d on the maintenance of hematopoietic cells on osteoblasts. When the anti-CD29 antibody that can interfere in the binding between CD29 and CD106 was added to the coculture of CD34+ bone marrow cells and osteoblasts, subsequent hematopoietic cell proliferation was clearly reduced (fig. 6). Identical results were observed for the anti-CD49d antibody (fig. 6).

Since CD184 (CXCR4) has been suggested to contribute to the homing of hematopoietic cells, we investigated the effect of osteoblasts on CD184 expression by hematopoietic cells. As shown in figure 7a, human CD34+ cells purified from bone marrow did not express CD184. When cultured on osteoblasts, however, expression of CD184 was significantly increased and the hematopoietic cells were divided into several fractions based on the diverse patterns of CD184 and CD33 co-expression (fig. 7).

In the absence of osteoblasts, on the other hand, each cytokine also induced CD184 expression by hematopoietic cells in a distinct manner (fig. 7). When CD34+ bone marrow cells were cultured in the presence of osteoblasts, addition of cytokines further enhanced CD184 expression (fig. 7). We also examined the effect of SDF-1, a ligand for CD184, and anti-SDF-1 antibody on the proliferation and differentiation of CD34+ bone marrow cells cultured on osteoblasts in the presence of each cytokine, but no significant effect was observed (data not shown).