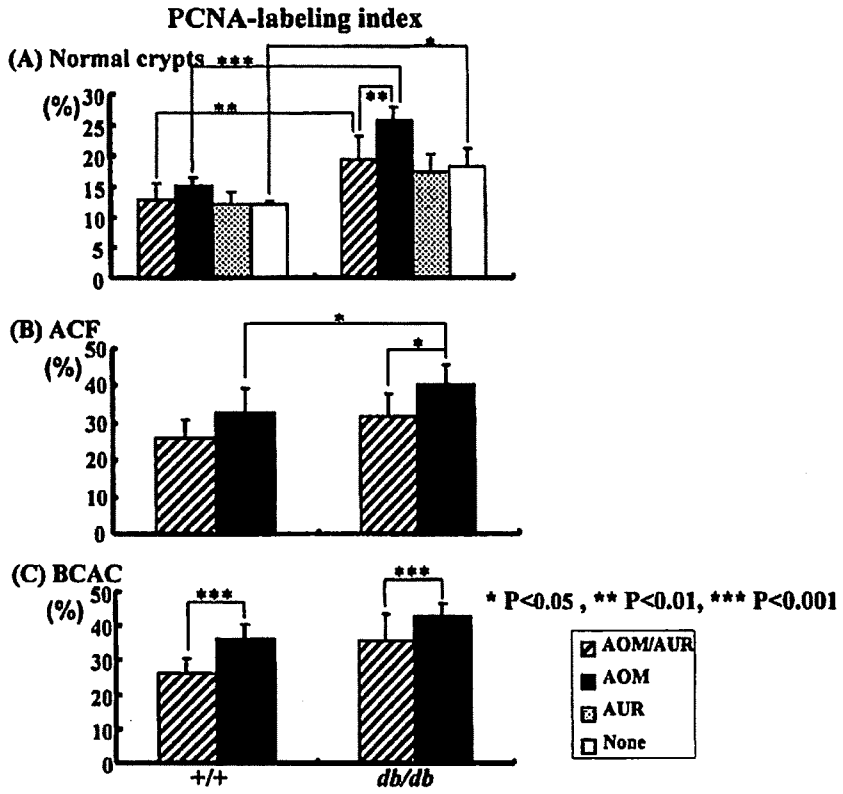

Figure 3. PCNA-labeling indices in the normal crypts, ACF, and BCAC in the colon of *db/db* and +/+ mice. (A) In the normal crypts, PCNA labeling indices of the AOM/AUR, AOM alone, and untreated groups of *db/db* mice were significantly greater than those of +/+ mice. While AUR feeding did not affect the PCNA-labeling index of the normal crypts of +/+ mice, the treatment significantly lowered the index in *db/db* mice. (B) The PCNA-labeling index of ACF of *db/db* mice was greater than that of +/+ mice, and feeding with AUR significantly decreased the index of each phenotype. (C) Feeding with AUR significantly lowered the PCNA-labeling index of BACA that developed in each phenotype. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

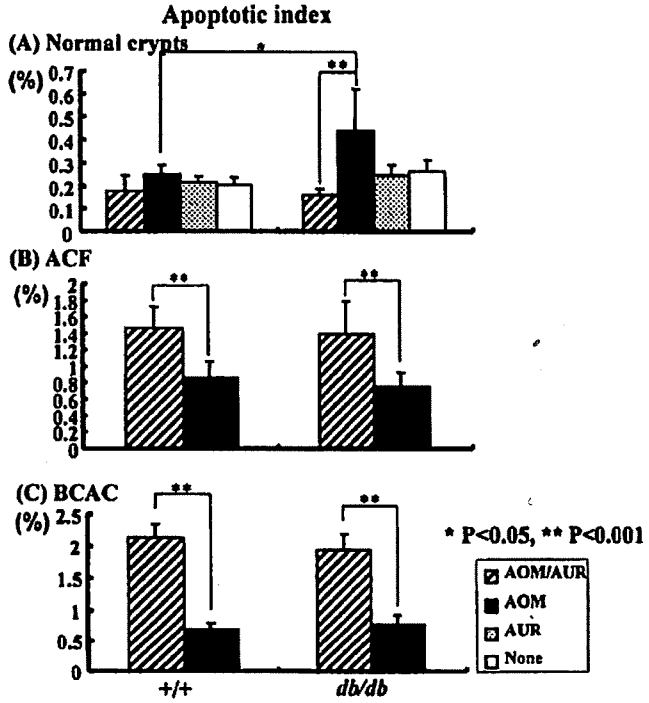

Figure 4. Apoptotic indices in the normal crypts, ACF, and BCAC in the colon of *db/db* and +/+ mice. (A) In the normal crypts, apoptotic index of the AOM alone group of *db/db* mice was significantly greater than that of +/+ mice. While AUR feeding did not affect the apoptotic index of the normal crypts of +/+ mice, the treatment significantly lowered the index in *db/db* mice. (B) AUR feeding significantly increased the apoptotic indices of ACF in +/+ and *db/db* mice. (C) The treatment with AUR also significantly increased the index of BCAC of mice with each phenotype. $^{*}P < 0.05$ and $^{**}P < 0.001$.

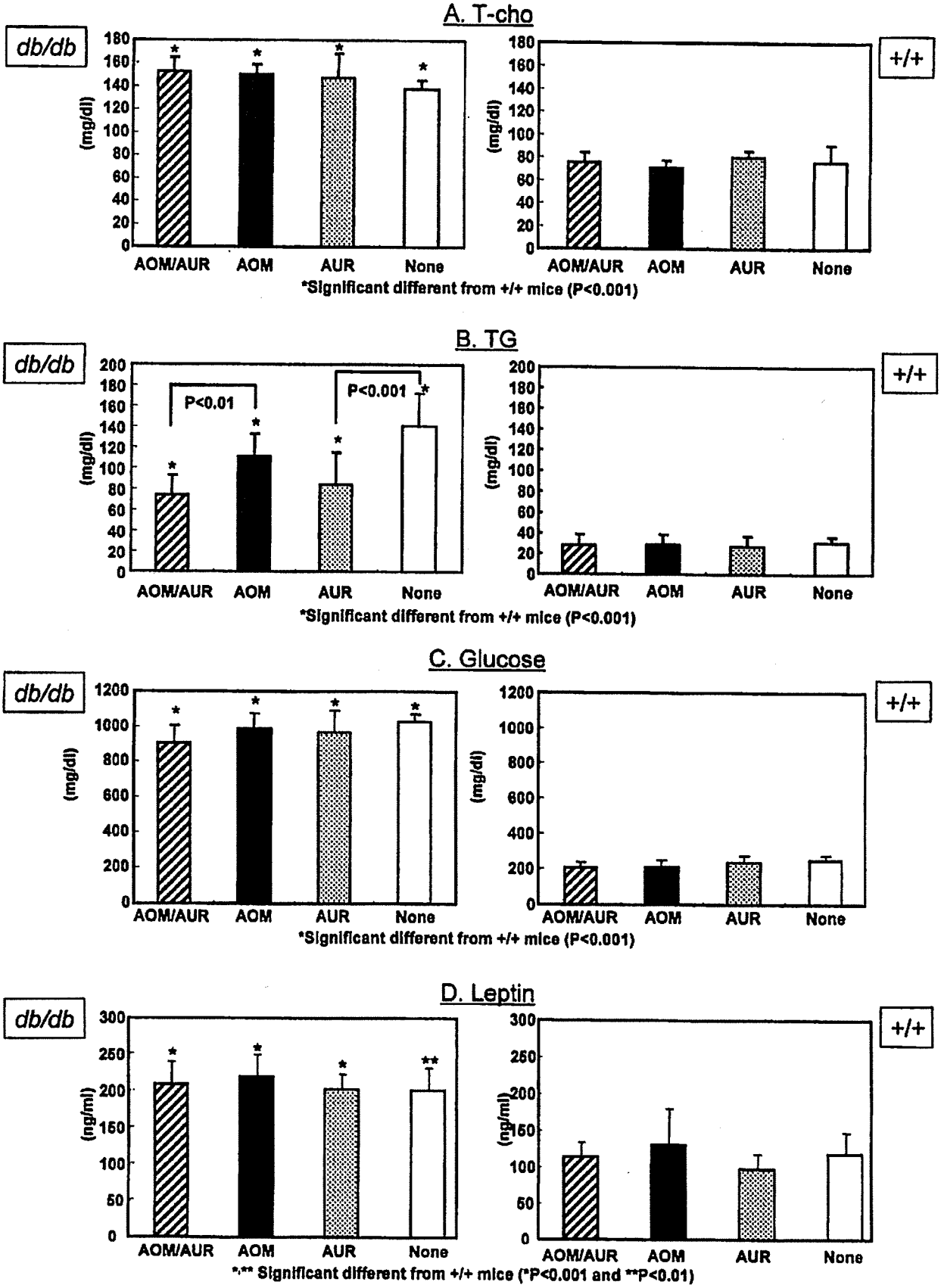

Figure 5. Clinical chemical profiles for (A) total cholesterol, (B) triglycerides, (C) glucose, and (D) leptin the serum of the *db/db* and +/+ mice. All the measurements were high in the *db/db* mice regardless of the treatments. AUR feeding significantly lowered the serum levels of triglycerides in the *db/db* mice.

between obesity/diabetes and colon tumorigenesis. Our findings also suggest that insulin resistance involves CRC development (43), although we were not able to determine serum insulin in this study because we did not get enough serum volumes to measure. The main purpose of the current study was to investigate the effect of AUR on the early phase of AOM-induced colon carcinogenesis in the *db/db* mice. Since ACF (37) and BCAC (38,44) are considered to be putative precursor lesions of colonic adenocarcinoma, the results described clearly indicate the inhibitory effects of the dietary administration of AUR on the early phase of colon carcinogenesis in the *db/db* mice as well as the +/+ mice.

In this study, all the serum measurements of total cholesterol, triglycerides, glucose, and leptin were greater in the *db/db* mice than those of +/+ mice, thus suggesting that these measurements may contribute to the high susceptibility of *db/db* mice to AOM-induced colon tumorigenesis. However, interestingly among the chemical profiles, only the triglycerides level lowered by feeding with AUR, and this was correlated with lower incidences of ACF and BACA in the *db/db* mice. These findings may suggest that a high level of serum triglycerides is the most important biological effect for developing colonic tumors in *db/db* mice, and a modification (lowering) of this measurement may thus lead the inhibition of colon tumorigenesis. In humans, a positive association between the serum triglycerides level and the risk of CRC development was reported (45). This association was also suspected by the findings in animal experiments (39,40,46), in which model animals for human familial adenomatous polyposis were used, Our recent study indicated that dietary citrus segment membrane (CUSM) is able to suppress the occurrence of BCAC in male *db/db* mice by lowering their serum triglycerides level (42).

Feeding a high fat diet, which is implicated to play a role in the stimulation of colonic cryptal cell proliferation while also promoting colon carcinogenesis (47), increases the circulating leptin level (48). In addition, dietary fiber that is known to suppress colon carcinogenesis (49) is reported to decrease serum leptin concentration and to lower the degree of cryptal cell proliferation (50). In this study, AUR feeding did not significantly affect serum leptin levels in the *db/db* mice, but the feeding reduced immunohistochemical expression of insulin-like growth factor-1 receptor (IGF-1R) in BCAC developed in the *db/db* mice (data not shown). Since insulin and IGF axis may be related to CRC development (51), IGF-1 may be able to influence both pre-malignant and cancer development. Insulin resistance is associated with hyper-insulinemia, increased levels of growth factors including IGF-1 (52).

In the current study, feeding with AUR reduced cell proliferation activity in the BCAC of +/+ mice and non-lesional crypts, ACF, and BCAC of *db/db* mice. In addition, AUR treatment induced apoptotic cells in the ACF and BCAC that developed in +/+ and *db/db* mice, suggesting that AUR is able to inhibit progression of precursor lesions for CRC, ACF, and BCAC, through inducing apoptosis and lowering cell proliferation. Many chemicals that are candidates for cancer chemopreventive agents are able to modulate apoptosis and cell proliferation activity in target tissues (14,41). Hence our findings on apoptosis and cell proliferation are important for clinical application of AUR as one of the cancer chemopreventive agents for obese.

In summary, our data provide further evidence that citrus AUR is able to inhibit the early phase of colon carcinogenesis in the genetically altered obese mice as well as the wild rodents. The effects may be caused by lowering lipid profiles in conjunction with modification of apoptosis and cell proliferation. Our finding also suggest that *db/db* mice are susceptible to AOM-induced carcinogenesis (3,42) and such *db/db* mice can thus be an appropriate animal model for the high risk group for CRC, such as obesity/diabetes (1,4). Since current study focused on the effects of obesity and AUR on the colonic pre-malignancies, further studies focusing the detailed mechanisms of inhibitory effects of AUR on the development of CRC in the *db/db* mice should be carried out for the prevention and treatment of the colonic malignancies associated with these conditions.

Acknowledgments and Notes

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ORIGINAL ARTICLE

Multi-step lymphomagenesis deduced from DNA changes in thymic lymphomas and atrophic thymuses at various times after γ-irradiation

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Whole-body γ-irradiation to mice causes thymic atrophy where a population of precancerous cells with mutation can be found. Thus, clonal growth and DNA changes at *Bcl11b*, *Ikaros*, *Pten*, *Notch1 and Myc* were examined in not only thymic lymphomas but also in atrophic thymuses at various times after irradiation. Clonal expansion was detected from the distinct patterns of rearrangements at the TCRβ receptor locus in a fraction of atrophic thymuses at as early as 30 days after irradiation. This expansion may be in part owing to the rearranged TCRβ signaling because the transfer of bone marrow cells with the rearrangement and the wild-type locus into severe-combined immunodeficiency mice showed preferential growth of the rearranged thymocytes in atrophic thymus. Loss of heterozygosity (LOH) at *Bcl11b* and trisomy of *Myc* were found at high frequencies in both lymphomas and atrophic thymuses, and in contrast, LOH at *Ikaros* and *Pten* were rare in atrophic thymuses but prevalent in lymphomas. *Notch1* activation was detected in lymphomas and in atrophic thymuses only at a late stage. Similar patterns of DNA changes were found in atrophic thymuses induced in *Bcl11b*$^{+/-}$ mice. These results suggest the order of genetic changes during lymphomagenesis, *Bcl11b* and *Myc* being at the early stage; whereas *Ikaros*, *Pten* and *Notch1* at the late stage.
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Keywords: multistep lymphomagenesis; γ-ray; atrophic thymuses; clonal growth; *Bcl11b*

Introduction

Cancers are the results of a series of somatic mutations, and some cases with genetic predisposition owing to inheritance of responsible mutations. Each successive mutation gives the cells a growth advantage, so that it forms an expanded clone, thus presenting a larger target for the next mutation (Vogelstein and Kinzler, 1993). On the other hand, normal cells possess multiple defence mechanisms to prevent uncontrolled cell division. Some of these are devices within the cell, such as those that limit cell-cycle progression or induce apoptosis, whereas others are signals from surrounding tissues that prompt a cell to remain within its supportive microenvironment (Hanahan and Weinberg, 2000). Ionizing radiation is a well-known carcinogen for a variety of tumors in human and in mouse, and can induce DNA damages in target cells and also disrupt their microenvironment.

Mouse thymic lymphomas are one of the classic models of ionizing radiation-induced malignancies (Kaplan, 1964; Ludwig *et al.*, 1968; Kominami and Niwa, 2006), and their developmental process may be conformed to the sequential model for mutation accumulation, proposed for the colorectal cancer development (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). Whole-body irradiation causes thymic atrophy and depletion of bone marrow (BM) cells, followed by a supply in an improper manner of cells from BM to the thymus. Most of the atrophic thymuses eventually develop lymphomas but some remain apparently unchanged. Thus, atrophic thymus may constitute a microenvironment that allows damaged thymocytes to find a population of proliferating and precancerous cells. Indeed, it was reported that prelymphoma cells develop in atrophic thymus within 2 weeks after irradiation (Muto *et al.*, 1987; Sado *et al.*, 1991). However, study of genetic changes of thymocytes in the atrophic thymus has not been performed.

We have identified previously two genes, *Ikaros* and *Bcl11b/Rit1/CTIP2*, that underwent bi-allelic DNA changes in mouse thymic lymphomas (Okano *et al.*, 1999; Shinbo *et al.*, 1999; Wakabayashi *et al.*, 2003a). Both genes encode zinc-finger transcription factors that regulate the development of lymphocytes (Georgopoulos *et al.*, 1992; Avram *et al.*, 2000; Wakabayashi *et al.*, 2003b). *Ikaros* has a proapoptotic function and its inactivation provides resistance to apoptosis, consistent with a predicted property for loss of tumor suppressor gene (Winandy *et al.*, 1995; Yagi *et al.*, 2002). In contrast, *Bcl11b*$^{-/-}$ mice exhibit a reduced cellularity and apoptosis of thymocytes (Wakabayashi *et al.*, 2003b), and this apoptotic phenotype seems to contradict the fact that Bcl11b being the tumor suppressor. Genetic changes of these two genes were also found in human

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hematopoietic malignancies (Sun *et al.*, 1999; Bernard *et al.*, 2001; Itoyama *et al.*, 2001; Yagi *et al.*, 2002; MacLeod *et al.*, 2003; Nagel *et al.*, 2003; Bezrookove and van Zelderen-Bhola, 2004; Przybylski *et al.*, 2005). Studies in other laboratories implicated several genes in the lymphoma development such as *Myc*, *Notch1* and *PTEN* (Klein, 1979; Graham *et al.*, 1985; Ellisen *et al.*, 1991; Mao *et al.*, 2003; Lin *et al.*, 2006; O'Neil *et al.*, 2006). On the other hand, mutation frequencies of mostly delineated *ras* and *p53* genes were low in radiogenic thymic lymphomas (Newcomb *et al.*, 1988; Brathwaite *et al.*, 1992).

Atrophic thymus induced by whole-body irradiation may comprise a subpopulation of clonally growing cells. If this is the case, DNA alterations may be detectable in thymocytes of those thymuses. This study has examined changes of the genes listed above in thymuses at various times after γ-irradiation. Here we show DNA alterations present in some of atrophic thymuses, and changes at *Bcl11b* and *Myc* occur earlier than at *Ikaros*, *Pten* and *Notch1*, suggesting the order of mutations during lymphomagenesis.

Results

Clonal growth of thymocytes in atrophic thymus

F_1 hybrids between BALB/c and MSM mice were γ-irradiated, and at 150–250 days after irradiation thymus was isolated from apparently 20 healthy mice. Most thymuses (85%) were atrophic, containing 10^6–10^7 cells, and some (15%) were hypertrophic, comprising more than 5×10^7 cells. Figure 1a displays an example of flow cytometric analysis of an atrophic thymus, showing the presence of a population of large-sized thymocytes. As overt thymic lymphomas consisted of large-sized lymphocytes (Figure 1a), this suggested the existence of a population of clonally growing cells. We thus examined clonality of thymocytes with a conventional protocol (Kawamoto *et al.*, 2003), which determines D–J recombination of the TCRβ gene locus using polymerase chain reaction (PCR). Three sets of primers were used, one set for recombination between the Dβ1 and Jβ1 loci, the second for the Dβ2 and Jβ2 loci and the third for the Dβ1 and Jβ2 loci (Figure 1b). DNA from brain provided only one large band with either Dβ1–Jβ1 or Dβ2–Jβ2 primer sets, reflecting no rearrangement. On the other hand, DNA from normal thymus, containing thymocytes of polyclonal origin, showed several smaller bands resulting from rearrangements between different Dβ and Jβ positions. In contrast, DNA from lymphomas showed only one or a few bands, indicating that lymphoma cells were of monoclonal (one prominent band detected) or oligoclonal origin (a few prominent bands detected). DNA from atrophic thymus showed that 13 of the 17 examined comprised one or few bands, and only four showed a pattern similar to normal thymus (Table 1). This suggests that a clonally growing population(s) is present in atrophic thymuses at a high frequency (76%).

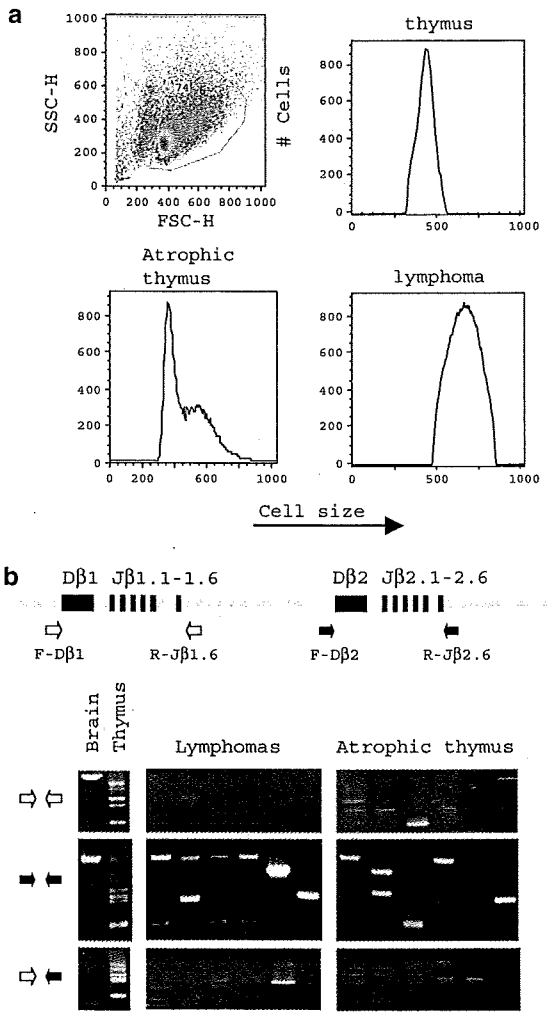

Figure 1 Clonal growth of thymocytes in atrophic thymuses after irradiation. (**a**) Flow cytometric analysis of the cell size of thymocytes from atrophic thymus, unirradiated thymus and thymic lymphoma. The atrophic thymus (under, left) contains a subpopulation of large-sized thymocytes, as found in lymphomas (under, right). (**b**) The upper panel shows a part of the TCRβ locus and the relative location of PCR primers used. The lower panel shows gel electrophoresis of PCR products with three different sets of primers. F-Dβ1 and R-Jβ1.6 (top), F-Dβ2 and to-Jβ2.6 (middle), and F-Dβ1 and R-Jβ2.6 (bottom).

DNA changes in atrophic thymuses

Existence of a clonally growing cell population(s) in atrophic thymuses raised a possibility that these thymocytes have already progressed to a preleukemic stage with loss of heterozygosity (LOH) of some tumor suppressor gene(s). Hence, we examined LOH at *Bcl11b*, *Ikaros* and *Pten* loci in the atrophic thymuses and lymphomas. Figure 2a shows examples of LOH analysis at *Bcl11b* and *Ikaros*, and Figure 2b (left) displays the distribution of lymphomas and atrophic thymuses in different band ratios of the BALB/c and MSM *Bcl11b* alleles. The MSM/BALB allele ratio was designated with a plus sign when it was >1, and with a minus sign when it was <1. The band ratio in normal tissues

Table 1 Frequencies of D–J recombination and DNA changes in various atrophic thymuses and lymphomas that were induced by γ-irradiation in Bcl11b wild-type mice

Samples	*Examined*	*DJ recombination*			*Bcl11b*	*Ikaros*	*Pten*	*Notch1*	*Myc*
		Mono	*Oligo*	*Poly*	*LOH*	*LOH*	*LOH*	*ID*	*Trisomy*
Atrophic thymus									
30 days	42	12 (5)	12 (5)	76 (32)	10 (4)	0	5 (2)	0	2 (1)
100 days	26	38 (10)	24 (6)	38 (10)	35 (9)	8 (2)	8 (2)	0	35 (9)
150–250 days	17	41 (7)	35 (6)	24 (4)	71 (12)	6 (1)	12 (2)	18 (3)	35 (6)
Thymic lymphomas	35	63 (22)	37 (13)	0	89 (31)	43 (15)	35 (11)	23 (8)	66 (23)

Abbreviation: LOH, loss of heterozygosity. Numbers indicate % change-positive samples, and numbers in parentheses are those detected.

ranged from 1.2 to −1.2 (data not shown), and hence LOH was judged as positive when the band ratio was >1.5 or <−1.5. Based on this criterion, we estimated that LOH frequency at *Bcl11b* was 71% in atrophic thymuses and 89% in lymphomas.

Similar analysis was performed at *Ikaros* and *Pten*. LOH frequency at *Ikaros* was only 6% in atrophic thymuses and 43% in lymphomas, whereas LOH frequency at *Pten* was 12% in atrophic thymuses and 35% in lymphomas (Table 1). Although this study did not examine mutation of remaining alleles of the three genes, frequent inactivation (50%) of the remaining *Ikaros* allele was reported in γ-ray-induced thymes lymphomas (Kakinuma *et al*., 2002). No lymphomas showed LOH at *p53* (data not shown), consistent with previous reports (Brathwaite *et al*., 1992; Okano *et al*., 1999). It is noteworthy that the LOH frequency at *Ikaros* markedly differed between atrophic thymuses and lymphomas. Recently, Tsuji *et al*. (2004) reported frequent intragenic deletion of *Notch 1* exon 1 in lymphomas and irradiated thymuses, resulting in the activation of Notch1. Accordingly, we examined *Notch 1* deletion with the PCR assay, which detected a DNA fragment resulting from the deletion. The deletion frequencies were 18 and 23% in atrophic thymuses and lymphomas, respectively. Trisomy of chromosome 15 harboring the *myc* gene was found at an early stage of thymic lymphoma development (McMorrow *et al*., 1988). We analysed the trisomy using the PCR assay, which detected a polymorphism between the two parental BALB/c and MSM alleles of *myc* (Figure 2c). Figure 2b (right) shows the distribution of lymphomas and atrophic thymuses in different band ratios of the BALB/c and MSM *Myc* alleles. Trisomy was judged by the BALB/c/MSM band ratio to be >1.2 or <−1.2, as the band ratio in normal tissues ranged from 1.2 to −1.2 (data not shown). This judgment may be less accurate than that of *Bcl11b* LOH, but sufficient to permit the comparison of the frequency between lymphomas and atrophic thymuses. The frequency of trisomy was 35% for atrophic thymuses and 66% for lymphomas (Table 1).

Accumulation of DNA changes and genetic association

We calculated the number of DNA changes at the five loci, *Bcl11b*, *Ikaros*, *Pten*, *Notch1* and *Myc*, in the 35 individual lymphomas. Lymphomas with the changes in four of the five genes were: five (17%), 3/5 were 13 (37%), 2/5 were 10 (29%), 1/5 was five (14%), and without change was one (2.9%). Interestingly, mutual exclusiveness of DNA changes in individual lymphomas was observed between *Pten* and *Notch1* and also between *Ikaros* and *Pten*. No lymphomas had changes in both *Pten* and *Notch1*, lymphomas having either of them were 19 (11 for *Pten* and eight for *Notch1*), and lymphomas having neither were 16 (χ^2 test, $P<0.001$) (see Supplementary Table S1), whereas lymphomas having LOH in both *Ikaros* and *Pten* were two, those having either of them were 22 (13 for *Ikaros* and 9 for *Pten*), and lymphomas having neither were 11 ($P=0.046$). *Notch1* and *Ikaros*, on the other hand, showed association of changes in lymphomas. Lymphomas having changes in both genes were six, those having either of them were 11 (two for *Notch1* and nine for *Ikaros*), and lymphomas having neither were 18 ($P=0.037$).

Changes in atrophic thymuses at earlier times

As LOH was detected in atrophic thymuses, we next examined atrophic thymuses at earlier times, 30 and 100 days, after the start of fractionated irradiation. Neither lymphomas nor hypertrophic thymus was observed at these stages, although it was reported that precancerous cells already exist at these stages (Muto *et al*., 1987). Figure 3a displays examples of flow cytomeric analysis of atrophic thymuses. Some of the 100-day samples comprised large-sized thymocytes together with small-sized ones, but none of the 30-day samples exhibited patterns containing large-sized thymocytes. Figure 3b displays 16 examples of clonality assay and *Bcl11b* LOH assay in atrophic thymuses 30 days later. Clonally growing population was detected in five of the 16, among which two showed LOH at *Bcl11b*. Table 1 summarizes these results. The frequency of clonal growth was 24% (12+12%) in the 30-day atrophic thymuses and 62% (38+24%) in the 100-day atrophic thymuses. On the other hand, LOH frequency at *Bcl11b* was 10 and 35%, respectively (see Figure 2b for distribution in the allele ratios). LOH at *Ikaros* and *Pten* was very low in those atrophic thymuses, and intragenic deletion of *Notch 1* was not detected, whereas *myc* trisomy was found in two and 35% of the 30 and

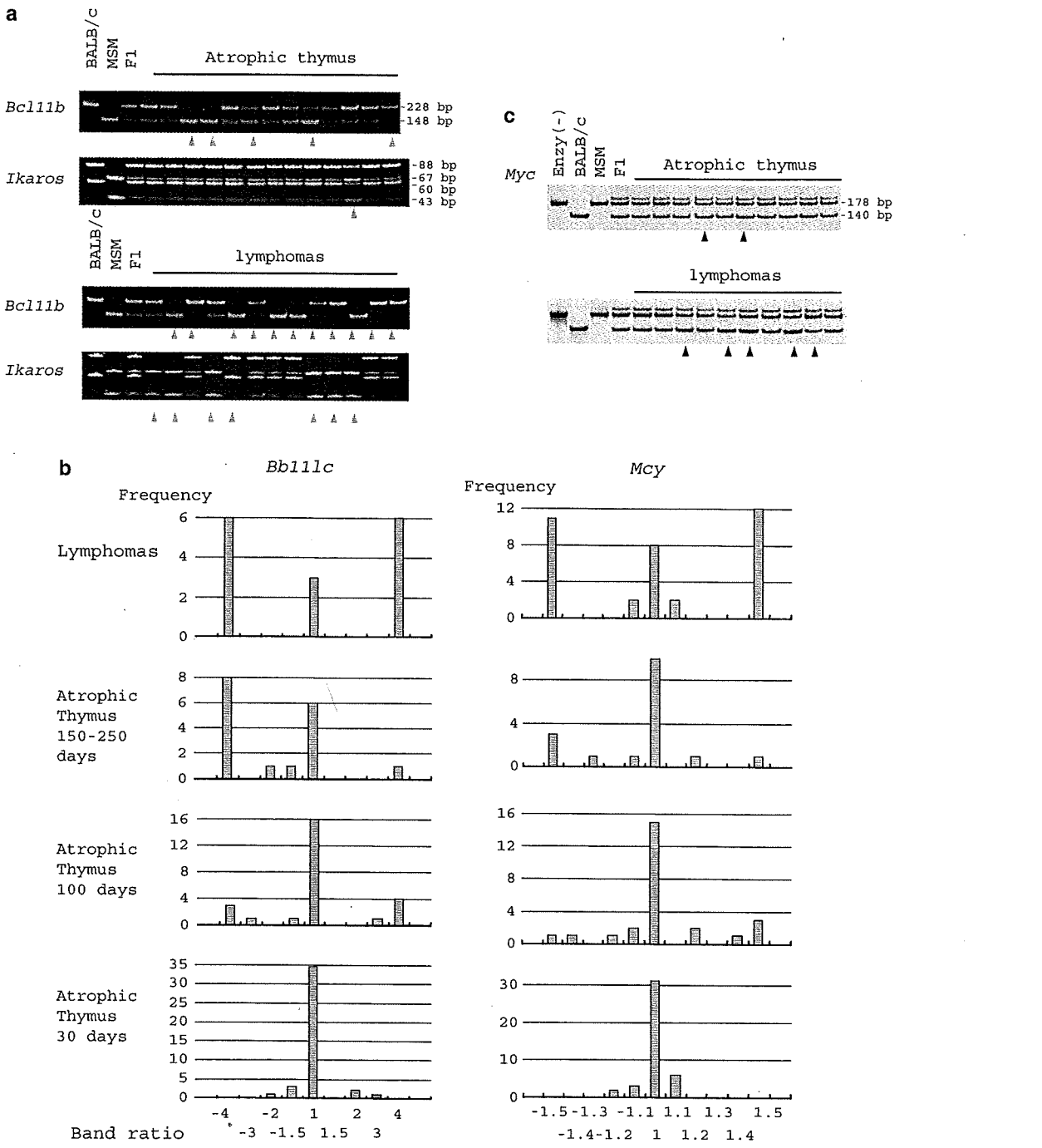

Figure 2 DNA alterations in atrophic thymuses. (**a**) Gel electrophoresis of PCR products after digestion with *Hin*fI for *Bcl11b* and with *Hap*II for *Ikaros*. Arrowheads indicate the presence of LOH. (**b**) Distribution of thymic lymphomas and atrophic thymuses in the ratios of BALB/c and MSM alleles: *Bcl11b* (left); *Myc* (right). Days after irradiation (left) and allele ratios (bottom) are indicated. The MSM/BALB allele ratio is designated with a plus sign when the ratio is > 1, and with a minus sign when the ratio of BALB/MSM is more than one. (**c**) PCR products after digestion with *Taq* I for *Myc*. Arrowheads indicate the presence of trisomy.

100-day atrophic thymuses, respectively (see Figure 2b for distribution of the allele ratios). These results suggest that changes at *Bcl11b* and probably at *Myc* occur at much earlier stages than changes at *Ikaros*, *Pten* and *Notch1*.

Changes in atrophic thymuses induced in Bcl11b-heterozygous mice

Bcl11b$^{+/-}$ mice developed radiogenic thymic lymphomas at a higher incidence and a shorter latency than those of *Bcl11b*$^{+/+}$ mice (Kamimura *et al.*, 2007). This tumor

susceptibility by *Bcl11b* heterozygosity may influence clonal growth and genetic changes in atrophic thymuses. Thus, we examined atrophic thymuses that were prepared from *Bcl11b*$^{+/-}$ mice using the same experimental protocols. Table 2 summarizes the results. The frequency of clonal growth was as high as 55% in the 30-day atrophic thymuses and 97% in the 100-day atrophic thymuses, consistent with a shorter latency of lymphomas in *Bcl11b*$^{+/-}$ mice. This suggests that most of the 30-day atrophic thymuses comprise a subpopulation(s) of developing prelymphoma. The frequency of loss of the *Bcl11b* wild-type allele, resulting in total inactivation of *Bcl11b*, was 40% in the 30-day atrophic thymuses, 47% in the 100-day atrophic thymuses and intriguingly, as high as 80% in the 150–250 day atrophic thymuses. The frequency of 80% was higher than 54% in lymphomas, suggesting that *Bcl11b* inactivation contributes to clonal growth but is not sufficient for the lymphoma development. Interestingly, the frequency of LOH at *Ikaros* was higher than that in *Bcl11b*$^{+/+}$ atrophic thymuses. This may reflect the acceleration of lymphoma development in *Bcl11b*$^{+/-}$ atrophic thymuses. The frequency of DNA changes at *Pten*, *Notch 1* or *Myc* did not markedly differ from that in *Bcl11b*$^{+/+}$ mice. These results suggest that *Bcl11b* heterozygosity accelerates the occurrence of clonal growth of thymocytes when irradiated, accompanying increases in the frequency of DNA alterations at *Ikaros* in atrophic thymuses, but such increase was not remarkable at the other loci.

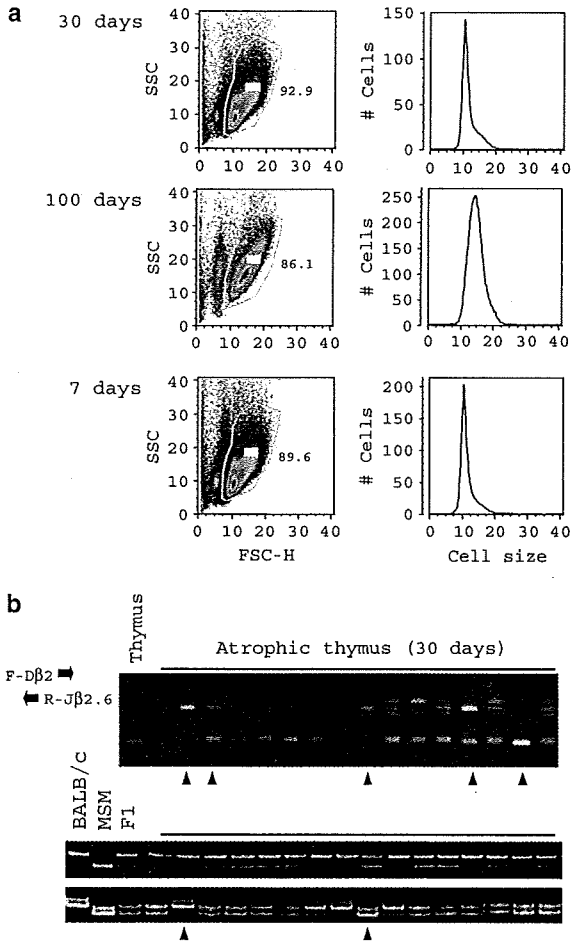

Figure 3 LOH in atrophic thymuses at 30 days after irradiation. (a) Flow cytometric analysis of the cell size of thymocytes from atrophic thymuses at indicated days after irradiation. (b) The upper panel shows gel electrophoresis of PCR products using the F-Dβ2 and R-Jβ2.6 primer set. Arrowheads indicate the presence of clonally growing subpopulation of cells. The lower panel shows gel electrophoresis of two different PCR products at *Bcl11b* locus. One (top) is the *Bcl11b* products after digestion with *Hin*fI, and the other (bottom) the products amplified with D12Mit181 microsatellite primers. Arrowheads indicate the presence of LOH.

Contribution of the pre-TCR receptor signaling to clonal growth

We observed D–J recombination of the *TCRβ* gene in atrophic thymuses at a higher frequency than LOH at *Bcl11b*. Success in the recombination produces a pre-TCR receptor complex on the cell surface that stimulates cell proliferation and survival (Shinkai *et al*., 1993; Newton *et al*., 2000; Kabra *et al*., 2001). Therefore, signaling through the receptor might be a step on the selection pathway of carcinogenesis. To test this possibility, we produced mice harboring mixed hematopoietic stem cells of unarranged and rearranged *TCRβ* genes and examined whether gene-rearranged cells are preferentially selected in clonally growing cells in atrophic thymus. BM cells were isolated from C57BL/10(B10) mice and transgenic C57BL/6(B6) mice expressing the rearranged Vβ8.1 T-cell receptor gene (Murphy *et al*., 1990) and the two cell preparations were mixed in

Table 2 Frequencies of D–J recombination and DNA changes in various atrophic thymuses and lymphomas that were induced by γ-irradiation in Bcl11b KO/+ mice

Samples	*Examined*	*DJ recombination*			*Bcl11b*	*Ikaros*	*Pten*	*Notch1*	*Myc*
		Mono	*Oligo*	*Poly*	*LOH*	*LOH*	*LOH*	*ID*	*Trisomy*
Atrophic thymus									
30 days	20	30 (6)	25 (5)	45 (9)	40 (8)	10 (2)	10 (2)	0	0
100 days	36	50 (18)	47 (17)	3 (1)	47 (17)	31 (11)	14 (5)	0	33 (12)
150–250 days	20	45 (9)	50 (10)	5 (1)	80 (16)	20 (4)	0	30 (6)	5 (1)
Thymic lymphomas	25	69 (18)	31 (7)	0	56 (14)	52 (13)	12 (3)	56 (14)	36 (9)

Abbreviations: LOH, loss of heterozygosity, KO, knockout. Numbers indicate % change-positive samples, and numbers in parentheses are those detected.

the ratios 1:1, 4:1 and 16:1. An aliquot of those mixed cells was then transferred into irradiated severe-combined immunodeficiency (SCID) mice, and 5 weeks after the transfer, the mice were subjected to a single whole-body irradiation of 2.5 Gy, followed by isolation of the thymus 30 days later. Figure 4a shows PCR analysis of D–J recombination in thymocytes. Thymus of mice receiving cells mixed at 1:1 showed the intense band of transgenic Vβ8.1 gene and the unrearranged band. Thymus of 4:1 or 16:1 cells showed developmentally rearranged bands in addition to the transgenic-derived band. Figure 4b shows PCR analysis using a set of primers detecting a polymorphism between B10 and B6 DNA. This experiment included thymus and leukocytes, and also as a control, the two tissues that were obtained from mice receiving wild-type B10 and B6 BM cells were mixed at 1:1. The band ratio of B10 and B6 in the wild-type cell-transferred mice was similar between thymocytes and leukocytes. In contrast, the band ratio markedly differed in the mice that were transferred with wild-type and transgenic BM cells. The band ratio in each of different mixtures (1:1, 4:1 and 16:1) was much lower in thymocytes than in leukocytes. These results indicated a preference of cells expressing the rearranged Vβ8.1 T-cell receptor gene to grow in thymus relative to wild-type cells, suggesting that the pre-TCR receptor signaling contributes to clonal growth of thymocytes in irradiated mice.

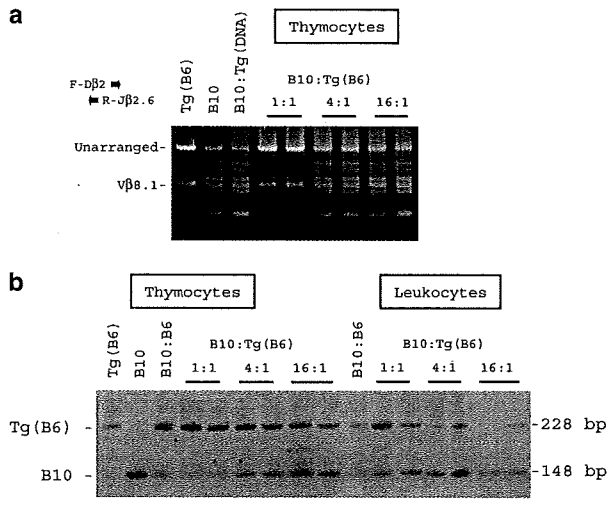

Figure 4 Growth reference of thymocytes having rearranged T-cell receptor gene in irradiated thymus. (**a**) Gel electrophoresis of PCR products using the Dβ2 and Jβ2.6 primer set. DNA was isolated from thymus of the SCID mice at 30 days after irradiation, which had received mixtures of BM cells from C57BL/10(B10) mice and transgenic C57BL/6(B6) mice expressing the Vβ8.1 rearranged T-cell receptor gene. The ratio mixed of B10 with B6 is indicated on gel lane. (**b**) Gel electrophoresis of *Mbo*I digests of PCR products that were amplified using a set of primers detecting polymorphism at an *Mbo*I cleavage site between B10 and B6 DNA. DNA used was isolated from thymus and peripheral blood leukocytes of the SCID mice described above. Also, this experiment included the two tissues obtained from the SCID mice that had received wild-type B10 and B6 BM cells mixed at 1:1.

Discussion

This study has analysed genetic changes at *Bcl11b*, *Ikaros*, *Pten*, *Myc* and *Notch1* in γ-ray-induced mouse thymic lymphomas and atrophic thymuses isolated at different times after irradiation. *Bcl11b* and *Myc* frequently underwent genetic changes at the early stage during lymphomagenesis, whereas alterations of *Ikaros*, *Pten* and *Notch1* occurred mostly at the late stage. The stage difference in accumulation of DNA changes suggests the order of mutations and can be conformed to the sequential model, proposed to describe the accumulation of mutations necessary for the colorectal cancer development (Fearon and Vogelstein, 1990). Furthermore, results lead to an implication of their contribution to lymphomagenesis.

Examination for recombination at the TCRβ receptor locus demonstrated clonal proliferation and expansion in a fraction of atrophic thymuses even 30 days after irradiation. In the atrophic thymuses of wild-type mice, *Bcl11b* exhibited the highest LOH frequency at the earliest time, which was followed by *Pten* and *Ikaros*. *Myc* also showed alterations, and *Notch1* was the latest among them to accumulate mutation. Of *Bcl11b*$^{+/-}$ mice, loss of the wild-type *Bcl11b* allele was detected at high frequencies (40–80%) in atrophic thymuses and lymphomas, consistent with a tumor-suppressor activity of *Bcl11b*. These results suggest that *Bcl11b* changes are the critical and possibly the first mutation in the multi-step evolution of thymic lymphomas. Our previous studies showed that a marked phenotype of *Bcl11b*-deficient thymocytes is prevalence of apoptosis (Wakabayashi *et al*., 2003b; Inoue *et al*., 2006). This apoptosis may be triggered by the strong cell proliferation stimulus of committed thymocytes because hyperplastic or dysplastic cells in precancerous lesions often exhibit apoptosis and accompanying high mitotic index (Bartkova *et al*., 2005; Gorgoulis *et al*., 2005). Consistently, 80% of the 150–250-day atrophic thymuses induced in *Bcl11b*$^{+/-}$ mice underwent loss of the wild-type allele and showed clonal growth but did not yet develop lymphomas. Therefore, thymocytes in atrophic thymus may well undergo cell proliferation and apoptosis at the same time through a balancing mechanism between cell division and cell death that is probably controlled by surrounding normal tissues (Hanahan and Weinberg, 2000).

The pre-TCR receptor on the cell surface is signaling molecules to stimulate thymocyte proliferation and survival (Shinkai *et al*., 1993; Newton *et al*., 2000; Kabra *et al*., 2001). The p56^{LCK} tyrosine kinase downstream of the receptor plays a key role in transducing mitogenic signals via cyclin D3 (Mombaerts *et al*., 1994; Sicinska *et al*., 2003), and transgenic mice overexpressing p56^{LCK} are highly prone to T-cell lymphomas (Abraham *et al*., 1991). Interestingly, this signaling is required for *Notch*-driven leukemogenesis (Sicinska *et al*., 2003). We observed D–J recombinations of the *TCRβ* gene in atrophic thymus at a higher frequency than genetic changes at *Bcl11b* locus. This suggests that this signaling followed by chromosomal rearrangement may lead to not only proliferation of normal thymocytes

but also the expansion of prelymphoma cells in atrophic thymus. Therefore, the pre-TCR receptor signaling can be a key factor to start clonal growth of thymocytes and hence the DNA rearrangement may be one of the first DNA changes contributing to lymphomagenesis. Indeed, our result showed that thymocytes expressing the rearranged Vβ8.1 T-cell receptor gene prefer to grow in irradiated thymus relative to wild-type thymocytes.

Trisomy of *Myc* was found in as high as one-third of the 100-day atrophic thymuses. This suggests that *Myc* trisomy tends to take place at an early time similar to that of *Bcl11b* inactivation. Increase in Myc expression contributes to cell proliferation, and at the same time, however, triggers apoptosis by increasing the levels of proapoptotic proteins such as p53. Accordingly, transgenic Myc-induced lymphomas exhibit frequent inactivating mutations of p53 or p19ARF (Eischen *et al.*, 1999).

In contrast to *Bcl11b* and *myc*, DNA alterations of *Ikaros*, *Pten* and *Notch1* occurred mostly at the late stage. LOH frequency at *Ikaros* was very low in atrophic thymus, though high in thymic lymphomas. Ikaros has a proapoptotic function and inactivation of *Ikaros* contributes to resistance for apoptosis *in vitro* (Yagi *et al.*, 2002; Ruiz *et al.*, 2004). Analysis of the knockout mice also implicates *Ikaros* in lymphomagenesis (Winandy *et al.*, 1995; Dumortier *et al.*, 2006). Therefore, it may be reasonable to speculate that *Ikaros* inactivation rescues prelymphoma cells from the apoptosis provided by *Bcl11b* inactivation (Wakabayashi *et al.*, 2003b; Inoue *et al.*, 2006) or *Myc* activation (Eischen *et al.*, 1999), leading to rapidly progressive tumor phenotype. Likewise, *Pten* inactivation may provide rescue from apoptosis as shown by *in vitro* and *in vivo* studies (Mayo *et al.*, 2002; Freeman *et al.*, 2003) and thereby may confer susceptibility to thymic lymphomas. Mutual exclusiveness of mutations observed between *Ikaros* and *Pten* may reflect their common function of conferring apoptosis.

Activating mutations in *Notch1* are frequently found in human T-cell leukemias (Weng *et al.*, 2004) and in mouse T-cell lymphomas (Tsuji *et al.*, 2004; Dumortier *et al.*, 2006; Lin *et al.*, 2006; O'Neil *et al.*, 2006). Our study demonstrated activation of *Notch1* by intragenic deletion in some of thymic lymphomas and atrophic thymuses at 150–250 days after irradiation, but not in atrophic thymuses at earlier stages (30–100 days). This suggests that activated Notch1 may contribute only to expansion of late preleukemic cells. Retroviral mutagenesis studies conducted with *c-myc* transgenic mice showed that a high frequency of T-cell leukemias occurring with shortened latencies had proviral integrations into *Notch1*, suggesting that c-myc and Notch1 act through independent, complementary pathways to promote pre-T-cell transformation (Girard *et al.*, 1996; Hoemann *et al.*, 2000). However, preference of *Notch1* activation to lymphomas with *Myc* trisomy was not found in our study. On the other hand, mutual preference of mutations was observed between *Notch1* and *Ikaros*, suggesting that their alterations synergistically contribute to lymphomagenesis. This is consistent with the finding that introduction of Ikaros inhibits the activated Notch1-driven leukemogenesis (Dumortier *et al.*, 2006). Note that mutual exclusiveness of mutations was observed between *Notch1* and *Pten*, although their relationship is not clear. Altogether, these results suggest genetic association among *Ikaros*, *Pten* and *Notch1* genes, changes of which contribute to the late stage of lymphomagenesis.

Hanahan and Weinberg (2000) depicted the key factors in the transition from a human normal cell to a malignant cancer cell. These are the acquisition of six specific capabilities: independent of external growth signals, insensitivity of anti-growth signals, ability to avoid apoptosis, infinite replication, angiogenesis, and tissue invasion and metastasis. Furthermore, Hahn and Weinberg (2002) described fewer genetic changes in mouse models for the induction of tumors than in comparative tissue sites in humans. Therefore, the mouse thymic lymphomas may be induced without some of the capabilities. These capabilities may be the last three: the mouse has long telomeres and active telomerase capable to replicate adequately, thymic lymphomas are free from necrosis, and the mouse tumors metastasize very rarely. On the other hand, the first three capabilities are probably required during lymphomagenesis. As described above, inactivation of *Bcl11b* or *Myc* activation found in atrophic thymuses may render thymocytes independent of external growth stimulation, whereas *Ikaros* or *Pten* inactivation may confer ability to avoid apoptosis. It is not clear, however, which gene inactivation overcomes the possible growth-inhibitory signal from the thymic microenvironment. *Notch1* and *Myc* might be the candidates, because expression of these genes is tightly-regulated, such that their expression is highly sensitive to external cellular cues. Notch ligands are expressed on thymic stromal cells and play key roles in proliferation and differentiation of thymocytes during T-cell development (Gray *et al.*, 2005). Myc activity is also regulated during the transition from an undifferentiated to a differentiated state. A switch from Myc–Max to Mad–Max complexes is known to occur and the Mad–Max complexes then elicit differentiation-inducing signals (Hanahan and Weinberg, 2000; McArthur *et al.*, 2002). Another possibility is that the insensitivity of anti-growth signals is not required for γ-ray-induced thymic lymphomas, because atrophic or disorganized thymus may be a microenvironment incapable to give anti-growth signals.

Our results showed that 31% of the thymic lymphomas underwent DNA changes in two of the five genes (*Bcl11b*, *Ikaros*, *Myc*, *Pten* and *Notch1*), 43% underwent in three, and 17% underwent in four. As discussed above, the development of mouse thymic lymphomas may require only two (if the insensitivity of anti-growth signals is not required) or three specific capabilities. If this is the case, these five genes are major targets for mutation, and mutation in two or three of the five is sufficient for genesis of most of the thymic lymphomas. This idea does not ignore the involvement of epigenetic (possibly secondary genetic) changes of many other genes following these mutations.

A small fraction (9%) of lymphomas was free from DNA changes or contained only one of the five genes. This suggests either the presence of as yet identified mutations or epigenetic inactivation of some of the five candidate genes.

Materials and methods

Mice

Bcl11b$^{+/-}$ mice of the BALB/c background were generated as described (Wakabayashi *et al.*, 2003b). *TCRβ* transgenic mice of the C57BL/6 background expressing a rearranged TCRβ receptor gene (V 8/D 1/J 1.1 gene; Murphy *et al.*, 1990) were purchased from (Taconic M&B, Hudson, NY, USA) and BALB/c and CB17 SCID mice were from CLEA Japan Inc. (Tokyo, Japan). MSM mice were kindly supplied from Dr Shiroishi (NIG at Mishima, Japan). Mice used in this study were maintained under specific pathogen-free conditions in the animal colony of the Niigata University.

Induction of atrophic thymus and lymphoma development

Bcl11b$^{+/+}$ and *Bcl11b*$^{+/-}$ BALB/c mice were mated with MSM mice and their progeny were subjected to γ-irradiation of 2.5 Gy four times at a weekly interval, starting at the age of 4 weeks. Thymus was isolated at 30, 100 and 150–250 days after the start of irradiation. Development of thymic lymphoma was diagnosed by inspection of labored breathing and palpable induration of thymic tumor. Existence of tumors was confirmed upon autopsy of the mice. Thymic lymphomas composed of approximately 2×10^8 cells and atrophic thymuses at 30 days after irradiation composed of approximately 10^6 cells. Genotyping of *Bcl11b* alleles was carried out as described previously (Wakabayashi *et al.*, 2003b). All animal experiments comply with the guidelines by the animal ethics committee for animal experimentation of Niigata University.

Flow cytometry

The cell size of thymocytes was determined on a FACSAria (BD Biosciences, San Jose, CA, USA), and data were analysed using the Flow-Jo software (Tree-Star). Dead cells and debris were excluded by appropriating the gating of forward light scatter and side light scatter.

Stem cell transfer

BM cells were isolated from wild-type C57B6(B6) mice, *TCRβ* transgenic B6 mice and wild-type C57B10(B10) mice, and suspended in Eagle's medium supplemented with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 2% fetal calf serum. After washing three times with medium, BM cells of wild-type B6 and B10 were mixed at 1:1, and BM cells of transgenic B6 and wild-type B10 were mixed at 1:1, 1:2, 1:4 and 1:16 ratios. An aliquot of cell mixtures ($1–2 \times 10^7$ cells) was injected into the tail vein of SCID mice. Five weeks after the transfer, the mice were subjected to a single whole-body irradiation of 2.5 Gy, followed by isolation of the thymus and peripheral blood leukocytes 30 days later.

DNA isolation

DNA was isolated from brain, lymphomas, thymocytes and peripheral blood leukocytes using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA).

PCR analysis of D–J recombination in TCRβ locus

PCR for TCRβ rearrangements was performed as described (Kawamoto *et al.*, 2003). Template DNA was used at three different concentrations in each experiment to ensure linearity of the PCR signal. As a control for the amount of template DNA, aliquots of a PCR mixture were amplified with primers specific for *α-catenin* (5′-GCATGGCTACAGTTACTAATG-3′) and (5′-TGAGCCCGATGGTGAATTTG-3′). The reaction was processed through 32 cycles of 94°C for 30 s, 63°C for 1 min and 72°C for 2 min.

PCR analysis of allelic differences, intergenic deletions and gene amplifications

PCR was performed as described previously (Wakabayashi *et al.*, 2003a, b). Primers were: *Bcl11b*, 5′-GGCTGAATTTA CAGGATGAGG and 5′-CTTGAACCCCAACTTCTGTG; *Ikaros*, 5′– TGAGAAGCCCTTCAAATGCC and 5′– CTG GGAACATGGAACACATG; *Myc*, 5′-GCAGACACTTCT CACTGGAA and 5′-GTTTCCAACGCCCAAAGGAA. Primers for *Notch1* deletion were 5′-GGAATGCCTACTTTG TATGAGG and 5′-ATTTCTCCTTTAGGTTCCTTTGAG. Primers to discriminate between C57B6 and C57B10 genomes were 5′-GCTACACAAAGAAACTCGCCC and 5′-ATGCT GAGAGTACGCAGATGC. PCR products were analysed for SNPs by gel electrophoresis after appropriate restriction enzymes. *Hin*fI digests of *Bcl11b* products gave 256 and 228 bp for BALB/c and 256, 148 and 83 bp for MSM. *Hap*II digests of *Ikaros* products gave 88 and 60 bp for BALB/c and 67, 43, 21 and 17 bp for MSM. *Taq*I digests of *Myc* products gave 178 bp for BALB/c and 140 and 38 bp for MSM. *Mbo*I was used for detection of a SNP between C57B6 and C57B10 genomes, which gave 171 and 32 bp for B6 and 105, 66 and 32 bp for B10. PCR products using Mit markers (*D12Mit181* for *Bcl11b*; *D19Mit64* for *Pten*) were analysed by gel electrophoresis. PCR bands were stained with ethidium bromide and band intensities were quantitated with a Molecular Imager FX (Bio Rad, Hercules, CA, USA). Ratio of BALB/c and MSM alleles or of MSM and BALB/c alleles in lymphomas and atrophic thymus was normalized with that of normal brain DNA. The BALB/c and MSM allele ratio was designated with a plus sign when it was > 1, and with a minus sign when it was < 1.

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Full Paper

Blockade of Leukotriene B_4 Signaling Pathway Induces Apoptosis and Suppresses Cell Proliferation in Colon Cancer

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Abstract. We investigated whether leukotriene B_4 (LTB_4) and its signaling pathway play an important role in the progression of human colon cancer via a direct stimulation of cancer cell proliferation. Remarkable expression of LTB_4 receptor 1 (BLT1) in human colon cancer tissues was detected by immunohistochemistry, and Western blot analysis revealed the BLT1 expression in cultured human colon cancer cell lines, Caco2 and HT29. The 5-lipoxygenase inhibitor AA-861 and LTB_4-receptor antagonist U75302 showed negative effects on survival and proliferation of both Caco2 and HT-29 cells. The inhibition of cell proliferation is due to the apoptosis because nuclear condensation and increased annexin V expression were observed in the cells treated with AA-861 and U75302. Knockdown of BLT1 by small interfering RNA caused the suppression of BLT1 protein, resulting in the inhibition of cancer cell proliferation. Blockade of BLT1 by the receptor antagonist significantly suppresses the LTB_4-stimulated extracellular signal-regulated kinase (ERK) activation in colon cancer cells. These results indicate that the blockade of the LTB_4-signaling pathway induces apoptosis via the inhibition of ERK activation in colon cancer cells. The LTB_4-signaling pathway might be a new therapeutic target for colon cancer.

Keywords: colon cancer, leukotriene B_4 (LTB_4), 5-lipoxygenase (5-LOX), carcinogenesis, apoptosis

Introduction

Chronic inflammation of the colon and rectum has been implicated in dysplasia and colon carcinogenesis (1 – 3). In addition, chronic inflammation has been reported to promote the conversion of premalignant colonic adenoma cells into malignant adenocarcinomas in nude mice (4). Thus, chronic and excessive inflammatory conditions may lead to the formation of malignant adenocarcinoma in the colon. However, it is still unknown what kinds of inflammatory mediators are involved in colon carcinogenesis.

Leukotriene B_4 (LTB_4), a potent chemotactic lipid mediator in inflammation, plays important roles in controlling inflammatory responses via LTB_4-receptor signaling. Two types of receptors, LTB_4 receptor 1 (BLT1) and receptor 2 (BLT2) are known, and BLT1 is mainly involved in inflammatory responses (5, 6). There are two major metabolic pathways for the arachidonic acid cascade: cyclooxygenase (COX) and lipoxygenase (LOX) pathways; LTB_4 is generated from arachidonic acid via the LOX pathway (6). The COX pathway, particularly including COX-2, has been widely studied because it is well known that the COX-2 and its metabolites play important roles in carcinogenesis in the colon (7 – 9). In contrast, little is known about the role of the LTB_4-signaling pathway on the carcinogenesis of colon cancer. Recently, several studies have demonstrated that inhibition of the 5-LOX pathway suppresses the proliferation of various cancer cells such as pancreatic, urological, and prostate cancer cells (10 – 12). In addition, Gregor et al. reported that combination of

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COX-2 and 5-LOX inhibition significantly decreased liver metastasis in ductal pancreatic cancer cells compared to single inhibition of COX-2 in Syrian hamster (13).

Based on the above evidences, we hypothesized that LTB_4 and its signaling pathway may play an important role in the progression of human colon cancer via a direct stimulation of cancer cell proliferation. To clarify the hypothesis, we showed the marked expression of BLT1 in both human colon cancer tissues and colonic cancer cell lines. In addition, we clearly showed that LTB_4 significantly stimulated the proliferation of colonic cancer cells, whereas the 5-LOX inhibitor AA-861 and selective LTB_4-receptor antagonist U75302 inhibited the proliferation and induced apoptosis. Furthermore, knockdown of BLT1 by small interfering RNA resulted in inhibition of cancer cell proliferation. These results presented in this study clearly suggest that the LTB_4-receptor signaling pathway directly controls the proliferation of tumor cells in the colon.

Materials and Methods

Reagents and antibodies

The polyclonal BLT1 receptor antibody was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The DAB substrate kit, 3,3-diaminobenzidine, and the Vectastain Elite ABC kit (rabbit IgG) for immunohistochemical staining were from Vector Laboratories (Burlingame, CA, USA). Mayer's hematoxylin solution was from Wako Pure Chemical Industries (Osaka). Fetal bovine serum (FBS) was from ICN Biomedicals (Aurora, OH, USA). AA-861, U75302, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bis-benzimide (Hoechst 33342), LTB_4, the Annexin-V FITC Apoptosis Detection kit, and the anti-phospho-ERK 1/2 were from Sigma Chemical Co. (St. Louis, MO, USA). The cell proliferation ELISA kit (BrdU, colorimetric) was purchased from Roche Diagnostics (Mannheim, Germany). Dye reagent concentrate for protein assay and Ready gels J were from Bio-Rad Laboratories (Hercules, CA, USA). The membrane optimized for protein transfer and the ECL Western Blot analysis system were from Amersham Biosciences Co. (Piscataway, NJ, USA). The anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) rabbit polyclonal antibody was from Trevigen, Inc. (Gaithersburg, MD, USA). Lipofectamine 2000 and The Stealth RNAi negative control kit were from Invitrogen Japan KK (Tokyo).

Tissue samples

Ten samples of colon adenocarcinoma tissue were randomly selected from the surgical specimens of patients who underwent surgery at Yokohama City University Hospital after obtaining their informed consent. Ten normal colon tissues were also obtained from patients as controls. All specimens were processed for histology by conventional methods, and 4-μm tissue sections were prepared from paraffin blocks and mounted on glass slides.

Immunohistochemistry for LTB_4 receptor

After deparaffinization, the slides were immersed in methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to quench endogenous peroxidase activity. The slides were then washed in phosphate-buffered saline (PBS) and incubated for 20 min at room temperature with PBS containing 1.5% normal goat serum. Next, the slides were incubated for 18 h at 4°C with the primary antibody. The primary antibody against BLT1 (rabbit, polyclonal) was used at dilutions of 1:400 in PBS. The slides were then washed again in PBS and incubated for 30 min at room temperature with biotinylated secondary antibody against rabbit primary antibody. The biotinylated secondary antibody was used at dilutions of 1:200 in PBS containing 1% normal goat serum. Next, the slides were washed in PBS, and after incubating for 30 min at room temperature with the Vectastain ABC reagent, the brown immunoperoxidase reaction was developed with the DAB substrate kit. Counterstaining was performed with Mayer's hematoxylin solution. To ensure the specificity of the primary antibodies, other tissue sections were processed without primary antibodies, as controls.

Cell culture and treatment with reagents

The human colon adenocarcinoma cell lines Caco2 and HT-29 cells were cultured in Dulbecco's modified Eagle medium and McCoy's 5A medium, respectively, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown as monolayers in 10-cm culture dishes and incubated at 37°C under a humidified atmosphere of 5% CO_2 in air.

Cell survival and cell proliferation analysis

Cell survival and cell proliferation were determined by the tetrazolium dye (MTT) assay and 5-bromo-2'-deoxyuridine (BrdU) incorporation assay, respectively. Cells were plated in 96-well, flat-bottom microtiter plates at a density of 2×10^4 cells/well and allowed to attach. Twenty-four hours later, the cells were incubated in medium in the absence or presence of the specified experimental conditions as indicated in the figure captions. After treatment, the medium was removed. For MTT assay, serum-free medium containing MTT was

added to each well, and the cells were incubated for another 4 h. The crystalline precipitate of the formazan product generated by the living cells was dissolved in 20% SDS for 18 h at room temperature, and the absorbance of the solution was read on the ELISA reader at 595 nm. For the BrdU incorporation assay, 10 μM BrdU was added to each well, and the cells were incubated for 2 h to label them with BrdU. The labeling medium was then removed, and cells were fixed and incubated with anti-BrdU-POD solution for another 90 min. After the antibody-conjugate was removed, cells were washed three times. The substrate solution (tetramethyl-benzidine) was then added to each well, and incubation was continued at room temperature until color development. The peroxidase reaction was stopped by adding 25 μl of 1M H_2SO_4 to each well and mixing thoroughly. The absorbance of the samples was measured in the ELISA reader at 450 nm, and the reference wavelength was 690 nm.

Western blot analysis

To analyze of LTB_4-receptor expression, Caco2 and HT-29 cells were scraped out into medium and pelleted by centrifugation at 1200 rpm for 10 min. The supernatant was removed, and the cells were resuspended in Chaps cell extract buffer. The cell suspensions were frozen and thawed three times and then centrifuged at 14,000 rpm for 20 min. To analyze extracellular signal-regulated kinase (ERK) activation, cells were lysed in lysis buffer [50 mM Tris (pH 7.5), 100 mM EDTA, 0.5% Triton X-114, 0.5% Triton X-100, 10 μl/ml protease inhibitor cocktail, and 1 mM PMSF] by incubating for 20 min at 4°C. The protein concentration was determined with dye reagent concentrate. The samples were heated with SDS sample buffer at 95°C – 100°C for 5 min. After cooling on ice, the samples were centrifuged for 5 min. The samples were then loaded onto 12% SDS-polyacrylamide gel, and the proteins were electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk solution and incubated with primary antibodies overnight at 4°C. The primary antibodies were used at dilutions of 1:1000 in 3% dry milk. The membranes were washed three times in TBS and 0.05% Tween 20 and incubated with secondary antibody for 1 h at room temperature. After washing three times in TBS and 0.05% Tween 20, the blots were visualized with an ECL Western Blot analysis system.

Hoechst 33342 staining for nuclear condensation

Apoptotic cells were detected by nuclear staining with Hoechst 33342 as described previously (14). Briefly, cells were seeded in 24-well plates at a density of 10^5 cells/well and allowed to attach. After 24 h, the cell culture medium was replaced with serum-free medium. The cells were then treated for 48 h with AA-861 (30 μM) serum-containing medium, and both detached and attached cells were harvested with 0.25% trypsin/EDTA. The cells were then pelleted by centrifugation at 1,000 rpm for 10 min, and after fixation in 4% paraformaldehyde, they were stained with 5 μg/ml of Hoechst 33342.

Flow cytometry for detection of apoptotic cells

HT-29 cells (2×10^6) were seeded in 6-cm culture dishes, and after incubation at 37°C for 48 h, the cells were treated for 24 h with AA-861 (30 μM) or U75302 (5 μM) in serum-containing medium. The cells were then scraped out into medium, and after pelleting by centrifugation at 3000 rpm for 10 min, the supernatants were removed, and the pelleted cells were resuspended in binding buffer. Annexin V-FITC conjugate and propidium iodide (PI) solution was added to each cell suspension, and the samples were incubated at room temperature for 10 min in the dark. Cell fluorescence was measured immediately with a FACScan flow cytometer from BD Biosciences (San Jose, CA, USA). At least 12,000 cells were examined in the gated region used for calculation. Dual parameter cytometric data were analyzed by using CellQuest software from BD Biosciences.

LTB_4-receptor siRNA transfection experiments

Stealth small interfering RNAs (siRNA) corresponding to BLT1 genes were obtained and annealed commercially (Invitrogen Japan KK). The following LTB_4-receptor gene-specific sequences were chemically synthesized: stealth siRNA-LTB4R sense 5'-UACUCC ACACCACAAAGCUGUUGCC-3' and antisense 5'-GGCAACAGCUUUGUGGUGUGGAGUA. Non-specific siRNA, stealth RNAi negative control kit, was used as a negative control. LTB_4-receptor siRNA was transfected into Caco2 and HT-29 cells with Lipofectamine 2000 reagent. To put it briefly, 2×10^5 cells were plated in 6-cm culture dishes and incubated in serum-containing medium without antibiotics for 24 h. Lipofectamine 2000 was diluted in Opti-MEM I medium without serum and incubated for 5 min. LTB_4-receptor siRNA and negative control siRNA were each diluted in Opti-MEM I medium without serum and added to the diluted Lipofectamine 2000. The mixture was incubated for 20 min at room temperature to allow the siRNA /negative control siRNA: Lipofectamine 2000 complexes to form. An 80-μl aliquot of complexes was added to each dish, and cells were incubated for another 48 h without replacement of the medium.

Finally, LTB_4-receptor expression in treated cells was analyzed by Western blotting as described above.

Statistical analyses

Statistical comparisons were made using Student's *t*-test or Scheffe's method after an analysis of variance (ANOVA). The results were considered significantly different at $P<0.05$.

Results

Expression of LTB_4 receptor in human colonic cancer tissues and cultured cell lines

The immunohistochemical studies revealed the strong expression of BLT1 in all of the human colon cancer tissues (10 of 10 samples positive, Fig. 1A). The staining was seen not only in lymphocyte-infiltrated areas but also in adenocarcinoma cells. In contrast, the expression was not seen in any of the normal colon tissues (none of 10 samples are positive). BLT1 expression was also detected in the tissues from most adenomas (8 of 10 samples are positive), but the staining intensity was much weaker than that in the cancer tissue (data not shown).

Western blot analysis revealed that strong expression of BLT1 protein was observed in established human colon cancer cell lines, Caco2 and HT-29 (Fig. 1B). These results for expression of the BLT1 in both colon cancer tissues and cultured cancer cell lines indicate that the LTB_4-receptor signaling pathway may play an important role in colon cancer.

Effect of LTB_4 on the proliferation of colon cancer cells

To investigate the effect of LTB_4 signaling on the proliferation of colon cancer cells, we applied LTB_4 itself to HT-29 and Caco2 cells. As shown in Fig. 2, LTB_4 stimulated the proliferation of both Caco2 and HT-29 cells (Fig. 2: A and B, respectively). Stimulated proliferation by LTB_4 was time-dependent (data not shown), although it seemed to show a bell-shaped dose-response curve. Namely, the proliferation rate of HT-29 cells was 137%, 128%, and 119 % at the concentration of 10^{-12}, 10^{-10}, and 10^{-8} M, respectively (Fig. 2A). Similar results were observed when Caco2 cells were treated with LTB_4 (Fig. 2B). However, the stimulatory effect of LTB_4 on cancer cell proliferation was not so dramatic in our present study.

Effect of 5-LOX inhibition on the survival and proliferation of colon cancer cells

As supplementation of LTB_4 itself showed weak stimulation of cell proliferation, we therefore investigated the effect of the inhibition of LTB_4 synthesis. As the 5-LOX is a critical enzyme to synthesize LTB_4, we examined the effect of the specific 5-LOX inhibitor AA-861 on the survival of colon cancer cells (15). Treatment with AA-861 significantly decreased both Caco2 and HT-29 cell survivals in a dose-dependent manner (Fig. 3A). In addition, we investigated the effect of 5-LOX inhibition by AA-861 on colon cancer cell proliferation using the BrdU incorporation assay. As shown in Fig. 3B, AA-861 showed a dose-dependent inhibition of proliferation on Caco2 and HT-29 cells proliferation. These results indicated that 5-LOX metabolites, especially LTB_4, might play important roles of colon cancer cell survival and proliferation.

Effect of the LTB_4-receptor blockade on human colon cancer cell survival and proliferation

To clarify whether the inhibition of proliferation observed in the cancer cells treated with 5-LOX inhibitor is due to the reduction of LTB_4 and its receptor signaling, we investigated the effect of the selective BLT1 antagonist U75302 on colon cancer cell proliferation and survival. As shown in Fig. 3C, U75302 dose-dependently decreased the survival of both Caco2 and HT-29 cells. Similarly, U75302 inhibited the proliferation both of Caco2 and HT-29 cells as assessed by the BrdU incorporation assay (Fig. 3D). These results clearly indicated that the blockade of LTB_4-receptor signaling induced the suppression of cell survival and proliferation.

Induction of colon cancer cell apoptosis by 5-LOX inhibitors and BLT1 antagonist

To determine whether the 5-LOX-induced inhibition of cell proliferation and decrease in cell survival were attributable to apoptosis, we investigated nuclear condensation, a specific morphological change associated with apoptosis, by chromatin staining with Hoechst 33342 on colon cancer cells treated with AA-861. As shown in Fig. 4A, Hoechst 33342 staining clearly showed the nuclear condensation in HT-29 cells treated with AA-861. To confirm the apoptotic effect of AA-861, we also performed flow cytometric analysis by double staining with Annexin V-FITC and PI. As shown in Fig. 4B, the percentage of Annexin V-positive/PI-negative cells, that is, apoptotic cells, was increased to 24.7% when cells were treated with AA-861.

Similar results about the chromatin condensation and Annexin V expression were observed when cells were treated with the BLT1 antagonist U75302 (Fig. 4: A and B). Based upon the results observed in our present study, it is strongly indicated that the blockade of LTB_4-receptor signaling caused apoptosis resulting in the decrease in cell survival.

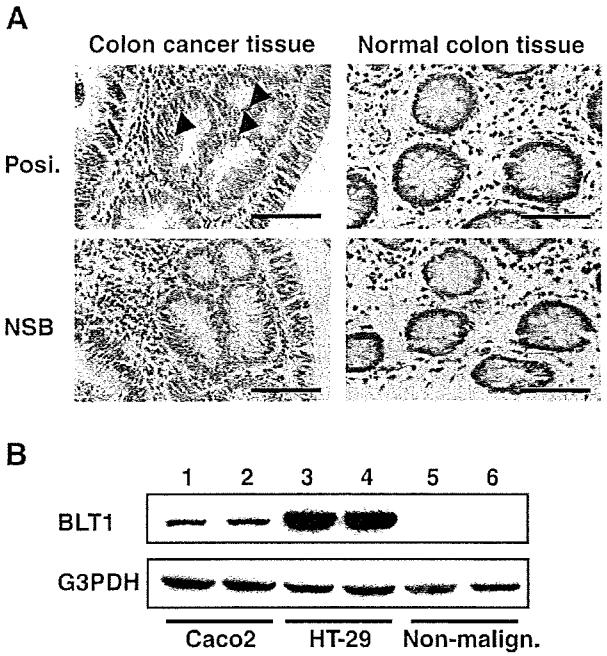

Fig. 1. Expression of LTB_4 receptor in human colon cancer tissues and cultured cell lines. A: Human colonic adenocarcinoma with strong positive staining (left panel, brown color, arrowheads) and the normal colon tissue (right panel). Lower two panels represent non-specific bindings (NSB). Counterstaining was performed with Mayer's hematoxylin solution. Scale bar: 100 μm. B: Expression of LTB_4-receptor 1 protein (BLT1) in human colon cancer cell lines. Lanes 1 and 2: Caco2, Lanes 3 and 4: HT-29, Lanes 5 and 6: neuronal cells differentiated from neural stem cells (non-malignant transformation). G3PDH is an internal control to adjust the sample loading.

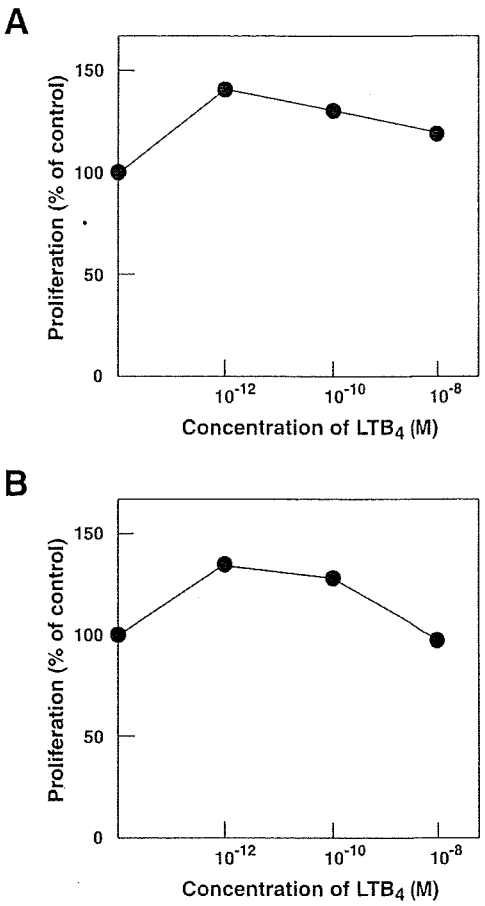

Fig. 2. Effect of LTB_4 on the proliferation of human colon cancer cells, HT-29 (A) and Caco2 (B). Cells were treated with 10^{-8} – 10^{-12} M of LTB_4 for 48 h. BrdU incorporation was used to determine cell proliferation rate. The results are each expressed as a percentage of the proliferation rate of the control. Each data point is expressed as the mean from 3 independent experiments.

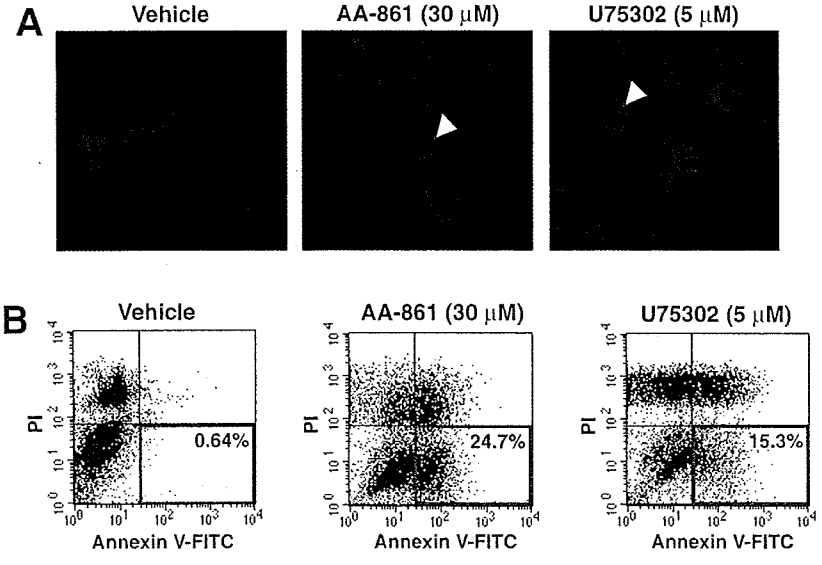

Fig. 4. Induction of colon cancer cells apoptosis by AA-861 and U75302. A: Evaluation of apoptosis by Hoechst 33342 staining. HT-29 cells were treated with 30 μM of AA-861 or 5 μM of U75302 for 48 h at 37°C. Both detached and attached cells were harvested, and stained with Hoechst 33342. The nuclear morphology of cells was observed by fluorescence microscopy to evaluate cell apoptosis. 400× original magnification. Apoptotic cells are indicated by arrowheads. B: Flow cytometric analysis with Annexin V-FITC / PI staining of human colon cancer cells. Numbers indicate the percentage of early apoptotic cells (Annexin V-FITC positive, PI negative) in bottom right quadrant (Blue squares). Typical data are representive of 3 independent experiments.