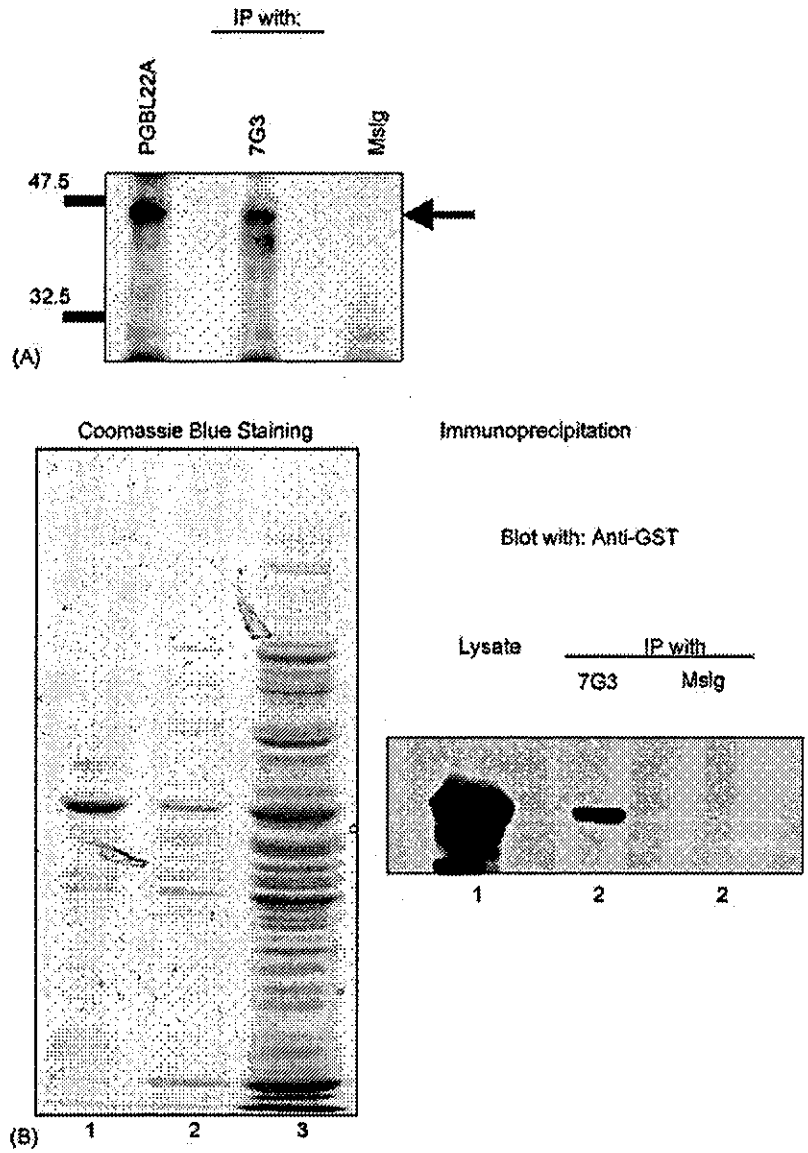

Fig. 4. Immunoprecipitation study using 7G3. (A) Immunoprecipitation was performed using 7G3 or PGBL22A in cell lysates extracted from biotinylated porcine peripheral blood leukocytes. After separation on an SDS-PAGE gel, the proteins were transferred onto a nitrocellulose membrane and probed with peroxidase-conjugated avidin. An unrelated mouse monoclonal antibody (MsIg) was used as a control. (B) GST fusion proteins of the full-length porcine TCR δ-chain were produced in *Eschericha coli* (lane 3). After purification with glutathione sepharose (lane 1), the proteins were eluted with reduced glutathione (lane 2). Each protein was separated by SDS-PAGE in a 10% acrylamide gel and visualized using Coomasie blue-staining. (C) Immunoprecipitation was performed on eluted purified GST fusion TCR δ-chain proteins as described in A followed by immunoblotting with an anti-GST antibody. Lysates of full-length porcine TCR δ-chain were produced in *E. coli* and used as a positive control for the anti-GST antibody. As a negative control, an unrelated mouse monoclonal antibody (MsIg) was used.

Fig. 3, a two-color analysis demonstrated that the staining pattern of 3E12 was similar to that of 7G3. All $3E12^+$ cells were included in the $CD3^+$ cells, but not in the $CD4^+$ cells, and a portion of the $3E12^+$ cells overlapped with the CD8 α-chain$^+$ cells. Further analysis revealed that the $3E12^+$ cells were always included in both $7G3^+$ and $PGBL22A^+$ cells, with approximately two-thirds of the $7G3^+$ cells being

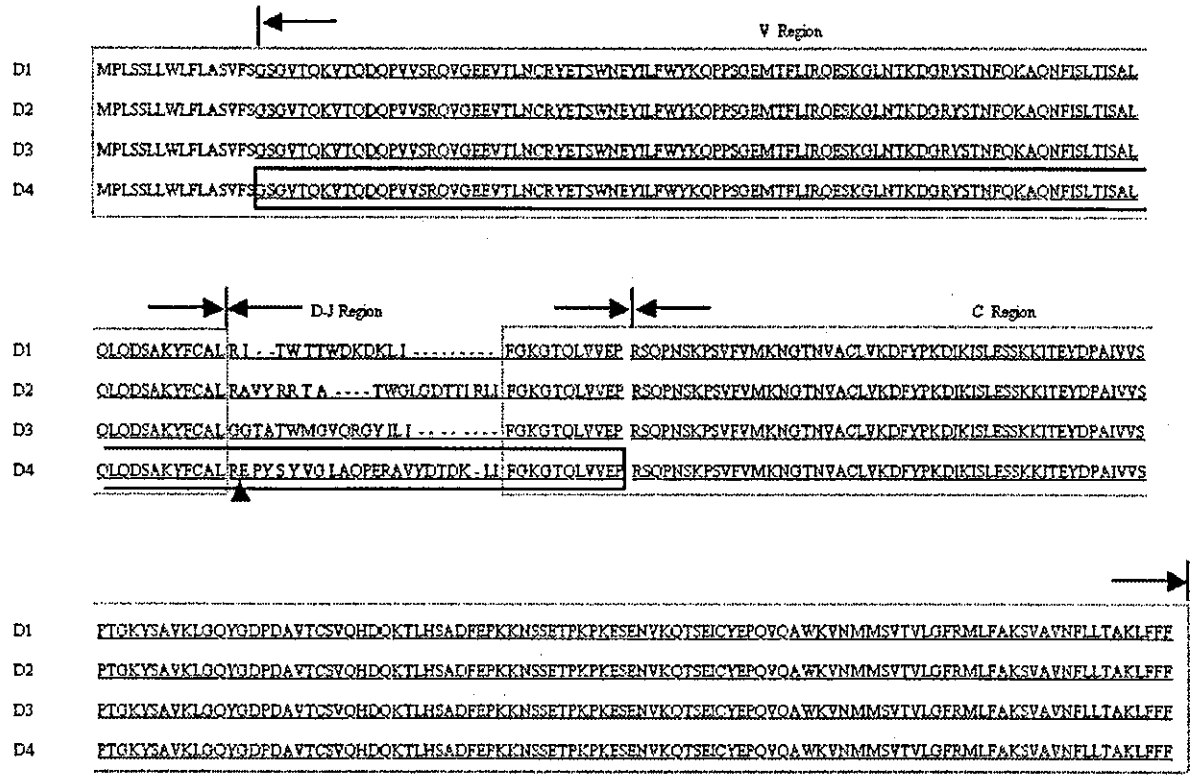

Fig. 5. Schematic presentation of TCR δ-chain cDNA. The nucleotide sequences of the TCR δ-chain cDNAs that were cloned from the 7G3$^+$ cell cDNA library are shown. The arrow-head indicates a termination codon introduced in TCR D4 to generate the TCR D4 V region construct. The solid box indicates the deleted site in the TCR D4 C region construct.

positive for 3E12. The 3E12$^+$ cells were almost identical to those detected by PT79A, and only partially overlapped with either PG92A$^+$ or PG94A$^+$ cells. The above data indicates that 3E12 recognizes a subpopulation of γδ T-cells.

3.2. Reactivity of 7G3 with TCR δ-chain protein

From the biotinylated cell lysates of PB lymphocytes, 7G3 immunoprecipitated a protein with a molecular weight of approximately 40 kDa under reducing conditions (Fig. 4A). A side-by-side comparison revealed that mAb PGBL22A, which has been reported to detect the TCR δ-chain, produced a band with an identical molecular weight.

To confirm that 7G3 reacted with TCR δ-chain, 7G3 was challenged to react with recombinant forms of the TCR δ-chain protein. In our cDNA libraries prepared from 7G3$^+$ cells and 6F10$^+$ cells, one TCR β-chain clone (TCR B), one TCR γ-chain clone (TCR G), two TCR α-chain clones (TCR A1 and A2) and four TCR δ-chain clones (TCR D1 through D4) (Fig. 5) full-length cDNAs were identified. These cDNAs were inserted into mammalian expression vector pME18S-FL3 and transfected into Cos-7 cells. After culturing for three days, the cells were stained with 7G3 using an immunohistochemical method. No staining was observed when the TCR β-chain (Fig. 6A), TCR α-chain or TCR γ-chain cDNAs were transfected (data not shown), but the Cos-7 cells that were transfected with one of the four different TCR δ-chain cDNAs stained positive for 7G3 (Fig. 6A, Table 3). An identical staining pattern to that of 7G3 was obtained when mAb PGBL22A was used (Table 3).

The above results prompted us to examine the reactivity of 7G3 with TCR δ-chain recombinant protein. For this purpose, we first produced a full-

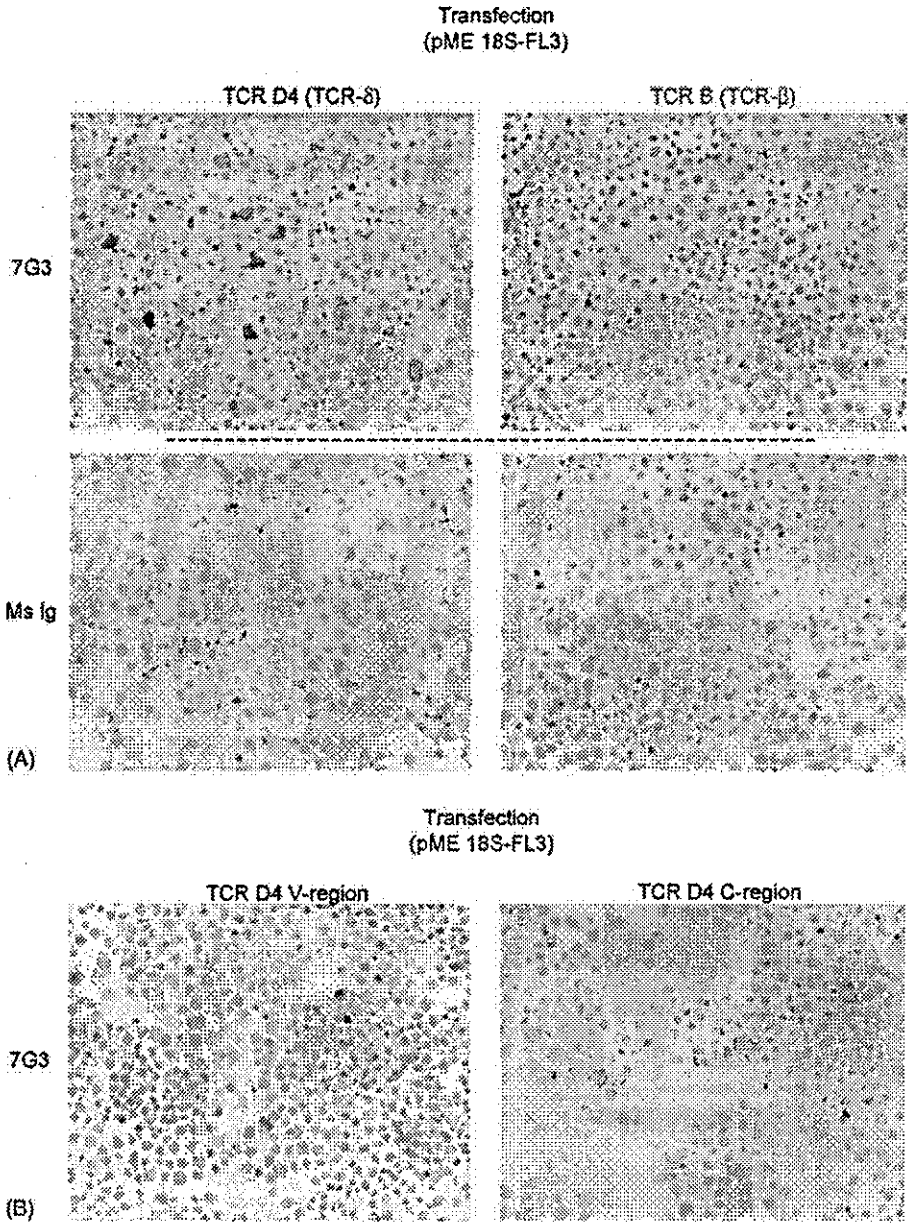

Fig. 6. Reactivity of 7G3 on Cos-7 cells transfected with TCR δ-chain cDNA. (A) Mammalian expression vectors containing either TCR δ-chain (TCR D4) or TCR β-chain (TCR B) were introduced into Cos-7 cells, and the cells were stained with 7G3 or a control antibody. (B) Expression vectors for the TCR δ-chain mutant that completely lack either the C region (TCR D4 V region) or the V region (TCR D4 C region) were introduced into Cos-7 cells, and the cells were stained with 7G3 or a control antibody.

length TCR δ-chain recombinant protein fused with GST in bacteria. As shown in Fig. 4B, a full-length TCR δ-chain-GST fusion protein with a molecular weight of 60 kDa was effectively produced (lane 3) and purified using glutathione beads (lanes 1 and 2). An immunoprecipitation experiment using 7G3 and the recombinant full-length TCR δ-chain-GST fusion protein, followed by immunoblotting with anti-GST

Table 3
Reactivities of monoclonal antibodies against Cos-7 cells transfected with various types of porcine TCR cDNA

	TCR D1	TCR D2	TCR D3	TCR D4	TCR A1	TCR A2	TCR G	TCR B
7G3	+	+	+	+	–	–	–	–
PGBL22A	+	+	+	+	–	–	–	–
PT79A	–	–	–	–	–	–	–	–
PG92A	–	–	–	–	–	–	–	–
PG94A	–	–	–	–	–	–	–	–
86D	–	–	–	–	–	–	–	–
3E12	–	–	–	–	–	–	–	–

Ab, clearly indicated that 7G3 reacted with the full-length TCR δ-chain protein (Fig. 4C).

Next, we determined whether 7G3 reacts with the C region or the V region of the TCR δ-chain. GST protein fused with the V region of the TCR δ-chain was successfully produced in bacteria, but was not reactive with 7G3 (data not shown). Since we failed to produce the C region of the TCR δ-chain protein in bacteria, we attempted to use a mammalian expression system and a deletion mutant of the TCR D4 clone. As shown in Fig. 6B, 7G3 reacted with the Cos-7 cells in which the N-terminal deletion mutant of the TCR D4 clone, containing the C region of the TCR δ-chain, was expressed. On the other hand, 7G3 did not react with Cos-7 cells in which the C-terminal deletion mutant of the TCR D4 clone, containing the V region of the TCR δ-chain, was expressed. Based on the above data, we concluded that 7G3 recognizes the C region of the TCR δ-chain.

3.3. Reactivity of 6F10 (CD8), 7G3 (TCR δ-chain) and 3E12 (γδ T-cells) in tissue sections

We next examined whether the newly established mAbs (6F10, 7G3, and 3E12) could be used for immunostaining on frozen sections. As shown in Fig. 7, all of the mAbs produced high-quality immunostaining results when used on frozen sections. In the thymus, 6F10 stained a significant number of both cortical and medullary thymocytes (Fig. 7). The strong staining pattern produced by 6F10 was striking when compared with the staining pattern produced by the commercially available CD8 α-chain mAb, the latter of which only weakly stained the thymocytes. Both 7G3 and 3E12 also stained small subpopulations of the thymocytes. $7G3^+$ cells were found mainly in the medulla, but a significant number of $7G3^+$ cells were also identified in the cortex of the thymus. In the medulla of the thymus, the $7G3^+$ cells tended to be found around Hassal's corpuscle. The $3E12^+$ cells were distributed in a similar manner as the $7G3^+$ cells, but the number of positive cells was lower than the number of $7G3^+$ cells. Commercially available mAbs against γδ T-cells were also examined, but none of these mAbs produced a satisfactory staining intensity. For example, mAb PGBL22A weakly stained only a small number of thymocytes. Similarly, the staining pattern produced by CD3 was not distinct on the frozen sections.

In lymph node (data not shown) and spleen tissues (Fig. 7), 6F10-stained cells were mainly distributed in the parafollicular area, which was rich in $CD2^+$ T-cells. In addition, a few $6F10^+$ cells were identified in the lymphoid follicles, which were visualized by the B-cell marker CD21 (data not shown). In the lymph node and spleen tissues, the $7G3^+$ and $3E12^+$ cells were scattered mainly in the parafollicular area.

4. Conclusions

In this paper, we report the development of a battery of murine mAbs that were reactive with porcine lymphocytes, including one Ab probably recognizes CD8 (6F10) and two anti-γδ T-cell Abs (7G3 and 3E12). All three of the newly characterized mAbs produced high-quality results when used for immunostaining on frozen sections, compared with the results obtained using commercially available anti-CD8 and anti-γδ T-cells mAbs.

The pig is unique with regard to the distribution of TCR in its peripheral organs. In mice and humans, from which a modern immunological scheme has been developed, the number of γδ T-cells in

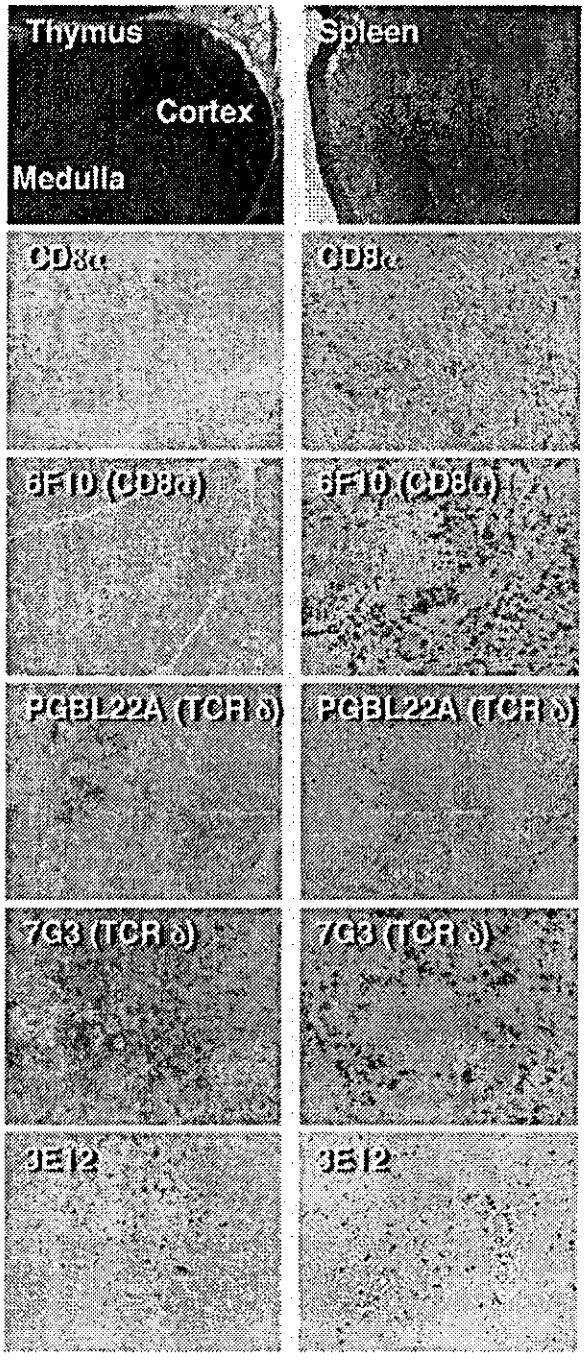

Fig. 7. Reactivity of 7G3, 3E12 and 6F10 on frozen sections of porcine tissues. Frozen sections of porcine thymus (left panels) and spleen (right panels) were stained with the monoclonal antibodies, as indicated.

peripheral lymphoid organs is very small, usually less than a few percent of the lymphocyte population (Carding and Egan, 2002). Instead, γδ T-cells tend to reside in the epithelia of various organs, such as the intestine, uterus and skin, that are often directly subjected to external Ag stimuli. Artiodactyls, including pig, however, are known to be abundant in γδ T-cells in their PB (Hein and Mackay, 1991). Davis et al., previously showed by using mAb PGBL22 that about 30–50% of porcine PB mononuclear cells are γδ T-cells (Davis et al., 1996, 1998, 2001). Indeed, five pigs we investigated also gave high proportion of γδ T-cells with an average of 24.7% with our new mAb 7G3, ranging from 16.1% to 32.7% and the value with PGBL22A was essentially the same with 7G3 in each pig. This striking feature prompted us to reconsider the biological role of γδ T-cells in defense immunity against direct Ag attacks from the external world. For this purpose, a precise characterization of porcine γδ T-cells, as well as αβ T-cells, is indispensable, and the data described here will serve to improve our understanding of the porcine immune system.

mAb 7G3 was selected based on its limited reactivity in PB lymphocytes and was found to react with a subpopulation of $CD3^+$ T-cells using two-color flow cytometry. Since this reactivity was quite similar to the previously reported proportion of γδ T-cells in porcine PB (Davis et al., 1998) we decided to compare our results with those obtained using established, commercially available mAbs. Among the mAbs against γδ T-cells that were tested, 7G3 exhibited a nearly identical distribution to that of mAb PGBL22A. The evidence that 7G3 immunoprecipated a protein with an identical molecular weight (approximately 40 kDa) to that produced by PGBL22A under reduced conditions further indicated that 7G3 and PGBL22A recognized the same molecule. In a previous report by another group, a biochemical analysis revealed that one TCR δ-chain with a molecular weight 40 kDa and three distinct TCR γ-chains with molecular weights of 37, 38, and 46 kDa were distributed in different subsets of porcine γδ T-cells (Hirt et al., 1990; Saalmuller et al., 1990; Thome et al., 1994). Based on the similarity of the identified molecular weights, we hypothesized that 7G3 reacts with the TCR δ-chain. To confirm this idea, we employed a molecular

biology approach. Fortunately, we were able to identify full-length cDNA clones of all TCR α-, β-, γ- and δ-chains in our cDNA libraries generated from $7G3^+$ cells and $6F10^+$ cells. Using a mammalian expression system, 7G3 was shown to react with Cos-7 cells in which all four TCR δ-chains cDNA had been introduced. Furthermore, 7G3 was shown to bind with a recombinant full-length TCR δ-chain protein fused with GST. These results confirmed that 7G3 reacts with the porcine TCR δ-chain.

Next, we investigated whether 7G3 reacts with the V or the C region of the porcine TCR δ-chain. Among the mAbs previously shown to react with γδ T-cells, PGBL22A seemed to have a wider range of reactivity than the other mAbs. PGBL22A was known to react with the TCR δ-chain, as proved using immunochemistry, but whether the mAb would react with a recombinant protein and the region of the TCR δ-chain that it recognized where uncertain. Using flow cytometry and various mAbs against porcine TCR γ-, δ-chains, the reactivities of 7G3 and PGBL22A were shown to be identical and were wider than those of the other mAbs. Thus, the possibility that both 7G3 and PGBL22A recognize a common framework in the TCR δ-chain (Davis et al., 1998, 2001) was considered, although the exact determinants are clearly different since these two mAbs can be used to perform a two-color analysis. The pig Vδ region sequences can be grouped into five families (Vδ1–5); one family, Vδ1, consists of a large number of members, whereas the other families have a limited number of members, as determined by cDNA sequencing as well as genomic Southern blotting (Yang et al., 1995). The TCR Vδ1 family was seen in 25 out of 34 cDNA clones in γδ T-cells obtained from a 1-month-old, germfree pig. In fact, four of the cDNAs for the TCR δ-chain found in the $7G3^+$ cell cDNA library contained an identical V region (Vδ1), and the only differences were seen in the joint (J) region (Fig. 5). Therefore, 7G3 may recognize Vδ1, and not the C region, of the porcine TCR δ-chain. Consequently, we conducted an experiment to determine the region of the TCR δ-chain to which 7G3 reacts. Ideally, recombinant proteins of both the V region and the C region should have been produced, but we were only able to produce a GST-fusion TCR δ-chain mutant, in which the C region was completely deleted; this protein was unreactive with 7G3. These results does not necessary mean, however, that 7G3 does not bind TCR δ-chain V-domain because the folding of the V region peptide might be different from GST-fused full length TCR δ-chain in *Escherichia coli*.

We therefore attempted to use a mammalian expression system. Consistent with the results obtained using recombinant TCR δ-chain V-domain protein fused with GST, 7G3 did not reacted with the Cos-7 cells in which the expression vector for V region of the TCR δ-chain was transfected. It should be noted that the TCR δ-chain V region construct lacked membrane anchoring domain, and the peptides produced might be secreted out of Cos-7 cells. This experiment, therefore, cannot rule out the possibility of 7G3 binding to TCR δ-chain V-domain. We finally showed, however, that 7G3 clearly reacted with the Cos-7 cells in which the C region of the TCR δ-chain was expressed and the strength of staining with this construct was comparable to the Cos-7 cells with full length TCR δ-chain constructs. Taken together, the present evidence strongly suggest that at least a part of the epitope recognized by mAb 7G3 resides within the peptide coded by TCR δ-chain C region. Indeed, the published structure of a human γ δ TCR suggests a portion of TCR δ-chain C region to be accessible to antibody.

In this study, we also developed another mAb, 3E12, that reacts with a subset of γδ T-cells. Since the $3E12^+$ subpopulation was always included in the population of $7G3^+$ cells, namely the TCR δ-chain-expressing cells, and was never found in $7G3^-$ cells, 3E12 likely recognizes a molecule expressed specifically on γδ T-cell subpopulation, but not the common framework of TCR γδ-chains. Furthermore, 3E12 did not react with the four TCR δ-chain proteins (TCR D1–4) or the one TCR γ-chain protein (TCR G) examined in this study. Comparing the reactivity of 3E12 and 7G3, it seems possible that 3E12 may recognize one of the three distinct TCR γ-chains other than one we cloned (Hirt et al., 1990; Saalmuller et al., 1990; Thome et al., 1994). Alternatively, 3E12 may recognize a Vδ family other than Vδ1 or a novel molecule related to the TCR γ- and δ-chain complex that is specifically expressed in a subset of γδ T-cells. A detailed investigation of the 3E12 Ag, including whether 3E12 recognizes the same molecule as that recognized by mAb PT79A, is now underway.

mAb 6F10 was found to react probably with the CD8 α-chain using flow cytometry. The major benefit of using 6F10 in research on the porcine immune system is its applicability to immunohistochemical analyses. Compared with the commercially available CD8 mAb, 6F10 reacts strongly on frozen sections. This benefit is also seen with the mAbs against γδ T-cells. mAb 7G3 distinctly stains TCR δ-chain$^+$ cells on frozen sections. Similarly, mAb 3E12 strongly stains a subset of γδ T-cells. Immunohistochemical analyses are particularly important for studying pathological conditions; thus, the mAbs developed in this study are expected to improve our understanding of not only the fundamental aspects of immune systems in general, but the immunological reactions involved in various porcine diseases.

In conclusion, we report the development of three mAbs that recognize distinct subsets of porcine T lymphocytes, all of which produced high-quality immunostaining results on tissue sections. In particular, mAb 7G3, which was confirmed to recognize the C region of the porcine TCR δ-chain, should facilitate the study of porcine γδ T-cells not only in the field of porcine immunity, but also in the field of comparative immunological evolution.

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Dietary bioflavonoids induce apoptosis in human leukemia cells

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Abstract

Dietary bioflavonoids are secondary metabolites of plants that are known to have a variety of bio-effects, including anti-cancer activity. In this study, we examined the effects of flavonoids on the growth of human leukemia cells and found that certain flavonoids induce apoptosis in a variety of human leukemia cells. The apoptosis induced by bioflavonoids was dose-dependent and was accompanied by a disruption of the mitochondrial transmembrane potential and the activation of caspase. Our data suggests that dietary bioflavonoids may be useful chemotherapeutic reagents for leukemia patients.

Keywords: Bioflavonoid; Apoptosis; Acute lymphoblastic leukemia; Precursor-B-cell

1. Introduction

Flavonoids are ubiquitously occurring and widely consumed secondary metabolites of plants [1,2]. Flavonoids can be divided into three main groups: Flavones, Flavonones (2,3-dihydroflavones), and isoflavones, which differ in structure and ring substitutions [3]. They have diverse pharmacological properties, including antioxidant, cytoprotective, and anti-inflammatory activities [1,2], and have also been reported to display anti-viral [4] and anti-parasitic [5] activities.

Moreover, some flavonoids are known to act as anti-cancer reagents. For example, Yoshida et al. reported that Quercetin markedly inhibited the growth of human gastric cancer cells [6]. Record et al. also described the inhibition of B16 melanoma cells by Genistein, both in vivo and in vitro [7]. Huang et al. demonstrated that Luteolin and Quercetin significantly inhibited the proliferation of epidermoid carcinoma A431 cells with an overexpression of epidermal growth factor receptor [8]. Indeed, some bioflavonoids like Quercetin and Genistein have already been used as chemotherapeutic agents in phase trials [9,10].

In an attempt to examine the effects of flavonoids on the growth of human leukemic cells, we challenged cultured human leukemic cell lines with several kinds of flavonoids. In the present study, we demonstrated that certain flavonoids can induce significant apoptosis in a variety of human leukemia cells.

2. Materials and methods

2.1. Cells and reagents

The cell line BV-173 that were established from a patient in an acute relapse who most likely had Ph1-positive chronic myelogenous leukemia [11]; the acute-phase of chronic myelogenous leukemia-derived cell lines K-562 (Japanese Cancer Research Resources Bank, JCRB, Tokyo, Japan)

Abbreviations: ALL, acute lymphoblastic leukemia; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PC-5, PE-Cy-5; PC-7, PE-Cy-7; topo, topoisomerase

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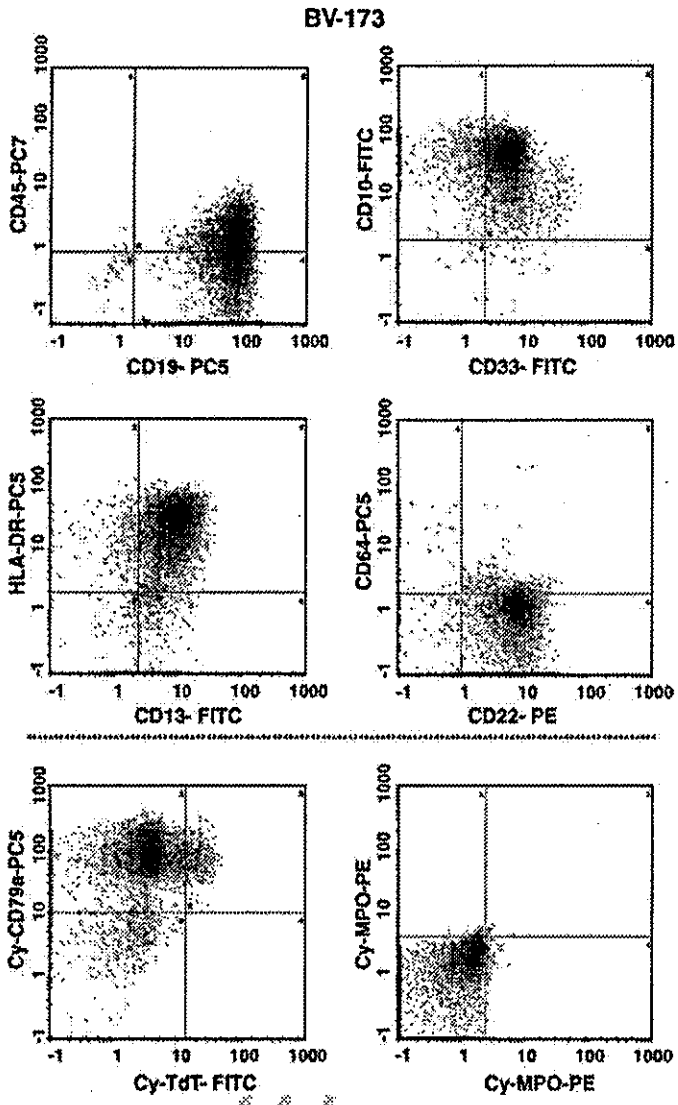

Fig. 1. Immunophenotypic analysis of BV-173 cells. The BV-173 cells were stained with fluorescence-labeled specific monoclonal antibodies against leukocyte antigens, as indicated, and analyzed by flow cytometry. The resulting histograms are shown. (Cy-) Cytoplasmic antigen stained after cell-permeabilization treatment.

and KU-812 (Institute for Fermentation, Osaka, Japan); precursor-B-acute lymphoblastic leukemia (ALL)-derived cell lines, including NALM-16, NALM-20, HPB-NULL and NALM-17 [12]; Burkitt's lymphoma-derived cell lines, Daudi and Ramos (JCRB); the histiocytic lymphoma-derived cell line U-937 (JCRB) and the acute monocytic leukemia-derived cell line THP-1 (JCRB) were used. Cells were cultured in RPMI1640 supplemented with 10% FCS at 37 °C in a humidified 5% CO_2 atmosphere.

Fluorescence-labeled monoclonal antibodies against leukocyte antigens were obtained from Beckman/Coulter Inc. (Westbrook, MA). Bioflavonoids, including Flavone, Genistein, Genistin, Quercetin, Fisetin, Luteolin, Apigenin and Rutin, and the anti-cancer drug VP-16 (which exhibits topoisomerase (topo) II-inhibitor activity) were purchased from Sigma–Aldrich (St. Louis, MO). The peptide-inhibitors for the caspases were obtained from Calbiochem-Novabiochem Co. (San Diego, CA). Bioflavonoids, VP-16 and the caspase inhibitors were desorbed in DMSO and then added to the cell cultures. All other chemical reagents were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), unless otherwise indicated.

2.2. Immunofluorescence study and detection of apoptosis

A multi-color immunofluorescence study was performed using a combination of fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy-5 (PC-5) and PE-Cy-7 (PC-7). Cells were stained with fluorescence-labeled monoclonal

antibodies and analyzed by flow cytometry (EPICS-XL, Beckman/Coulter), as described previously [13]. Staining of the cytoplasmic antigens was performed using Cytofix/Cytoperm™ Kits (Becton Dickinson), according to the manufacturer's protocol.

To quantitate the incidence of apoptotic cells, cells were stained with FITC-labeled annexin V using a MEBCYTO®-Apoptosis Kit (Medical & Biological Laboratories (MBL) Co. Ltd., Nagoya, Japan) and then analyzed by flow cytometry according to the manufacturer's protocol. Experiments were performed in triplicate, and the mean ± S.D. of the cells that bound annexin V are shown. Caspase-3 activity was assessed with a PhiPhiLUX™ G1D2 kit (MBL) and analyzed by flow cytometry according to the manufacturer's protocol. The disruption of the mitochondrial transmembrane potential was detected by the MitoCapture Apoptosis Detection Kit (MBL) and analyzed by flow cytometry according to the manufacturer's protocol.

2.3. Examination of morphological appearance

BV-173 cells were immobilized onto glass slides with Cytospin 2 (Shandon Inc., Pittsburg, PA), Giemsa-stained, and their morphological appearance was examined by light microscopy (BX-61, Olympus, Tokyo, Japan).

3. Results

3.1. Immunophenotypic analysis of BV-173 cells

First, we examined the cell surface and cytoplasmic antigens expressed in BV-173 cells originally derived from a patient with Ph1-positive acute leukemia. As shown in Fig. 1, the BV-173 cells expressed B-cell antigens, such as cluster of differentiation (CD)19, CD22 and cytoplasmic CD79a, as determined by flow cytometry. Together with the expression of CD10 and HLA-DR (Fig. 1) and the absence of surface IgM (data not shown), the cell line was thought to have originated from a precursor-B-cell. However, flow cytometric analysis also revealed that the BV-173 cells simultaneously expressed myeloid antigens, including CD13 and CD33 (Fig. 1). Therefore, BV-173 was thought to exhibit biphenotypic leukemia characteristics with both precursor-B-cell and myeloid lineages. This cell line was mainly used in the following experiments.

3.2. Dietary bioflavonoids induce apoptosis in BV-173 cells

Next, we tested whether the administration of dietary bioflavonoids induced any cytotoxic effects on BV-173 cells. When BV-173 cells were treated with 200 μM of Flavone for 24 h and then examined morphologically by light microscopy, a portion of the cells exhibited condensation (arrow-head) and cleavage (arrow) of the nuclei, findings that are typical

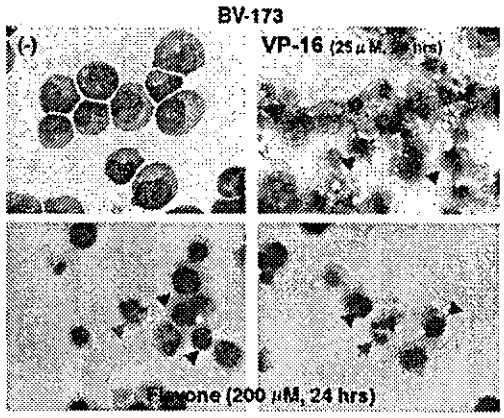

Fig. 2. Morphological examination of Flavone-treated BV-173 cells. BV-173 cells were cultured in the presence or absence of either Flavone or VP-16, as a positive control of apoptosis induction, for 24 h as indicated in the figure, then cytocentrifuged on the slide glasses. After Giemsa-staining, the morphological appearance of the cells was examined using light microscopy. The arrowheads indicate nuclear condensation. Typical apoptotic cells, characterized by cleaved nuclei, are indicated by the arrows. Magnification 400×.

of apoptosis (Fig. 2). No such figures were observed in untreated cells (Fig. 2). The following results clearly show that the administration of Flavone indeed induced apoptosis in BV-173 cells. First, DNA prepared from BV-173 cells treated with Flavone for 24 h showed oligonucleosomal ladder fragmentation on agarose gel electrophoresis (Fig. 3). Second, the number of cells binding to annexin V increased significantly after Flavone-treatment (Fig. 4). As shown in Fig. 4, other bioflavonoids, including Luteolin, Genistein, Quercetin, and

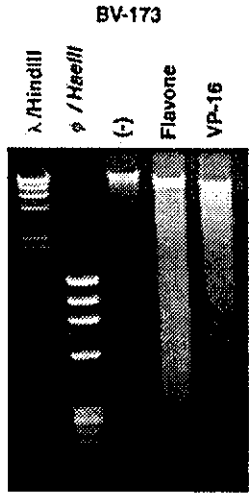

Fig. 3. DNA ladder formation in BV-173 cells after Flavone-treatment. BV-173 cells were treated with (lane 4) or without (-, lane 3) 200 μM of Flavone for 24 h. The extracted DNA (1.5 μg per lane) from each sample was separated by 1% agarose gel electrophoresis. The λ/*Hin*dIII and φ/*Hae*III DNA markers were applied to the same gel (left two lanes). As a positive control for DNA ladder formation, DNA extracted from BV-173 cells treated with 25 μM of VP-16 for 24 h was also examined (VP-16, lane 5).

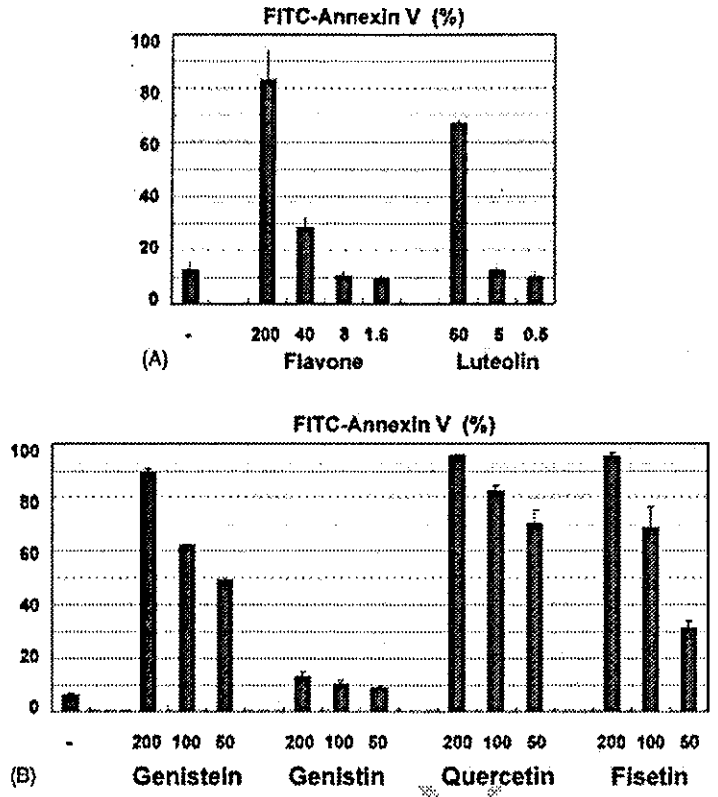

Fig. 4. Detection of annexin V binding cells after flavonoid-treatment. After culturing for 24 h in the presence of the indicated concentrations of each flavonoid, BV-173 cells were incubated with FITC-conjugated annexin V and then analyzed by flow cytometry. Experiments were performed in triplicate and the means + S.D. of the percentages of annexin V bound cells are indicated.

Fisetin, but not Genistin, also induced an increase in the number of cells binding to annexin V.

3.3. Characterization of bioflavonoid-induced apoptosis

The apoptosis induced by bioflavonoids in BV-173 cells was further characterized. In healthy cells, MitoCapture, a cationic dye, accumulates and aggregates in the mithocondria, giving off a bright red fluorescence. When the mitochondrial transmembrane potential is disrupted, however, this dye remains in the cytoplasm in its monomoer form, fluorescing green. As shown in Fig. 5, flow cytometric analysis revealed that treatment with Flavone significantly increased the number of cells with green fluorescence indicating that the mitochondrial transmembrane potential was disrupted after the induction of Flavone-induced apoptosis. In addition, Luteolin and Apigenin, but not Rutin, also disrupted the mitochondrial transmembrane potential (Fig. 5). Next, we examined the activation of caspase-3 in the process of bioflavonoid-induced apoptosis. Flow-cytometric analysis with PhiPhiLux^{TM} G1D2 indicated that treatment with Flavone significantly increased the number of cells in which caspase-3 was activated (Fig. 6). The incidence of caspase-3-activated cells induced by Flavone-treatment was much higher than that induced by VP-16-treatment (Fig. 6). Luteolin and Apigenin also increased the number of cells in which caspase-3 was activated (Fig. 6).

We further examined whether the activation of caspase was indeed involved in the bioflavonoid-induced apoptosis. As shown in Fig. 7, when BV-173 cells pretreated with either z-DEVD-fmk (a tetrapeptide inhibitor of caspase-3), z-IETD-fmk (a tetrapeptide inhibitor of caspase-8), or z-VAD-fmk (a tripeptide inhibitor of a broad range of caspases), a reduction in annexin V-positive cells after Flavone treatment was observed (Fig. 7). Of these inhibitors, z-VAD-fmk produced the most significant inhibition of Flavone-induced apoptosis in BV-173 cells.

3.4. Bioflavonoids induce apoptosis in other leukemia cell lineages

Next, we tested the effect of dietary bioflavonoids on other leukemia cell lines. As shown in Fig. 8, all of the leukemia cell lines tested in this study, including the pro-B cell lines NALM-16 and NALM-20, the pre-B cell lines HPB-NULL and NALM-17, the Burkitt's lymphoma cell lines Ramos and Daudi, the erythroleukemia cell line K-562, the basophilic leukemia cell line KU-812, the histiocytic lymphoma cell line U-937, and the acute monocytic leukemia cell line THP-1, were found to be sensitive to the apoptosis-inducing effect

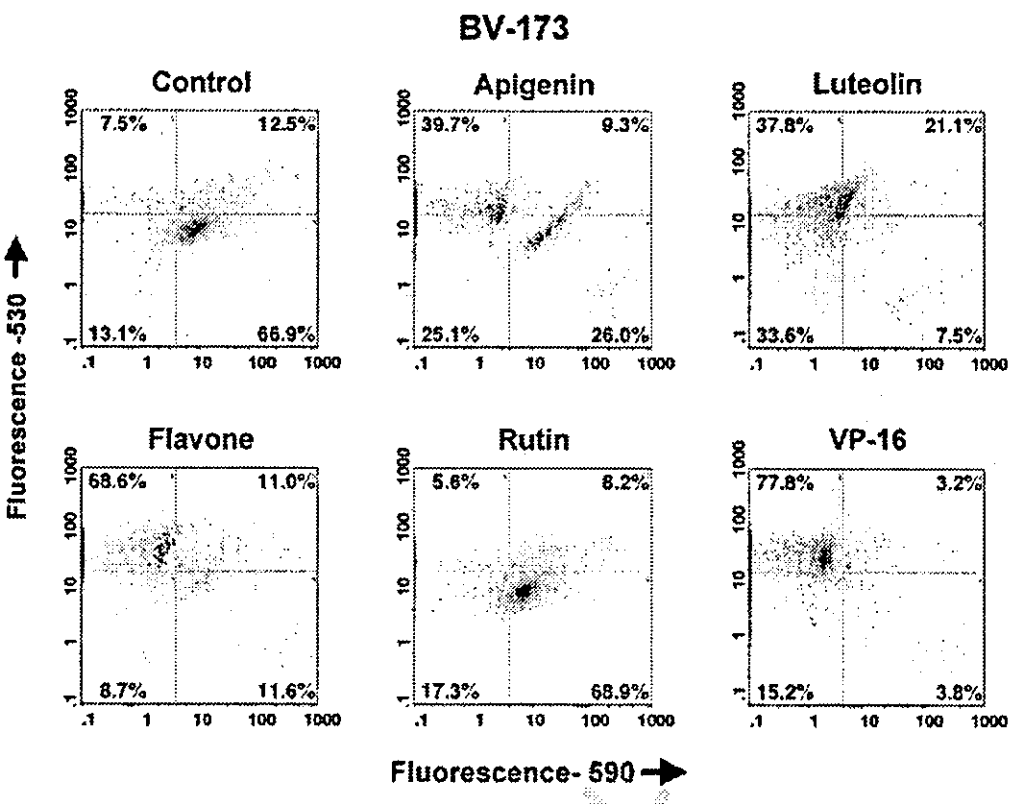

Fig. 5. Disruption of the mitochondrial transmembrane potential in BV-173 cells after bioflavonoid-treatment. BV-173 cells treated with or without flavonoids (Apigenin, 200 μM; Luteolin, 50 μM; Flavone, 200 μM; Rutin, 200 μM) or VP-16 (25 μM) for 24 h were examined using a MitoCapture Apoptosis Detection Kit and analyzed by flow cytometry. The resulting histograms are shown. *X*-axis, intensity of fluorescence -590 (red); *Y*-axis, intensity of fluorescence -530 (green). A shift in the fluorescence from red to green indicates the disruption of the mitochondrial transmembrane potential.

of dietary bioflavonoids. Among these cell lines, however, NALM-6 and K-652 exhibited a relatively lower sensitivity. Although the Burkitt's cell lines showed a limited sensitivity to VP-16 cytotoxicity, they showed a very high sensitivity to Flavone-mediated apoptosis induction.

4. Discussion

Our data clearly indicates that certain, but not all, bioflavonoids induce apoptosis in a variety of human leukemia cell types. As shown in the present study, Flavone, Luteolin, Genistein, Quercetin, and Fisetin induced significant apoptosis in BV-173 cells, while Genistin and Rutin did not. The apoptosis-inducing effect of Apigenin was intermediate. As demonstrated in the present study, all of the human leukemia cells that were tested were effectively induced to undergo apoptosis after bioflavonoid treatment. The bioflavonoid-induced apoptosis occurred in a dose-dependent manner and was accompanied by the disruption of the mitochondrial transmembrane potential and the activation of caspase-3 and perhaps caspase-8. Indeed, the apoptosis was diminished by pretreatment of the cells with anti-caspase inhibitors.

A number of studies have reported the potential ability of bioflavonoids to act as anti-cancer drugs. The precise mechanism of this phenomenon, however, remains unclear, although several effects of bioflavonoids on cell growth and cell death have been reported. For example, bioflavonoids are reported to have topo inhibitor activity. Luteolin is reported to inhibit both topo I and II and induces apoptosis in Leishmania cells [5,14]. Strick et al. [15] reported that certain bioflavonoids induce MLL gene cleavage through the inhibition of topo II.

Some topo II-inhibitors, such as VP-16 and doxorubicin, are widely used as anti-cancer reagents and have been linked with therapy-related leukemia induction due to topo II-inhibition. The effect of topo II-inhibiting substances on cells is thought to consist of two stages [16,17]. During the first stage, topo II-inhibitors stabilize topo II-cleavable complexes by forming drug:topo II:DNA ternary complexes on chromosomal DNA. This stage is reversible by DNA religation or by DNA repair. However, cellular processing of the accumulating ternary complexes triggers the initiation of apoptotic DNA cleavage, an irreversible process (secondary stage of the pathway). At this stage, it is reported that caspase-8 is activated through FADD/TRADD-dependent mechanism and plays a critical role in caspase-3 activation and apop-

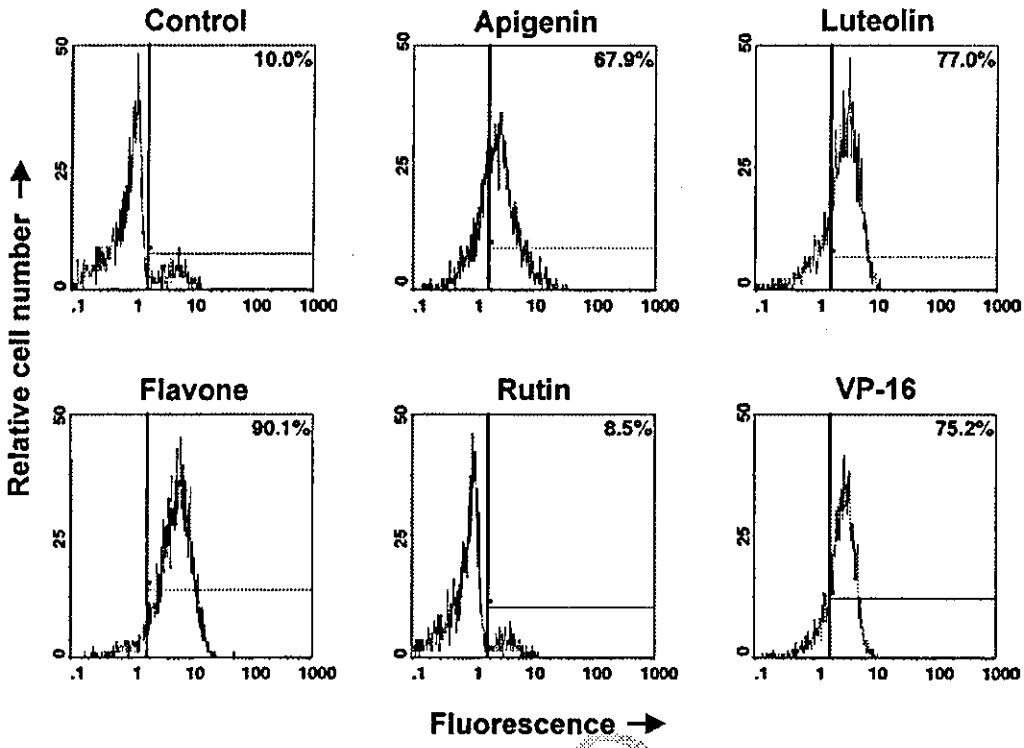

Fig. 6. Caspase-3 activity in BV-173 cells after bioflavonoid-treatment. To measure caspase-3 activity in BV-173 cells after bioflavonoid-treatment, cells prepared as in Fig. 5 were examined using PhiPhiLux^{TM} G1D2 and analyzed by flow cytometry. The resulting histograms are shown. *X*-axis, fluorescence intensity; *Y*-axis, relative cell number.

totic cell death [18]. In parallel, caspase-9 is also activated by apoptosome-mediated mechanism as a result of mitochondrial dysfunction. However, latter pathway seems to play a much less role in caspase-3 activation [18]. Considering the above evidence, including similar activation pattern of caspase pathway (Figs. 6 and 7), it seems reasonable to assume that the anti-cancer effect of the bioflavonoids originated in their topo II-inhibitor activity.

However, as we presented in Fig. 8, Ramos Burkitt's cells, which were resistant to VP-16, were sensitive to Flavone-induced apoptosis. In addition, Strick et al. have reported that Luteolin exhibits more strong topo II-inhibitory effect than that of Flavone [15], whereas apoptosis-inducing effect of Flavone is not lower than that of Luteolin (Fig. 8). Therefore, the induction of apoptosis by flavonoids cannot be explained solely by their topo II-inhibitory effect, and additional possible anti-cancer effects may be involved.

Of note, K-562 cells have been reported to be topo II-resistant [19], whereas our data indicated that K-562 cells are sensitive to VP-16-induced apoptosis and show over than 70% annexin V-positive cells, similar to the flavonoids used. The precise reason for the discrepancy between the previous reports and our data is presently unclear. However, it is also reported that K-562 cells show delay in the VP-16-induced caspase activation in compared with HL-60 cells, leading to a long latent period before initiation of apoptosis, and once the active phase of apoptosis is initiated, a similar proportion of cells are ultimately killed in both cell lines [20]. Therefore, K-562 cells are not completely resistant to topo II and the sensitivity to VP-16-mediated apoptosis may vary among the stocks of K-562 cells in different laboratories.

On the other hand, some bioflavonoids, such as Genistein and Quercetin inhibited tyrosine kinase activity both in vitro and in vivo [21]. Since the overactivation of tyrosine kinases is thought to be involved in oncogenesis in many types of cancer, it seems reasonable that bioflavonoids

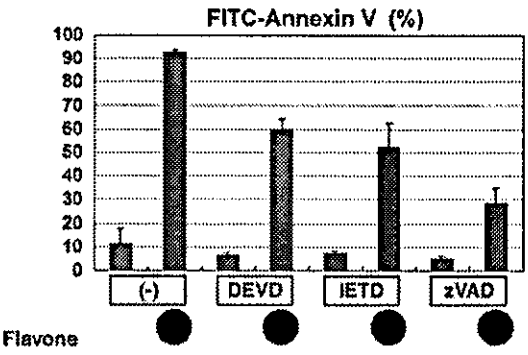

Fig. 7. Effect of caspase-inhibitors on Flavone-induced apoptosis. BV-173 cells pretreated with 50 μM of caspase-inihibitor, as indicated, were treated with 200 μM of Flavone for 24 h, as in Fig. 4. Subsequent apoptotic cells were detected by annexin V binding and analyzed by flow cytometry and are indicated as shown in Fig. 4.

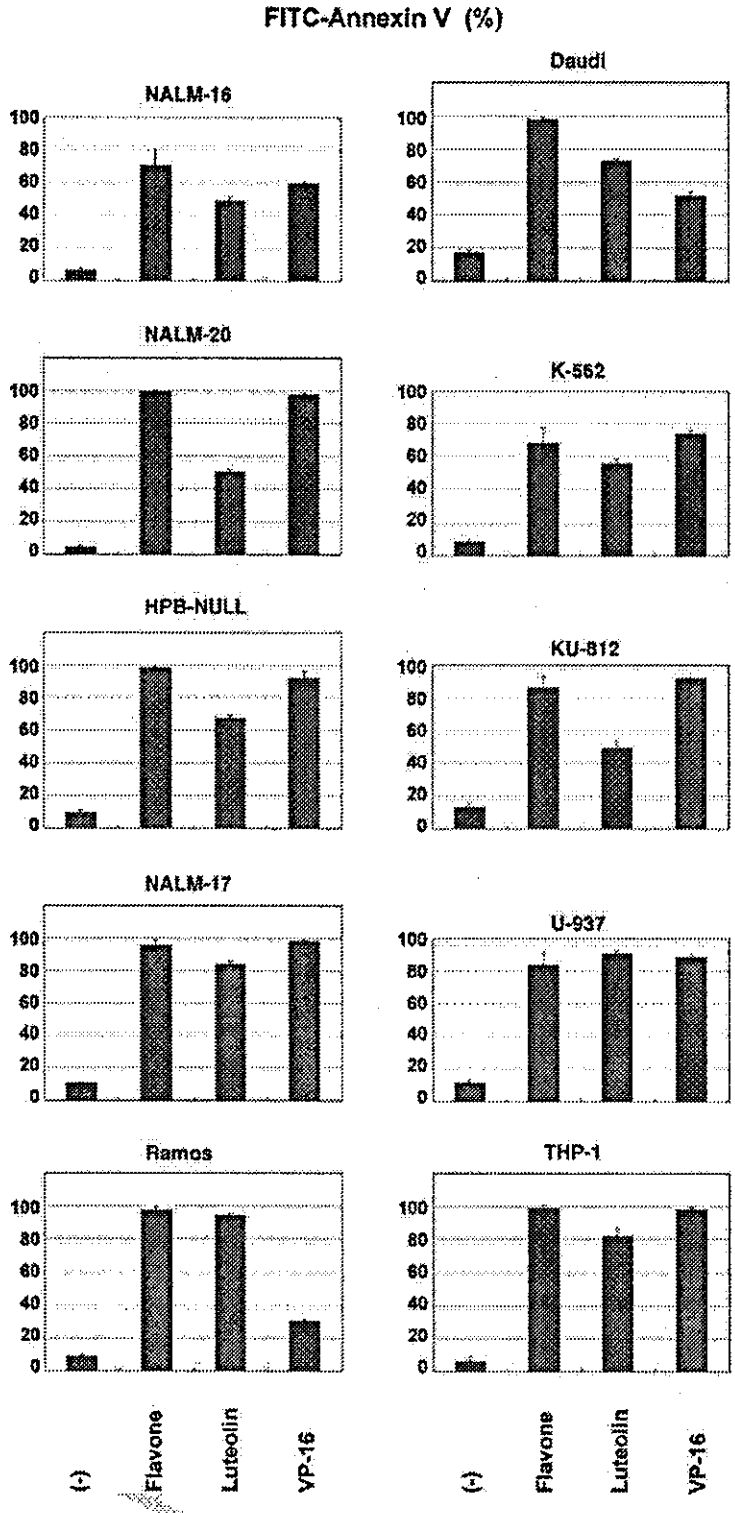

Fig. 8. Apoptosis-inducing effect of bioflavonoids in other leukemia cell lines. A variety of leukemia cell lines, as indicated in the figure, were treated with 200 μM of Flavone, 50 μM of Luteolin, or 25 μM of VP-16 (as a positive control for apoptosis induction), as in Fig. 4. Subsequent apoptotic cells were detected by annexin V binding and analyzed by flow cytometry and are indicated as shown in Fig. 4.

with anti-tyrosine kinase activity would exhibit an anti-cancer effect.

Bioflavonoids have also been reported to be involved in cell cycle regulation. For example, Quercetin was found to downregulate the expression of mutant p53 protein in human breast cancer cell lines, leading to an arrest of the cells in the G2–M phase of the cell cycle [21]. In the case of human leukemic T-cells, Quercetin was found to arrest the cells in late G1 phase. In addition, Luteolin has been reported to arrest the cell cycle in the G1 phase of human melanoma cells [22], and Genistein induces cell cycle arrest at the G2–M stage and the inhibition of cdc2 kinase activity [23]. The arrest of the cell cycle in turn reduces cell growth and results in apoptosis induction.

Another explanation for the anti-cancer activity of bioflavonoids is suggested by their ability to interact with hormone receptors [21]. Certain bioflavonoids have been reported to bind to estrogen binding sites in estrogen receptors, there by interrupting estrogen binding. Indeed, flavonoids, such as Daidzein, Genistein, Quercetin, and Luteolin, were found to suppress the induction of the proliferation-stimulating activity of environmental estrogens in human breast cancer cell lines [24]. However, whether estrogen binding induces the proliferation of leukemic cells has not been reported. Alternatively, flavonoids may interact with the binding sites of growth factors other than estrogen, thereby inhibiting the growth of leukemia cells.

In conclusion, dietary bioflavonoids exhibited an apoptosis-inducing effect in various human leukemia cells. Although further studies must be performed to elucidate the mechanism by which bioflavonoids induce apoptosis in leukemia cells, the present data indicates that dietary bioflavonoids might be useful chemotherapeutic reagents for leukemia patients.

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