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Leukemia Research xxx (2005) xxx–xxx

Leukemia Research

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Dietary bioflavonoids induce apoptosis in human leukemia cells

Jun Matsui, Nobutaka Kiyokawa*, Hisami Takenouchi, Tomoko Taguchi, Kyoko Suzuki, Yusuke Shiozawa, Masahiro Saito, Wei-ran Tang, Yohko U. Katagiri, Hajime Okita, Junichiro Fujimoto

Department of Developmental Biology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 154-8535, Japan

Received 21 July 2004; accepted 11 November 2004

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.
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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

* Corresponding author. Tel.: +81 3 3417 2496; fax: +81 3 3417 2496.
E-mail address: nkiyokawa@nch.go.jp (N. Kiyokawa).

0145-2126/$ – see front matter © 2004 Published by Elsevier Ltd.
doi:10.1016/j.leukres.2004.11.015

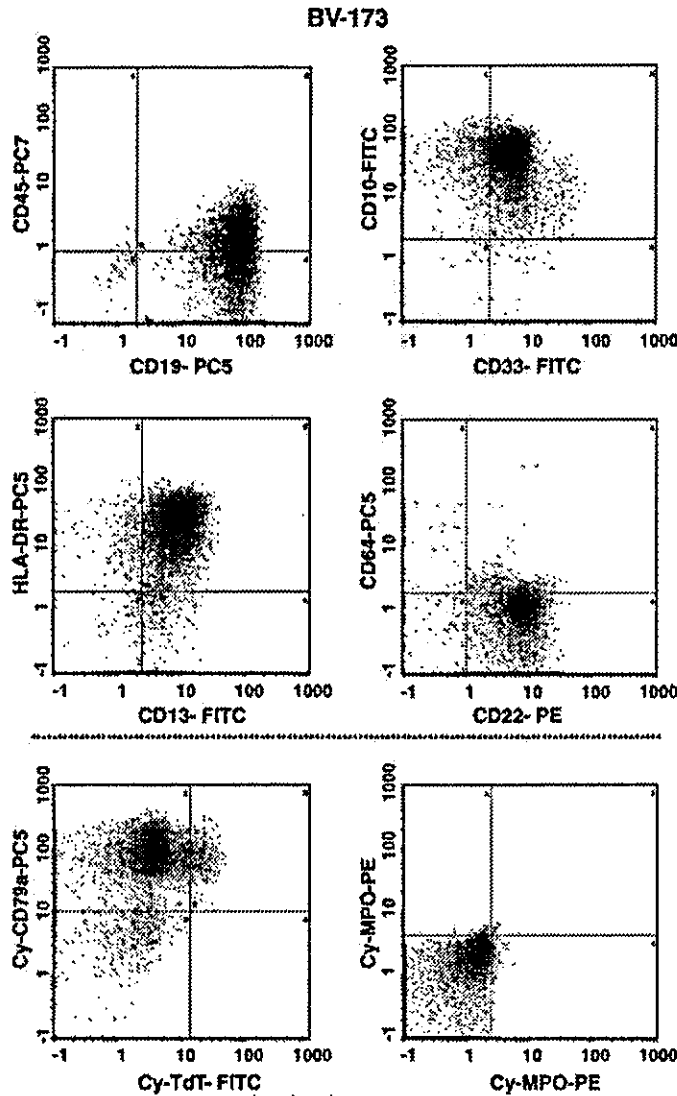

Fig. 1. Immunophenotypic analysis of BV-173 cells. The BV-173 cells were stained with fluorescence-labeled specific monoclonal antibodies against leukocyte antigens, as indicated, and analyzed by flow cytometry. The resulting histograms are shown. (Cy-) Cytoplasmic antigen stained after cell-permeabilization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

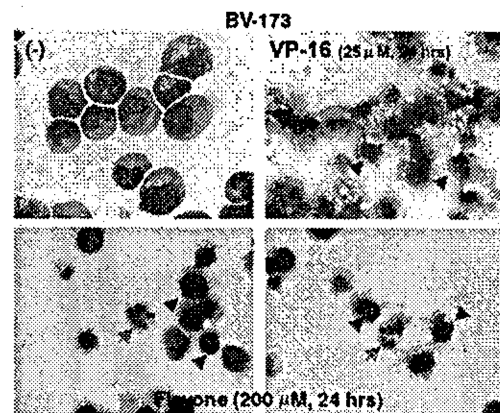

Fig. 2. Morphological examination of Flavone-treated BV-173 cells. BV-173 cells were cultured in the presence or absence of either Flavone or VP-16, as a positive control of apoptosis induction, for 24 h as indicated in the figure, then cytocentrifuged on the slide glasses. After Giemsa-staining, the 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×.

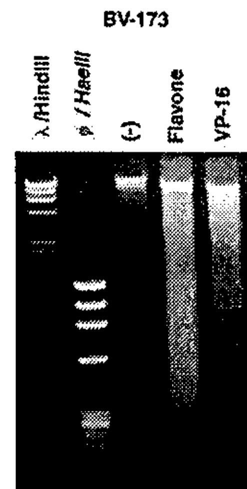

Fig. 3. DNA ladder formation in BV-173 cells after Flavone-treatment. BV-173 cells were treated with (lane 4) or without (-, lane 3) 200 µM of Flavone for 24 h. The extracted DNA (1.5 µg per lane) from each sample was separated by 1% agarose gel electrophoresis. The λ/HindIII and φ/HaeIII DNA markers 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).

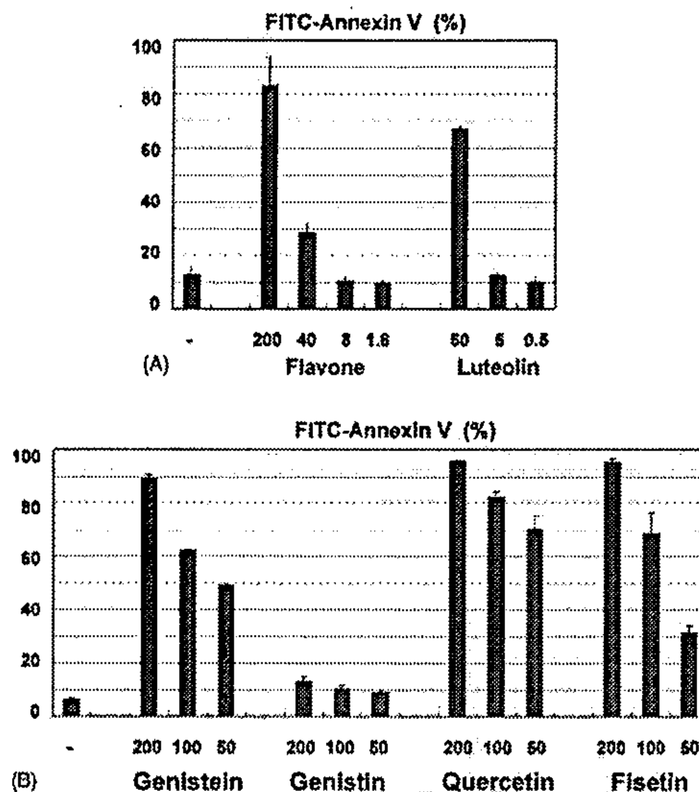

Fig. 4. Detection of annexin V binding cells after flavonoid-treatment. After culturing for 24 h in the presence of the indicated concentrations of each flavonoid, BV-173 cells were incubated with FITC-conjugated annexin V and then analyzed by flow cytometry. Experiments were performed in triplicate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

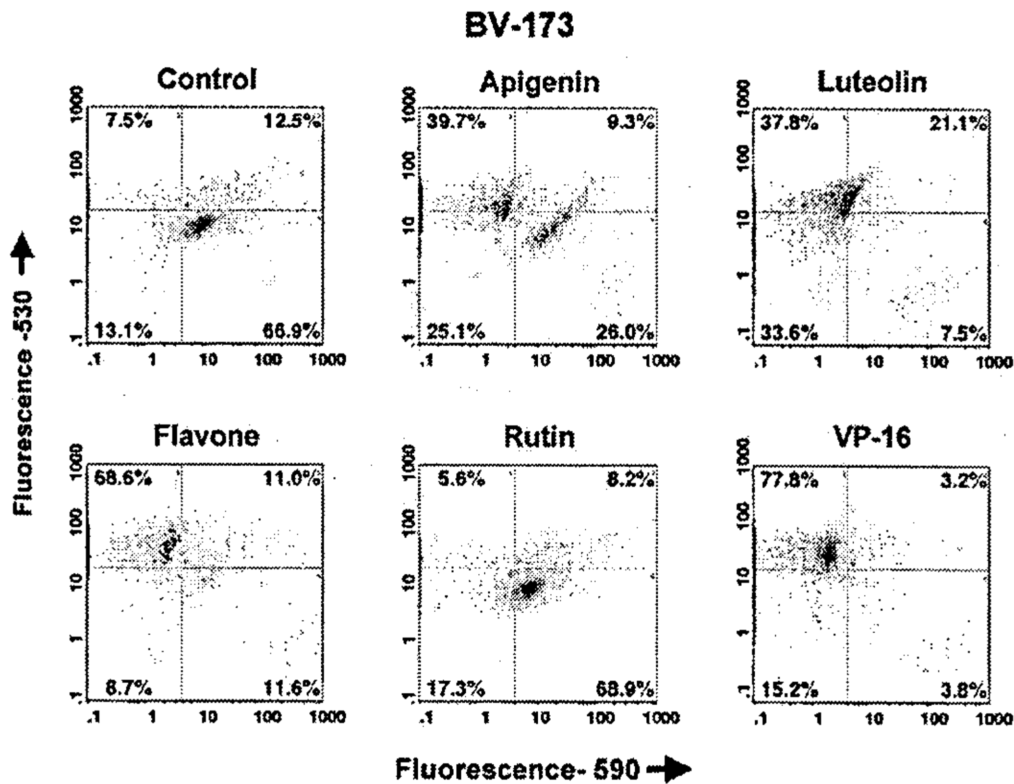

Fig. 5. Disruption of the mitochondrial transmembrane potential in BV-173 cells after bioflavonoid-treatment. BV-173 cells treated with or without flavonoids (Apigenin, 200 μM; Luteolin, 50 μM; Flavone, 200 μM; Rutin, 200 μM) or VP-16 (25 μM) for 24 h were examined using a MitoCapture Apoptosis Detection 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-

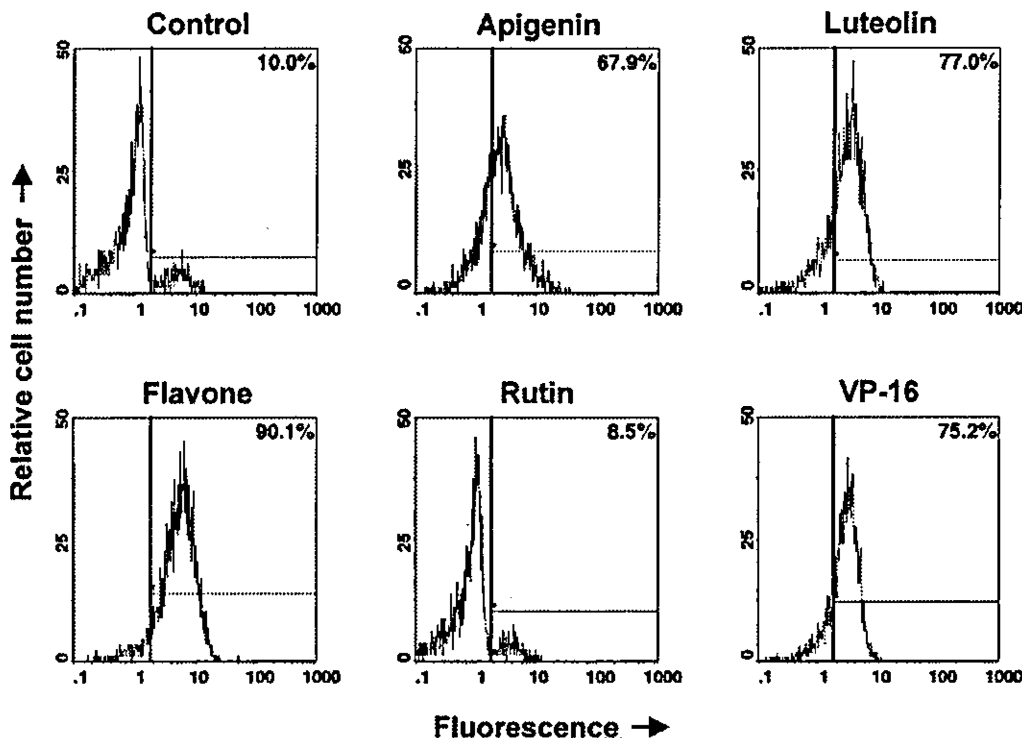

Fig. 6. Caspase-3 activity in BV-173 cells after bioflavonoid-treatment. To measure caspase-3 activity in BV-173 cells after bioflavonoid-treatment, cells prepared as in Fig. 5 were examined using PhiPhiLux^{TM} GiD2 and analyzed by flow cytometry. The resulting histograms are shown. X-axis, fluorescence 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

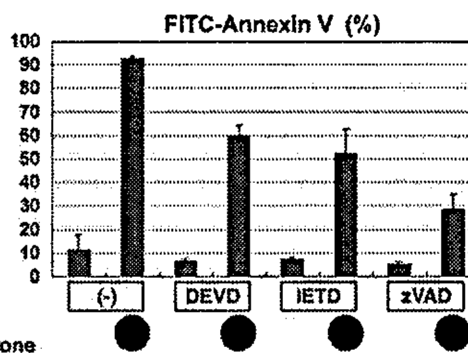

Fig. 7. Effect of caspase-inhibitors on Flavone-induced apoptosis. BV-173 cells pretreated with 50 μM of caspase-inhibitor, as indicated, were treated with 200 μM of Flavone for 24 h, as in Fig. 4. Subsequent apoptotic cells were detected by annexin V binding and analyzed by flow cytometry and are indicated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

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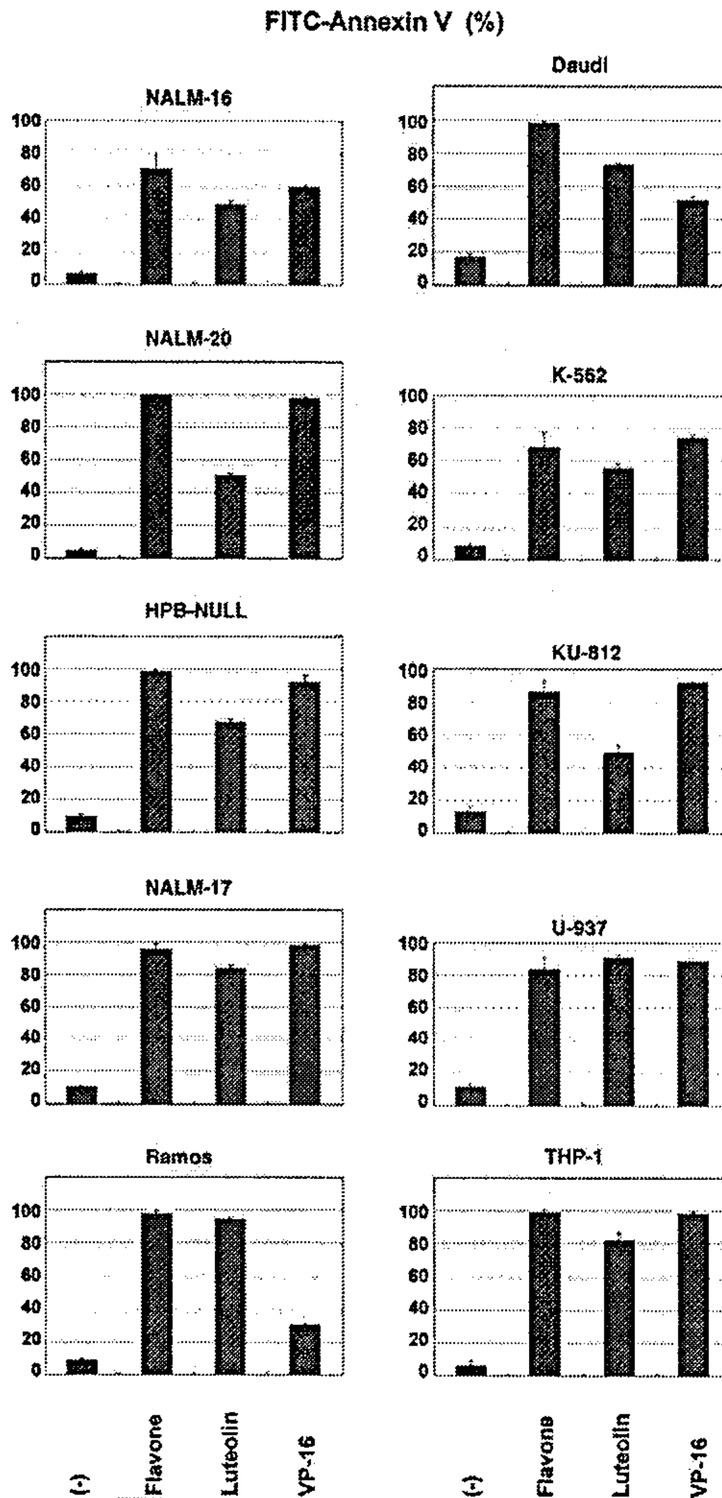

Fig. 8. Apoptosis-inducing effect of bioflavonoids in other leukemia cell lines. A variety of leukemia cell lines, as indicated in the figure, were treated with 200 μM of Flavone, 50 μM of Luteolin, or 25 μM of VP-16 (as a positive control for apoptosis induction), as in Fig. 4. Subsequent apoptotic 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.

Acknowledgements

We thank S. Yamauchi for her excellent secretarial work. This work was supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan, MEXT. KAKENHI 15019129, JSPS. KAKENHI 15390133 and 15590361, grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation, a grant from Sankyo Foundation of Life Science.

Contributions: J. Mat-mi contributed to the concept and design, interpreted and analyzed the data, provided drafting of the article, and gave final approval. N. Kiyokawa contributed to the concept and design, interpreted and analyzed the data, provided drafting of the article, and gave final approval, and obtained a funding source. H. Takeaouehi interpreted and analyzed the data, provided critical revisions and important intellectual content. T. Taguchi and K. Sutuki interpreted and analyzed the data. Y. Shiozawa provided administrative support, provided critical revisions and important intellectual content. M. Saito, M. Saito and W.-R. Tang provided administrative support. H. Okitu provided critical revisions and important intellectual content. J. Fuiimoto provided critical revisions and important intellectual content, and obtained a funding source.

References

[1] Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 1983;32:1141–8.

[2] Wollenweber E. Occurrence of flavonoid aglycones in medicinal plants. Prog Clin Biol Res 1988;280:45–55.

[3] Cody V. Crystal and molecular structures of flavonoids. Prog Clin Biol Res 1988;280:29–44.

[4] Vrijsen R, Everaert L, Boeye A. Antiviral activity of flavones and potentiation by ascorbate. J Gen Virol 1988;69:1749–51.

[5] Mittra B, Saha A, Chowdhury AR, Pal C, Mandal S, Mukhopadhyay S, et al. Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. Mol Med 2000;6:527–41.

[6] Yoshida M, Sakai T, Hosokawa N, Marui N, Matsumoto K, Fujioka A, et al. The effect of Quercetin on cell cycle progression and growth of human gastric cancer cells. FEBS Lett 1990;260:10–3.

[7] Record IR, Broadbent JL, King RA, Dreosti IE, Head RJ, Tonkin AL. Genistein inhibits growth of B16 melanoma cells in vivo and in vitro and promotes differentiation in vitro. Int J Cancer 1997;72:860–4.

[8] Huang YT, Hwang JJ, Lee PP, Ke FC, Huang JH, Huang CJ, et al. Effects of Luteolin and Quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. Br J Pharmacol 1999;128:999–1010.

[9] Ferry DR, Smith A, Malkhandi J, Fyfe DW, deTakats PG, Anderson D, et al. Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. Clin Cancer Res 1996;2:659–68.

[10] Mohammad RM, Al-Katib A, Aboukameel A, Doerge DR, Sarkar F, Kucuk O. Genistein sensitizes diffuse large cell lymphoma to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy. Mol Cancer Ther 2003;2:1361–8.

[11] Pegoraro L, Matera L, Ritz J, Levis A, Palumbo A, Biagini G. Establishment of a Ph1-positive human cell line (BV-173). J Natl Cancer Inst 1983;70:447–53.

[12] Tsuganezawa K, Kiyokawa N, Matsuo Y, Kitamura F, Toyama-Sorimachi N, Kuida K, et al. Flow cytometric diagnosis of the cell lineage and developmental stage of acute lymphoblastic leukemia by novel monoclonal antibodies specific to human pre B cell receptor. Blood 1998;92:4317–24.

[13] Kiyokawa N, Kokai Y, Ishimoto K, Fujita H, Fujimoto J, Hata J. Characterization of the common acute lymphoblastic leukemia antigen (CD10) as an activation molecule on mature human B cells. Clin Exp Immunol 1990;79:322–7.

[14] Chowdhury AR, Sharma S, Mandal S, Goswami A, Mukhopadhyay S, Majumder HK. Luteolin, an emerging anti-cancer flavonoid, poisons eukaryotic DNA topoisomerase I. Biochem J 2002;366:653–61.

[15] Strick R, Strissel PL, Borgers S, Smith SL, Rowley JD. Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukaemia. Proc Natl Acad Sci USA 2000;97:4790–5.

[16] Liu LF. DNA topoisomerase poisons as antitumor drugs. Annu Rev Biochem 1989;58:351–75.

[17] Beere HM, Chresta CM, Alejo-Herberg A, Skladanowski A, Dive C, Larsen AK, et al. Investigation of the mechanism of higher order chromatin fragmentation observed in drug-induced apoptosis. Mol Pharmacol 1995;47:986–96.

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[18] Chandra D, Choy G, Deng X, Bhatia B, Daniel P, Tang DG. Association of active caspase 8 with the mitochondrial membrane during apoptosis: potential roles in cleaving BAP31 and caspase 3 and mediating mitochondrion-endoplasmic reticulum cross talk in etoposide-induced cell death. Mol Cell Biol 2004;24:6592–607.
[19] Strissel PL, Strick R, Rowley JD, Zeleznik-Le NJ. An in vivo topoisomerase II cleavage site and a DNase I hypersensitive site colocalize near exon 9 in the MLL breakpoint cluster region. Blood 1998;92:3793–803.
[20] Martins LM, Mesner PW, Kottke TJ, Basi GS, Sinha S, Tung JS, et al. Comparison of caspase activation and subcellular localization in HL-60 and K562 cells undergoing etoposide-induced apoptosis. Blood 1997;90:4283–96.
[21] Lamson DW, Brignall MS. Antioxidants and cancer, part 3: quercetin. Altern Med Rev 2000;5:196–208.
[22] Casagrande F, Darbon JM. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. Biochem Pharmacol 2001;61:1205–15.
[23] Su SJ, Yeh TM, Lei HY, Chow NH. The potential of soybean foods as a chemoprevention approach for human urinary tract cancer. Clin Cancer Res 2000;6:230–6.
[24] Han DH, Denison MS, Tachibana H, Yamada K. Relationship between estrogen receptor-binding and estrogenic activities of environmental estrogens and suppression by flavonoids. Biosci Biotechnol Biochem 2002;66:1479–87.

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HYBRIDOMA AND HYBRIDOMICS
Volume 23, Number 3, 2004
© Mary Ann Liebert, Inc.

Development of Novel Monoclonal Antibody 4G8 against Swine Leukocyte Antigen Class I α Chain

WEI-RAN TANG,[1] NOBUTAKA KIYOKAWA,[1] TOMOKO EGUCHI,[3] JUN MATSUI,[1] HISAMI TAKENOUCHI,[1] DAISUK HONMA,[3] HIROSHI YASUE,[3] SHIN ENOSAWA,[2] KENICHI MIMORI,[1] MITSUKO ITAGAKI,[1] TOMOKO TAGUCHI,[1] YOHKO U. KATAGIRI,[1] HAJIME OKITA,[1] HIROSHI AMEMIYA,[2] and JUNICHIRO FUJIMOTO[1]

ABSTRACT

A mouse monoclonal antibody (MAb) was generated against swine leukocyte antigen (SLA) class I α chain. A newly developed series of MAb clones that react with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. Among them, MAb 4G8 was characterized by the following features: (1) 4G8 reacted with Cos-7 cells transfected with SLA class I α chain from the *d* haplotype, (2) 4G8 recognized epitopes that were different from those of commercially available anti-SLA class I MAbs 74-11-10 and PT85A, and (3) 4G8 could be used to immunostain frozen sections of thymus, spleen, lymph node, kidney, and liver tissues with good results.

INTRODUCTION

THE PORCINE SYSTEM has received much attention as a suitable model for transplantation medicine. Therefore, an accurate understanding of human immune responses to porcine tissues has become increasingly important. However, the details of the porcine immune system, especially those features that are novel to the pig, remain unclear. We thus attempted to develop new MAbs that could be used to analyze the porcine immune system.

The immune response to foreign antigens is determined by the expression of specific major histocompatibility complex (MHC) molecules that can bind and present peptide fragments of that protein to T cells. There are two different types of MHC gene products, termed Class I and Class II MHC molecules, and any given T cell recognizes foreign antigens bound to only one Class I or Class II MHC molecule. Antigens associated with Class I molecules are recognized by $CD8^{+}$ cytolytic T cells, whereas class II–associated antigens are recognized by $CD4^{+}$ helper T cells. Class I molecules are located on every nucleated cell surface, except those of neurons and trophoblasts. In contrast, the expression of Class II molecules is limited to certain cell types. In pigs, MHC molecules are known as swine leukocyte antigens (SLA). All SLA class I molecules contain two separate polypeptide chains: an MHC-encoded α chain of 45 kD and a non–MHC-encoded β chain of 12 kD.

Recently, the profound involvement of SLA Class I molecules in human anti-porcine cell reactions has been described. Several studies have shown that human T cells can directly recognize porcine MHC molecules and that such recognition can lead to the killing of the porcine cells. Porcine cells have recently been shown, moreover, to be targets for human NK cells. Since human MHC class I molecules deliver a negative signal to human NK cells, protecting syngeneic cells from lysis, we surmised that differences in the gene sequences of porcine MHC class I molecules may be responsible for the lack of recognition by human NK cell receptors and subsequent cytolysis of the porcine cells. In addition, it was reported that a single treatment with a monoclonal antibody (MAb) directed against the SLA class I provides an attractive approach to the induction of T cell tolerance, possibly enabling long-term graft survival in porcine-to-human cell transplantations.[1] These studies indicate that SLA class I molecules play critical roles in transplantation medicine.

Departments of [1]Developmental Biology and [2]Innovative Surgery, National Research Institute for Child Health and Development, Tokyo, Japan.
[3]Genome Research Department, National Institute for Agrobiological Science, Ibayaki, Japan.

Here, we report a novel MAb 4G8 against the SLA class I α chain that was proven to be different from four commercially available anti-SLA class I MAbs. The utilization of 4G8 in tissue sections was also examined.

MATERIALS AND METHODS

Animals and tissues

Landrace or (Landrace × Large White) F1 pigs were used in this study. Peripheral blood (PB) and tissues were obtained from anesthetized animals and were processed. PB was collected in acid citric buffer to avoid coagulation. Tissues were immediately snap frozen and kept in the deep freezer until use.

Monoclonal antibodies

PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. After washing twice in phosphate-buffered saline (PBS), approximately 1×10^8 cells were injected into the abdominal cavity of 8-week-old female Balb/c mice. Boost injections were performed twice at 2-week intervals. At 4 days after the last boost, splenocytes were fused with P3U1 mouse myeloma cells and incubated in hypoxanthine and thymidine (HAT) medium. Supernatants of growing hybridomas were screened on porcine PB leukocytes by flow cytometry and clones secreting antibodies reactive with porcine PB leukocytes were subcloned twice by limiting dilution. Clones were grown in the abdominal cavity of Pristane-treated Balb/c mice, and ascites were obtained. Purification of MAbs was performed by Protein-A or Protein-G column (Bio-Rad Laboratories, Hercules, CA). After purification, MAb was fluorescence isothiocyanate (FITC) conjugated as described previously.[2] Commercially available MAbs against SLA class I 74-11-10, PT85A, H17A*, H58A* (* indicates known as cross-reactive with pig and other species) were obtained from Veterinary Medical Research and Development (Pullman, WA).

Flowcytometry and immunohistochemistry

Flowcytometrical analysis of MAbs was carried out as follows. Briefly, aliquot of porcine PB was incubated with appropriate amount of MAb for 30 min at 4°C. After washing with PBS, cells were incubated with FITC-conjugated (Jackson Laboratory, West Grove, PA) for 30 min at 4°C. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter, Westbrook, MA).

Reactivity of MAbs on tissues were analyzed by immunohistochemistry on frozen sections. Briefly, porcine tissues were snap-frozen in optimal cutting temperature (OCT) compounds and frozen sections were made by cryostat apparatus. Sections were fixed by acetone for 15 min at 4°C. After washing in PBS and blocked with normal rabbit serum, sections were incubated with MAbs at appropriate dilutions for 30 min at room temperature. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)–conjugated rabbit anti-mouse antibodies (Jackson Laboratory) for 30 min at room temperature. After washing with PBS, color development was done in diaminobenzidine solution (10 mM in 0.05 M Tris-HCl, PH 7.5) with 0.003% H_2O_2.

Binding competition assay

Binding competition assay was carried out as follows. Briefly, after aliquot of porcine PB leukocytes were incubated with 2ug saturated amount of commercially available MAbs for 30 min at 4°C. The cells saturated with these commercially available MAbs were stained with FITC-4G8 for 30 min at 4°C. Then, PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. FITC-mouse immunoglobulin (MsIg) was used as control antibody. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter).

Cloning and expression of porcine cDNA library

As another purpose for analysis of $\gamma\delta$TCR against MAb (7G3) and CD8 against MAb (6F10), cDNA libraries of 7G3-positive as well as 6F10-positive PB leukocytes were first constructed. A brief description is shown below. Porcine PB labeled with FITC-7G3 antibody was incubated with magnetic-activated cell sorting (MACS) beads conjugated with anti-FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and was loaded onto AutoMACS cell separator (Miltenyi Biotec). 7G3-positive cells were positively selected and a cDNA library was constructed using the oligo-capping method[3] and plasmid vector pME18S-FL3, which contains the SR-α promoter for expression in mammalian cells. To 7G3-negative pass-through fractions, FITC-6F10 was added and labeled. These cells were also positively selected by AutoMACS and used for the cDNA library construction. Out of several thousand clones sequenced from both cDNA libraries, one clone was selected which exhibited homologies to known porcine MHC class I sequences from d haplotype and contained full-length open reading frames.

Complementary DNA coding for porcine MHC class I under SRα promoter was introduced into COS7 cells by lipofec-

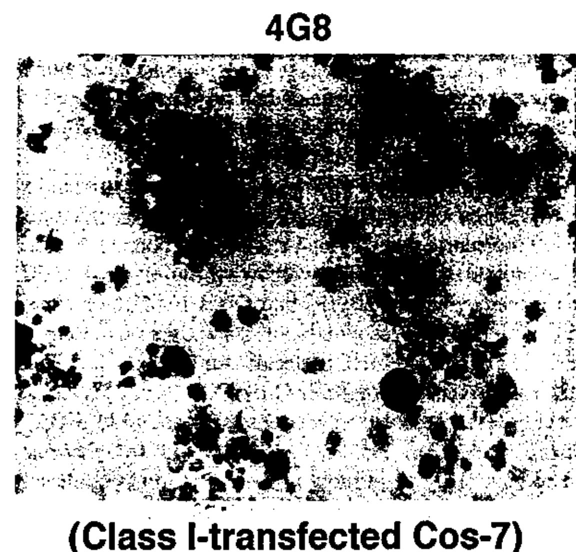

FIG. 1. Reactivity of 4G8 on Cos-7 cells transfected with SLA class I PD1. Mammalian expression vectors containing SLA class I PD1 were introduced into Cos-7 cells, and the cells were stained with 4G8 using immunohistochemistry.

tion (LIPOFECTAMIN, Invitrogen, Groningen, Netherlands) and cells were stained with 4G8 MAb after 3 days.

RESULTS AND DISCUSSION

Anti-SLA Class I MAb 4G8 recognizes a distinct epitope from those of commercially available antibodies

From one hybridization experiment, 45 hybridoma clones were established. The MAbs produced by these clones reacted differently to the porcine PB leukocytes, as revealed by flow cytometry (data not shown). To determine whether a MAb against SLA class I α chain was included among these clones, MAb clones that reacted with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. As shown in Figure 1, when a mammalian expression vector of SLA class I PD1 from d haplotype was transfected into Cos-7 cells, clone 4G8 was found to stain the cells, whereas control MsIg failed to stain the cells (data not shown). Therefore, 4G8 was considered to recognize the SLA class I α chain, including the d haplotype.

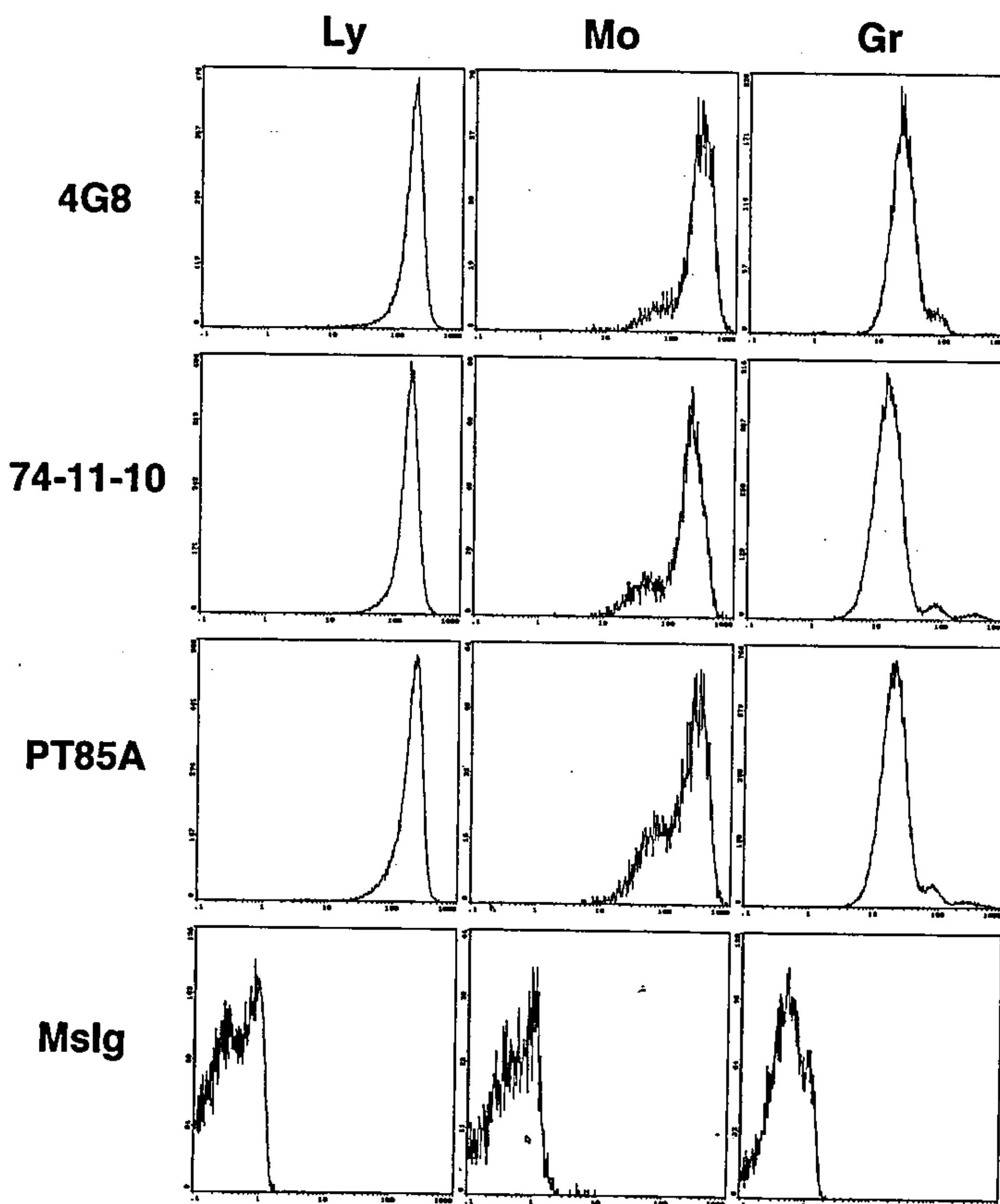

FIG. 2. Comparison of reactivity profiles of 4G8 and commercially available anti-SLA class I monoclonal antibodies. Porcine PB leukocytes were stained with 4G8 and commercially available anti-SLA class I MAbs, 74-11-10, and PT85A, using flow cytometry. MsIg was used as a control antibody.

Next, we compared the reactivity of 4G8 with commercially available anti-SLA class I Abs 74-11-10, PT85A, H17A, H58A to PB from outbred domestic pigs. As shown in Figure 2, flow-cytometrical analysis demonstrated that the reactivity of 74-11-10 and PT85A to the porcine PB was similar to that of 4G8. Although multiple samples from individual animals were tested, all of these MAbs always revealed a pan leukocyte positive staining pattern. In contrast, H17A did not react with the domestic porcine PB samples (data not shown), indicating that H17A is polymorphic in pigs. H58A, exhibited variable reactivity from one animal to another (data not shown). This data indicates that the reactivity of H17A and H58A is different from that of 4G8.

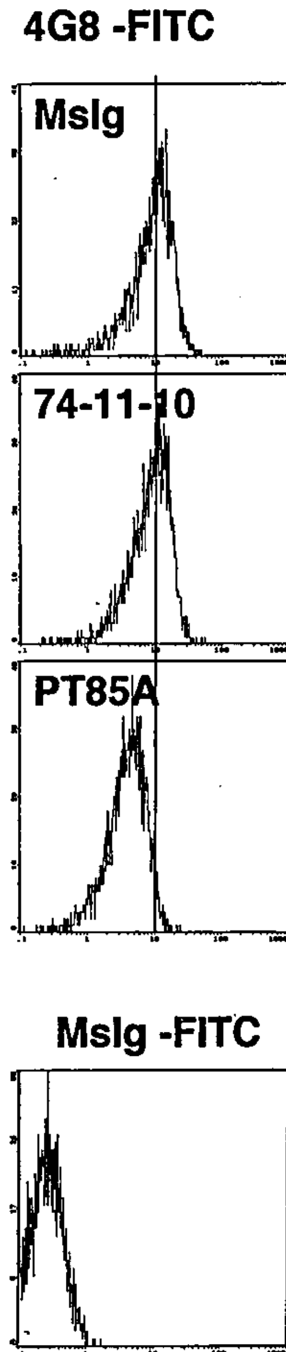

FIG. 3. Competition binding assay using flow cytometry. 4G8 continued to react with porcine PB leukocytes that had been saturated with commercially available MAbs (74-11-10 and PT85A). MsIg-FITC was used as a negative control.

To determine whether the epitope recognized by 4G8 was the same as that of 74-11-10 and PT85A, we examined whether 4G8 could still bind to the cells after the cells had been saturated with 74-11-10 or PT85A. As shown in Figure 3, 4G8 continued to react with PB leukocytes that had been saturated with 74-11-10 or PT85A. These results indicate that 4G8 recognizes a novel epitope distinct from those recognized by 74-11-10, PT85A, H17A and H58A. MHC class I molecules are extremely polymorphic, and polymorphism often occurs in the $\alpha 1$ domain, or the $\alpha 2$ domain, but the $\alpha 3$ domain is nonpolymorphic.[4] 74-11-10 and PT85A have been reported to require the PD1 $\alpha 1/\alpha 2$ domains, but not the $\alpha 3$ domain, to exhibit reactivity.[1] A precise analysis to clarify which domain is recognized by 4G8 is now underway.

Analysis of 4G8 using immunohistochemistry

We examined whether 4G8 could be used to immunostain frozen sections. As shown in Figure 4, 4G8 produced high-quality immunostaining results when used on frozen sections. In thymus tissues, 4G8 produced a dense and diffuse staining pattern in the medullar region and a lighter, scattered staining pattern in the cortex, suggesting that SLA class I molecules are mainly expressed on mature thymocytes in the medulla, but not on immature thymocytes in the cortex. In spleen, lymph node, kidney and liver tissues, 4G8 produced an ubiquitous staining pattern, as shown in Figure 4. These results demonstrate that 4G8 can be effectively used to immunostain frozen sections. Therefore, 4G8 may be a useful reagent for immunopathology studies and improving our general understanding of the porcine immune system.

In conclusion, a novel MAb, 4G8, that recognizes the SLA class I α chain has been identified and used to produce high-quality immunostaining results on tissues sections. 4G8 is expected to become a useful tool for investigating the immune system of domestic pigs.

ACKNOWLEDGMENTS

We thank M. Sone and S. Yamauchi for their excellent secretarial work. This research was supported in part by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan and MEXT (KAKENHI 15019129, JSPS. KAKENHI 15390133 and 15590361). Support was also provided by a grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation and a grant from the Sankyo Foundation of Life Science.

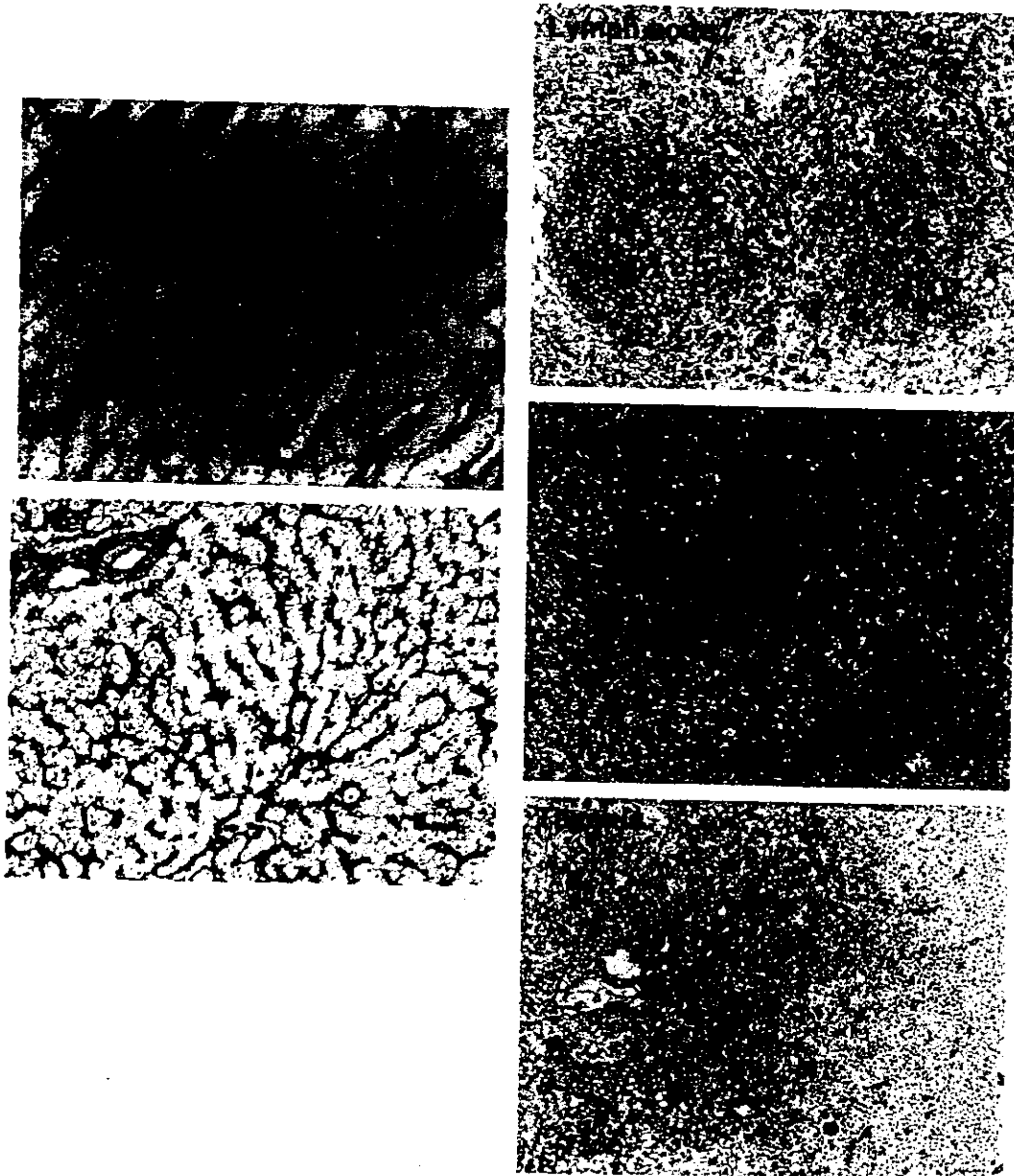

FIG. 4. Reactivity of 4G8 on frozen sections of porcine tissues. Frozen sections of porcine thymus, spleen, lymph node, kidney, and liver tissues were stained with 4G8 using immunohistochemistry.

REFERENCES

1. DerSimonian H, Pan L, Yatko C, Rodrigue-Way A, Johnson E, and Edge AS: Human anti-porcine T cell response: blocking with anti-class I antibody leads to hyporesponsiveness and a switch in cytokine production. J Immunol 1999;162:6993–7001.
2. Fujimoto J, Ishimoto K, Kiyokawa N, Tanaka S, Ishii E, and Hata J: Immunocytological and immunochemical analysis on the common acute lymphoblastic leukemia antigen (CALLA): evidence that CALLA on ALL cells and granulocytes are structurally related. Hybridoma 1988;7:227–236.
3. Maruyama K, and Sugano S: Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. Gene 1994;138:171–174.
4. Sullivan JA, Oettinger HF, Sachs DH, and Edge AS: Analysis of polymorphism in porcine MHC class I genes: alterations in signals recognized by human cytotoxic lymphocytes. J Immunol 1997;159:2318–2326.

Address reprint requests to:
Junichiro Fujimoto, Ph.D.
Department of Developmental Biology
National Research Institute for Child Health and Development
3-35-31, Taishido, Setagaya-ku
Tokyo 154-8567, Japan

E-mail: jfujimoto@nch.go.jp

Received for publication February 27, 2004. Accepted for publication March 24, 2004.

Immunology 2004 **112** 575–582 doi:10.1111/j.1365-2567.2004.01918.x

Deficiency of BLNK hampers PLC-γ2 phosphorylation and Ca^{2+} influx induced by the pre-B-cell receptor in human pre-B cells

TOMOKO TAGUCHI,*† NOBUTAKA KIYOKAWA,* HISAMI TAKENOUCH,* JUN MATSUI,* WEI-RAN TANG,* HIDEKI NAKAJIMA,* KYOKO SUZUKI,* YUSUKE SHIOZAWA,* MASAHIRO SAITO,* YOHKO U. KATAGIRI,* TAKAO TAKAHASHI,† HAJIME KARASUYAMA,‡ YOSHINOBU MATSUO,§ HAJIME OKITA,* & JUNICHIRO FUJIMOTO* *Department of Developmental Biology, National Research Institute for Child Health and Development, Setagaya-ku, Tokyo, †Department of Pediatrics, Keio University, School of Medicine, Shinjuku-ku, Tokyo, ‡Department of Immune Regulation, Tokyo Medical and Dental University, Graduate School, Tokyo, and §Fujisaki Cell Center, Hayashibara Biochemical Laboratories Inc, Fujisaki, Okayama, Japan

SUMMARY

B-cell linker protein (BLNK) is a component of the B-cell receptor (BCR) as well as of the pre-BCR signalling pathway, and $BLNK^{-/-}$ mice have a block in B lymphopoiesis at the pro-B/pre-B cell stage. A recent report described the complete loss or drastic reduction of BLNK expression in approximately 50% of human childhood pre-B acute lymphoblastic leukaemias (ALL), therefore we investigated BLNK expression in human pre-B ALL cell lines. One of the four cell lines tested, HPB-NULL cells, was found to lack BLNK expression, and we used these human pre-B ALL cell lines that express and do not express BLNK to investigate the intracellular signalling events following pre-BCR cross-linking. When pre-BCR was cross-linked with anti-μ heavy-chain antibodies, significant phosphorylation of intracellular molecules, including Syk, Shc, ERK MAP kinase, and AKT, and an activation of Ras were observed without regard to deficiency of BLNK expression, suggesting that BLNK is not required for pre-BCR-mediated activation of MAP kinase and phosphatidyl-inositol 3 (PI3) kinase signalling. By contrast, phospholipase C-γ2 (PLC-γ2) phosphorylation and an increase in intracellular Ca^{2+} level mediated by pre-BCR cross-linking were observed only in the BLNK-expressing cells, indicating that BLNK is essential for PLC-γ2-induced Ca^{2+} influx. Human pre-B cell lines expressing and not expressing BLNK should provide an *in vitro* model for investigation of the role of BLNK in the pre-BCR-mediated signalling mechanism.

Keywords B-cell receptor; B cells; signalling/signal transduction

INTRODUCTION

Signals transduced through antigen receptors play essential roles in B-cell development and fate determination. The B-cell antigen receptor (BCR), which consists of a μ heavy chain (HC), conventional light chain (LC), immunoglobulin α (Igα; CD79a), and Igβ (CD79b), mediates different biological responses in B cells, i.e. proliferation, differentiation, growth arrest, or induction of apoptosis, depending on the differentiation and activation stage of the B cell.[1–3]

In contrast to mature B cells, B-cell progenitors do not possess the complete forms of the BCR, but do express BCR-related components. For example, pro-B cells express the Igα/Igβ heterodimer in association with calnexin,[4] an integral membrane protein, and the surrogate light (SL) chain encoded by the VpreB (CD179a) and λ5 (CD179b) genes.[5,6] These molecules have been found to be competent for transducing differentiation signals for pro-B cells.[4]

In addition, pre-B cells that have successfully accomplished rearrangement of the HC genes start to express a premature form of the antigen receptor, i.e. a pre-B-cell

Received 15 December 2004; revised 21 April 2004; accepted 10 May 2004.

Correspondence: Dr Nobutaka Kiyokawa, Department of Developmental Biology, National Research Institute for Child Health and Development, 3-35-31, Taishido, Setagaya-ku, Tokyo 154–8567, Japan. E-mail: nkiyokawa@nch.go.jp

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receptor (pre-BCR) consisting of μ HC, SL chains and the Igα/Igβ heterodimers.[7-9] Several studies have shown the vital importance of pre-BCR as a mediator of pre-B-cell differentiation signals.[10-12] Expression of pre-BCR on the cell surface suppresses further recombination of μ HC genes and induces rearrangement of the conventional LC genes, indicating that signals through pre-BCR facilitate the proliferation of successfully developed pre-B cells.

Although the μ HC does not have any enzymatic activity in its cytoplasmic domain to transduce intracellular signals, the regulatory cascade of molecules is involved in BCR-mediated signalling.[2,3] The stimuli conveyed by antigens through BCR activate a number of BCR-associated cytoplasmic protein tyrosine kinases (PTKs), including the Src-family PTKs, Syk and Brutons tyrosine kinase (BTK).[13,14] These PTKs then phosphorylate numerous intracellular proteins and couple BCR stimulation to intracellular signallings, such as phosphoinositide hydrolysis, protein kinase C activation, and activation of Ras-mitogen-activated protein (MAP) kinase pathways.[2,3] A similar molecular cascade for signal transduction has been postulated for pre-BCR signalling.[4,15]

B-cell linker protein (BLNK), also known as SLP-65, BASH and BCA, is a B-cell adaptor molecule that links the cytoplasmic PTKs with phosphorylation of downstream effector molecules[16-18] and plays a crucial role in the BCR signalling system. Since BLNK does not encode any intrinsic enzymatic activity, its function is to serve as a scaffold for assembling molecular complexes that include enzymes and additional linker proteins. Upon BCR stimulation, BLNK couples activated Syk to phospholipase C-γ (PLC-γ), Vav, Grb2 and NCK.[19] In addition, it binds Btk[20,21] and is required for activation of the transcription factor NF-κB.[22] It has been reported consistently that B cells lacking BLNK fail to elicit Ca^{2+} influx following BCR cross-linking and exhibit attenuated activation of all three families of MAP kinases.[19]

BLNK has also been shown to play important roles in pre-BCR signalling, and BLNK-deficient mice show a partial block at the pre-B cell stage characterized by impaired developmental progression from large cycling CD43^{+} pro-B cells into small resting CD43^{-} pre-B cells,[23-26] suggesting an essential role of BLNK in pre-BCR signalling that mediates the growth and differentiation of B-cell precursors.

More importantly, it has been reported that some BLNK-deficient mice spontaneously develop pre-B-cell lymphomas that express large amounts of pre-BCR on their surface.[27,28] Consistent with this, approximately 50% of human childhood pre-B acute lymphoblastic leukaemias (ALL) show complete loss or drastic reduction of BLNK expression.[29] These findings indicate that BLNK functions as a tumour suppressor and that loss of BLNK and the accompanying block in pre-B-cell differentiation is one of the primary causes of pre-B ALL, although the precise mechanism is unknown.

We employed human pre-B cell lines that express and do not express BLNK and examined the intracellular signalling events following pre-BCR cross-linking in an attempt to investigate the role of BLNK in pre-BCR-mediated signalling. In this paper, we report the absence of Ca^{2+} influx following pre-BCR ligation in BLNK-negative human pre-B-cell lines, but not interference with pre-BCR-mediated phosphorylation of intracellular molecules. This suggests that BLNK is essential to Ca^{2+} signalling in human pre-B cells but not to other signalling cascades and it should provide an *in vitro* model for studying the role of BLNK in pre-BCR-mediated signalling.

MATERIALS AND METHODS

Cells and reagents

The human pre-B cell lines, NALM-17, HPB-NULL, P30/OHK[30] and NALM-6[31] were used in this study. The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum at 37° in a humidified 5% CO_2 atmosphere. The mouse monoclonal antibodies (mAbs) used were; anti-μ (G20-127), anti-κ (G20-193), and anti-λ (JDC-12) from Pharmingen (San Diego, CA); anti-BLNK (2B11), anti-Syk (4D10), and anti PLC-γ2 (B-10), from Santa Cruz Biotechnology (Santa Cruz, CA); anti-extracellular signal-regulated kinase (ERK)-1 (MK12) from Transduction Laboratories (Lexington, KY); anti-phosphotyrosine (PY) (4G10) from Upstate Biotechnology Inc. (Lake Placid, NY); anti-μ (AF6) from Beckman/Coulter Inc. (Westbrook, MA); anti-β actin (ZSA1) from Seikagaku Co. (Tokyo, Japan); and anti-μ (DA4.4) from the American Type Culture Collection (Rockville, MD). Anti-λ5 (HSL11), anti-Vpre-B (HSL96) and anti-conformational pre-BCR (HSL2) were also used.[30] As the negative control for flow cytometric analysis, isotype-matched mouse immunoglobulins, IgG1 (KOPC-31C) and IgG2a (G155-178), from Pharmingen were used. The rabbit polyclonal antibodies used were; $F(ab')_2$ fragment of anti-μ HC from Jackson Laboratory, Inc. (West Grove, PA); anti-PLC-γ1, anti-phospho-ERK, anti-phospho-MAP kinase/ERK kinase (MEK), anti-phospho-PLC-γ1, anti-phospho-PLC-γ2 and anti-phospho-AKT from New England Biolabs, Inc. (Beverly, MA); anti-PLC-γ2 from Pharmingen; and anti-Shc from Transduction Laboratories. The goat polyclonal anti-BTK antibody from Santa Cruz Biotechnology was also used. Secondary antibodies, including fluorescein-conjugated and enzyme-conjugated antibodies, were purchased from Jackson.

Immunofluorescence study

The cells were stained with mAbs and analysed by flow cytometry (EPICS-XL, Coulter) as described previously.[32] Staining of cytoplasmic antigens was performed with CytoStain™ Kits (Pharmingen) according to the manufacturer's protocol.

Immunoblotting and immunoprecipitation

Immunoblotting was performed as described previously.[33] Briefly, cell lysates were prepared by solubilizing the cells in lysis buffer (containing 20 mM Na_2PO_4, pH 7·4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 5 mM phenylmethylsulphonyl fluoride, 100 mM NaF and 2 mM Na_3VO_4). After centrifugation, supernatants were obtained and the protein concentration of each cell lysate was

determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of each cell lysate were electrophoretically separated on sodium dodecyl sulphate–polyacrylamide gel and transferred onto a nitrocellulose membrane using a semi-dry transblot system (Bio-Rad). After blocking, the membranes were incubated with the appropriate combination of primary and secondary antibodies as indicated, washed intensively, then examined using the enhanced chemiluminescence reagent system (ECL, Amersham Life Science, Buckinghamshire, UK). The results obtained from a 1-min exposure of the ECL-treated membrane to film are presented.

For the immunoprecipitation, 500 μg of the cell lysates was incubated with 1 μg of antibody and 50 μl of 50% protein-G agarose (Boehringer Mannheim Biochemica, Mannheim, Germany) for 1 hr. After intensive washing, the immunoprecipitates were separated by electrophoresis and analysed as described above.

To measure Ras activation, EZ-Detect™ Ras Activation Kits from PIERCE Biotechnology (Rockford, IL) were used according to the manufacturer's protocol.

Ca^{2+} mobilization assay

Intracellular levels of Ca^{2+} were measured by flow cytometry using Fluo 3-AM (Dojin, Kumamoto, Japan) after pre-BCR cross-linking with anti-μ antibodies. Ten million cells were washed and resuspended in 1 ml of OPTI-MEM containing 0·5% bovine serum albumin, and incubated with 1 mM of Fluo 3-AM for 30 min at 37°. After washing, the cells were resuspended in 10 ml of medium, stimulated by adding different concentrations of rabbit anti-human μ HC antibody as described in Figure 4 and the intracellular calcium concentration was measured by flow cytometry as described previously.[14] Calcium ionophore (ionomycin, Sigma-Aldrich Fine Chemicals, St Louis, MO) was used as the positive control. The data obtained were analysed using WINMDI software (distributed by Dr Joe Trotter) and presented as a kinetics line.

RESULTS

Expression of cell surface pre-BCR and BLNK in human pre-B-cell lines

It has been reported that some pre-B-cell lines express pre-BCR on their cell surface, even if not abundantly.[35] To identify cell lines that express pre-BCR on their cell surface, we tested a series of human pre-B-cell lines for surface expression of μ HC. Flow cytometry showed that all four cell lines tested, i.e. lines NALM-6, HPB-NULL, NALM-17, and P30/OHK, expressed μ HC on their cell surface, and that two of them, NALM-17 and HPB-NULL cells, expressed μ HC on their cell surface more abundantly than the others (Fig. 1a). To investigate the cell surface expression of pre-BCR, we examined reactivity to mAb against SL chains. The mAbs HSL11, HSL96 and HSL2 specifically recognize λ5, VpreB and the conformational epitope of pre-BCR, respectively.[30] When evaluated by flow cytometry, the cell lines stained positive with all three mAbs (Fig. 1a), but not with anti-κ and -λ mAb (data not shown), indicating that the pre-BCR is indeed expressed on the cell surface of some pre-B cell lines. It is noteworthy that although NALM-6 cells clearly reacted with both HSL11 (λ5) and HSL96 (VpreB), they revealed much weaker reactivity with HSL2 (conformational epitope of pre-BCR), for which the precise reason is unknown (Fig. 1a).

Since complete loss or drastic reduction of BLNK expression has also been reported in approximately 50% of childhood precursor-B ALL cases[29] we tested pre-B cell lines for expression of BLNK. Immunoblotting revealed abundant expression of BLNK by NALM-17 cells, whereas no BLNK expression was detectable in HPB-NULL cells (Fig. 1b). The NALM-6 and P30/OHK cells showed an intermediate amount of BLNK expression (Fig. 1b). We also investigated HPB-NULL and NALM-17 cells for intracellular BLNK expression by flow cytometry. When membrane-permeabilized cells were stained with fluorescein-labelled anti-BLNK mAb, clear expression of BLNK was observed only in NALM-17 cells (Fig. 1c), consistent with the results of immunoblotting. In contrast, when tested for expression of other B-cell-related signalling molecules, i.e. BTK, Syk, PLC-γ1 and PLC-γ2, by immunoblotting, all four molecules were comparably expressed in all four pre-B cell lines (Fig. 1b). Based on these findings, we decided to use NALM-17 and HPB-NULL cells in the following experiments as *in vitro* models of BLNK-positive and negative pre-B cells, respectively.

Phosphorylation of intracellular proteins induced by pre-BCR cross-linkage in both BLNK-positive and -negative pre-B cells

Next, we investigated whether cross-linking of pre-BCR with anti-μ antibodies would induce tyrosine phosphorylation of intracellular proteins in pre-B cell lines. Evaluation by immunoblotting with the anti-PY mAb, DA4.4 directed against human μ HC was found to cross-link pre-BCR strongly enough to induce tyrosine phosphorylation of intracellular proteins in HPB-NULL cells (Fig. 2a), but isotype-matched control mouse immunoglobulin did not (data not shown). Phosphorylation of the tyrosine residues peaked at 1–5 min after pre-BCR cross-linking and then decreased to a resting level by 30 min (Fig. 2a). Testing of other antibodies specifically reacting with μ HC, including the other clone of anti-μ HC mAb (AF6 and G20-127) and $F(ab')_2$ fragment of rabbit polyclonal anti-μ HC, yielded identical results (data not shown). Based on the results obtained under various conditions, incubation with 10 μg/ml of anti-μ mAb DA4.4 for 5 min was considered the optimal condition for pre-BCR cross-linking.

Next, immunoprecipitation and immunoblotting were used to identify signal transduction molecules located downstream in the pre-BCR signalling cascade in HPB-NULL cells. When Syk PTK (Fig. 2b) and Shc adapter molecules (Fig. 2c) were immunoprecipitated with specific antibodies, a pre-BCR-mediated increase in tyrosine phosphorylation was observed on anti-PY immunoblotting, and use of phospho-specific antibodies revealed that ERK MAP

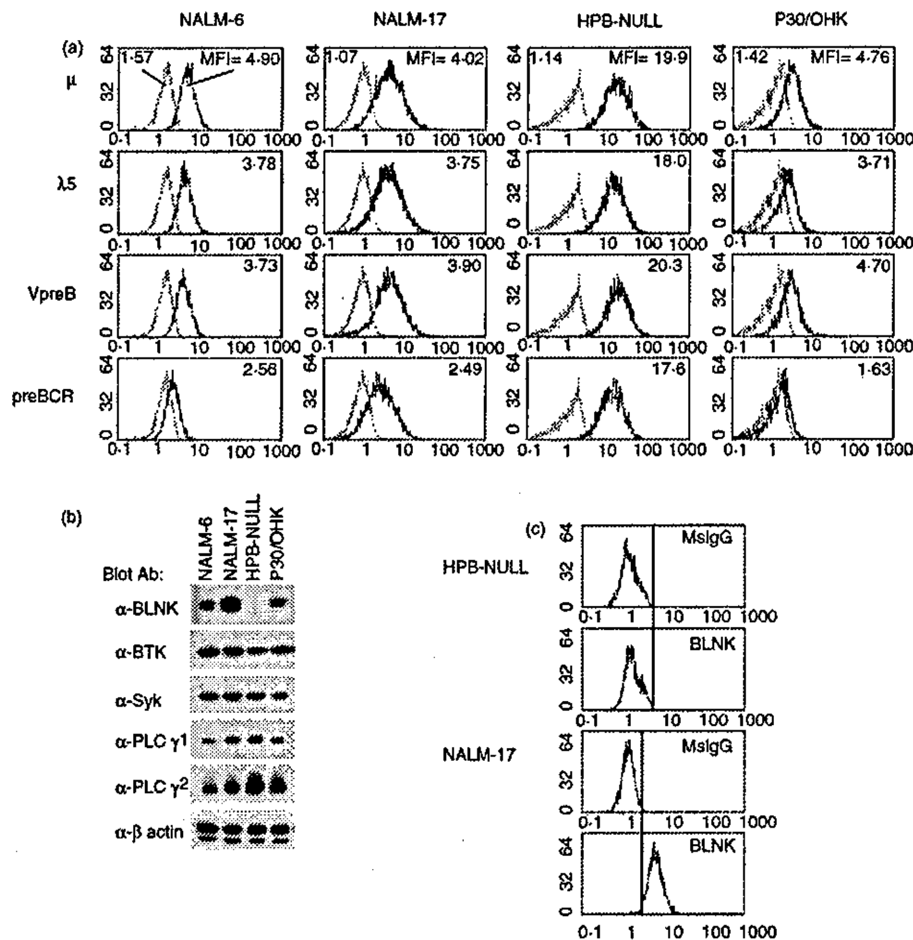

Figure 1. Expression of pre-BCR and BLNK in human pre-B-cell lines. (a) The pre-BCR-related molecules, i.e. μ heavy chain, $\lambda 5$, VpreB and conformational pre-BCR (preBCR), expressed on the cell surface of human pre-B cell lines, NALM-6, NALM-17, HPB-NULL, and P30/OHK, were stained with the specific monoclonal antibodies indicated and analysed by flow cytometry as described in the Materials and methods. The histograms obtained (solid lines) have been superimposed on those of the negative control (cells stained with isotype-matched control mouse immunoglobulin, IgG1 KOPC-31C, light broken lines). The mean fluorescence intensity (MFI) for each staining is presented in the top right hand corner of each panel. The MFI for each negative control staining is presented in the top left hand corner of the top panel of each cell line. The x-axis represents fluorescence intensity; the y-axis represents relative cell number. (b) Human pre-B-cell lines were tested for expression of BLNK and signal-transduction-related molecules by immunoblotting analysis with the specific antibodies indicated. For PLC-γ2 blotting, anti-PLC-γ2 monoclonal antibody (B-10) was used. (c) Human pre-B cell lines were tested for expression of BLNK by flow cytometry. Cells were permeabilized, stained with a combination of anti-BLNK monoclonal antibody and fluorescein isothiocyanate-conjugated secondary goat anti-mouse IgG, and analysed as described in the Materials and methods. As a negative control, cells were also stained with isotype-matched control mouse immunoglobulin (IgG2a G155-178, MsIgG).

kinase and MEK kinase were phosphorylated immediately after exposure to the anti-μ HC mAb DA4.4 (Fig. 2d). The time course of the phosphorylation state of MEK and MAP kinase showed kinetics similar to those of intracellular proteins detected with anti-PY mAb (Fig. 2a,d), and clear phosphorylation of PLC-γ1 and AKT was also detected in HPB-NULL cells after pre-BCR cross-linking (Fig. 3a). We also examined the activation of Ras, an upstream signalling molecule of ERK MAP kinase. As shown in Fig. 3(b), we observed an activation of Ras in HPB-NULL cells after pre-BCR cross-linking.

Similar testing of BLNK expressing NALM-17 cells revealed that pre-BCR cross-linking induced phosphorylation of these intracellular molecules as well as Ras activation that was as immediate and clear as we observed in HPB-NULL cells (Figs 3a,b). However, it is noteworthy that examination of phosphorylation of PLC-γ2 in the same manner showed that pre-BCR cross-linking induced phosphorylation of PLC-γ2 only in NALM-17 cells, and not in HPB-NULL cells (Fig. 3a).

A major mechanism by which BLNK has been proposed to regulate PLC-γ2 is through the juxtaposition of BTK and

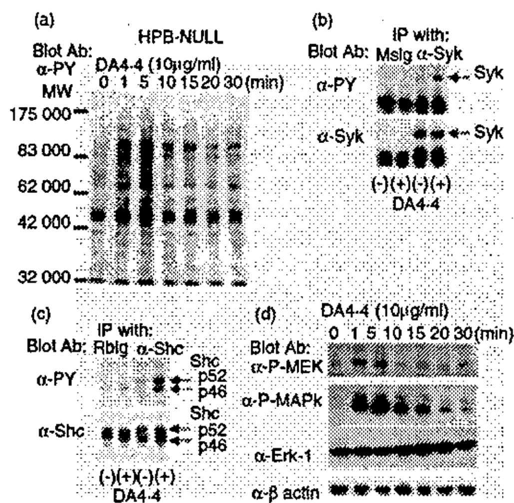

Figure 2. Increase in phosphorylation of intracellular proteins in HPB-NULL cells after exposure to anti-μ antibodies. (a) Immunoblot analysis with anti-phosphotyrosine (α-PY) monoclonal antibody was performed on cell lysates prepared from HPB-NULL pre-B cells exposed to 10 μg/ml of anti-μ mAb DA4.4 for the periods indicated. A molecular weight standard is indicated on the left. (b) Proteins immunoprecipitated with either isotype-matched control mouse immunoglobulin (MsIg, lanes 1 and 2) or mouse monoclonal antibody against Syk (α-Syk, lanes 3 and 4) from HPB-NULL lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis in duplicate. After transfer onto a nitrocellulose membrane, the samples were analysed by immunoblotting with either anti-phosphotyrosine (α-PY) monoclonal antibody (upper panel) or anti-Syk monoclonal antibody (lower panel). Corresponding bands for Syk are indicated by arrows. (c) An experiment similar to that in (b) was performed using a combination of HPB-NULL lysates and rabbit polyclonal anti-Shc antibody (α-Shc). (d) The cell lysates prepared from HPB-NULL as in (a) were also tested by immunoblotting with antibodies indicated.

PLC-γ2.[3,20,21,28] Therefore we examined the complex formation between BTK and PLC-γ2 in both cell lines. As shown in Fig. 3(c), immunoprecipitation revealed that a portion of BTK was detected in anti-PLC-γ2 immunoprecipitates from the lysates prepared from both NALM-17 and HPB-NULL cells without pre-BCR cross-linking. Interestingly, the total amount of BTK protein precipitated with anti PLC-γ2 antibody was increased after pre-BCR cross-linking in NALM-17 cells, while pre-BCR cross-linking did not affect the amount of BTK protein precipitated with anti PLC-γ2 antibody in HPB-NULL cells (Fig. 3c).

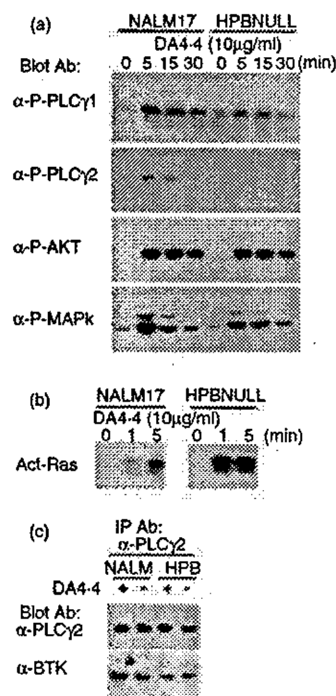

Figure 3. Pre-BCR-mediated signalling in pre-B cell lines. (a) Immunoblot analysis using phospho-specific antibodies on NALM-17 and HPB-NULL cells after exposure to anti-μ monoclonal antibody DA4.4. Cell lysates prepared from NALM-17 and HPB-NULL cells as in Fig. 2(a) were also tested by immunoblotting with the anti-phospho-specific antibodies indicated. (b) NALM-17 and HPB-NULL cells were exposed to DA4.4 and cell lysates were prepared as in (a). Active form of Ras (Ras-GTP) proteins were captured with Raf1-immobilized resin and detected by immunoblotting using anti-Ras antibody. (c) Cell lysates were prepared from NALM-17 and HPB-NULL cells treated with (+) or without (–) DA4.4 (10 μg/ml) for 5 min as in (a). Proteins were immunoprecipitated with rabbit anti-PLC-γ2 antibody from the cell lysates and were separated by SDS–PAGE in duplicate. Immunoblotting was performed with either mouse anti-PLC-γ2 monoclonal antibody (B-10, α-PLC-γ2, upper panel) or goat anti-BTK antibody (lower panel).

Ca^{2+} influx does not occur in BLNK-negative cell lines after cross-linking of pre-BCR

Next, we investigated whether the cross-linkage of pre-BCR leads to an increase in intracellular Ca^{2+} level. Measurement of the intracellular Ca^{2+} level of HPB-NULL cells by flow cytometry with Fluo 3-AM showed no significant increase in intracellular Ca^{2+} level after exposure to anti-μ antibodies, while treatment with calcium ionophore led to a clear increase in the intracellular Ca^{2+} level (Fig. 4). By contrast, however, when NALM-17 cells, which express BLNK were examined, an increase in intracellular Ca^{2+} level after pre-BCR cross-linkage was observed under identical experimental conditions (Fig. 4). We therefore concluded that the cross-linking of pre-BCR failed to increase intracellular Ca^{2+} in pre-B cells that lack BLNK expression.

DISCUSSION

Several different groups have reported that 5–10% of BLNK-deficient mice spontaneously develop pre-B cell leukaemia/lymphomas expressing large amounts of pre-BCR on their surface.[27,28] Injection of immunodeficient mice with a $BLNK^{-/-}$ pre-B-cell line has been found to result in the development of pre-B-cell leukaemia that was

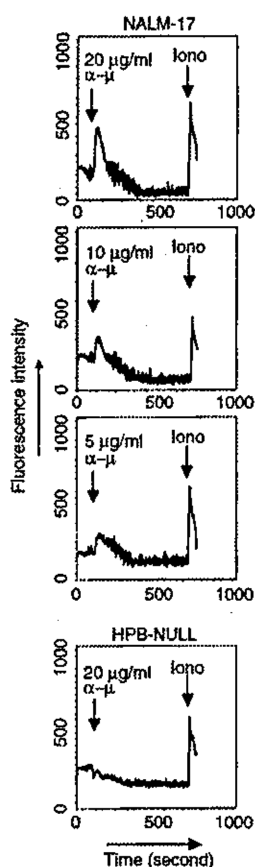

Figure 4. Flow cytometric analysis of Ca^{2+} mobilization in human B-cell lines. NALM-17 or HPB-NULL cells were loaded with Fluo 3-AM as described in the Materials and methods, and the intracellular Ca^{2+} levels were measured by flow cytometry. Rabbit anti-μ monoclonal antibody (α-μ, 5, 10, and 20 μg/ml) or the calcium ionophore ionomycin (Iono, 0·1 μg/ml) was added to the cells at the times indicated by the arrows. Other anti-μ antibodies were also used, and identical results were obtained (data not shown).

prevented by reconstitution of BLNK expression in $BLNK^{-/-}$ pre-B-cell line.[29] More important, it is also reported that 16 of 34 human childhood precursor-B ALL cases showed complete loss or drastic reduction of BLNK expression.[29] Thus, it was hypothesized that BLNK acts as a tumour suppressor and that somatic loss of BLNK and the accompanying block of pre-B-cell differentiation is one of the primary causes of childhood ALL.[27,28] Consistent with the above observations, the results of the present study demonstrated the existence of a human pre-B ALL cell line that lacks BLNK expression, and our findings indicate that the BLNK-deficiency phenotype in human precursor-B ALL cells is maintained in the cell lines established from these ALL cells.

The function of BLNK in pre-B cells is still a matter of controversy. Since, as stated above, reconstitution of BLNK expression in the $BLNK^{-/-}$ pre-B-cell line prevented the development of the leukaemia in immunodeficient mice, Jumaa *et al.* suggested that BLNK is essential to limiting pre-B-cell proliferation.[27,29] By contrast, Hayashi *et al.* observed that the pre-B cells that accumulate in BLNK-deficient mice are mostly non-cycling large pre-B cells, and they therefore concluded that BLNK is critical to pre-BCR signalling that induces proliferation of large pre-B cells.[28] Although the function of BLNK in pre-B ALL cell lines has not yet been clarified, the variable expression levels of BLNK in the pre-B ALL cell lines that we described in this study may mean that the level of expression of BLNK no longer critically affects growth and survival in established human pre-B ALL cell lines. In fact, we did not observe any significant difference in growth rate between the human pre-B ALL cell lines regardless of their BLNK expression level (data not shown).

In the present study we examined the downstream events mediated by cross-linking of pre-BCR in both BLNK-positive and -negative human pre-B-cell lines. The analysis with HPB-NULL cells that lack BLNK clearly indicated that the cross-linking of pre-BCR induces activation of Syk, Shc, Ras, ERK MAP kinase, and AKT, suggesting that the pre-BCR-mediated signalling in the MAP kinase pathway and the phosphatidyl-inositol 3 (PI3) kinase-AKT pathway do not require BLNK. In contrast, cross-linking of pre-BCR induced phosphorylation of PLC-γ2 and an increase in the intracellular Ca^{2+} level in NALM-17 cells alone, and not in HPB-NULL cells, suggesting that BLNK is essential to the pre-BCR-mediated Ca^{2+} influx via PLC-γ2 activation.

Kuwahara *et al.* analysed the downstream events of pre-BCR signalling in the human pre-B cell lines NALM-6 and 796 cells and reported a significant difference in comparison with the events mediated by the conventional BCR expressed on mature B cells, namely, less tyrosine phosphorylation of the cytoplasmic proteins, including Syk, and failure of Ca^{2+} mobilization.[35] However, the fact that the pre-B lines that they studied express only a small amount of pre-BCR on their cell surface may have been a limitation. Thus, their failure to detect signals following pre-BCR cross-linkage may have been because the signals were below the threshold of the detection system.[36] In fact, Nakamura *et al.* analysed the downstream signalling events after pre-BCR stimulation in μκ-transfected NALM-6 cells, which express larger amounts of the reconstituted BCR, and observed that cross-linkage of BCR on pre-B cells caused an elevation in intracellular Ca^{2+} that was qualitatively indistinguishable from the elevation following cross-linkage of BCR on mature B cells.[36]

In addition, we observed that NALM-6 cells clearly reacted with both anti-λ5 and anti-VpreB antibodies, while they revealed much weaker reactivity with antibody against conformational epitope of pre-BCR (Fig. 1a). Although the precise reason for the discrepancy is unknown, our finding may be related to the failure of Ca^{2+} mobilization by pre-BCR cross-linking in NALM-6 cells.

By contrast, our findings also indicated that cross-linkage of pre-BCR expressing on pre-B NALM-17 cells induces hyperphosphorylation of the tyrosine residues in numerous intracellular proteins as well as elevation of the intracellular Ca^{2+} level. Therefore, NALM-17 cells should

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