Ⅲ. 資　　料

4）慢性肉芽腫症に対する遺伝子治療の実績一覧

慢性肉芽腫症に対する遺伝子治療の実績一覧

2008年12月現在

症例	年齢	病型	実施国	実施時期	導入細胞	ベクター	前処置	効果	経過
1	26	gp91	ドイツ	2004	PBSC	SF71gp91phox	BU 8mg/kg	あり	死亡 (Mono7)
2	25	gp91	ドイツ	2003	PBSC	SF71gp91phox	BU 8mg/kg	あり	移植 (MDS)
3	5	gp91	スイス	2003	PBSC	SF71gp91phox	BU 8mg/kg	あり	生 (1.0%)
4	8	gp91	スイス	2008	PBSC	SF71gp91phox	BU 8mg/kg	あり	生 (90%)
5	12	gp91	イギリス	2001	PBSC	MFGS-gp91phox	Mel 140mg/kg	あり	生 (<0.1)
6	27	gp91	イギリス	2005	BMSC	SF71gp91phox	Mel 140mg/kg	なし	生 (<0.1)
7	6	gp91	イギリス	2006	PBSC	SF71gp91phox	Mel 140mg/kg	なし	生 (<0.1)
8	9	gp91	イギリス	2007	PBSC	SF71gp91phox	Mel 140mg/kg	あり	生 (<0.1)
9	16	gp91	韓国	2006	PBSC	MT-gp91	BU 8mg/kg	-	生 (<0.1)
10	9	gp91	韓国	2006	PBSC	MT-gp91	BU 8mg/kg	-	生 (<0.1)
11-15	37F 21M 18F 27M 27F	p47	米国	1995～	PBSC	MFGS-p47phox	なし	なし	生
16	20	gp91	米国	1998～2000	PBSC	MFGS-gp91phox	なし	なし	生
17-20	未発表	gp91	米国	1998～2000	PBSC	MFGS-gp91phox	なし	なし	生
21	28	gp91	米国	2006～	PBSC	MFGS-gp91phox	BU 10mg/kg	あり	生 (<1)
22	31	gp91	米国	2006～	PBSC	MFGS-gp91phox	BU 10mg/kg	なし	死亡(感染症)
23	37	gp91	米国	2008～	PBSC	MFGS-gp91phox	BU 10mg/kg	あり	生

・PBSC：G-CSF誘導末梢血由来CD34陽性細胞、BMSC：骨髄由来CD34陽性細胞
・SF71pg91phox： spleen focus forming virus由来レトロウイルスベクター
・MFGSgp91phox、MT-gp91： MoMLV由来レトロウイルスベクター
・BU：ブスルファン、Mel： メルファラン

Ⅳ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅴ．　研究成果の印刷物・別刷

Immunology
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IMMUNOLOGY ORIGINAL ARTICLE

B-cell-activating factor inhibits CD20-mediated and B-cell receptor-mediated apoptosis in human B cells

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Summary

B-cell-activating factor (BAFF) is a survival and maturation factor for B cells belonging to the tumour necrosis factor superfamily. Among three identified functional receptors, the BAFF receptor (BAFF-R) is thought to be responsible for the effect of BAFF on B cells though details of how remain unclear. We determined that a hairy-cell leukaemia line, MLMA, expressed a relatively high level of BAFF-R and was susceptible to apoptosis mediated by either CD20 or B-cell antigen receptor (BCR). Using MLMA cells as an *in vitro* model of mature B cells, we found that treatment with BAFF could inhibit apoptosis mediated by both CD20 and BCR. We also observed, using immunoblot analysis and microarray analysis, that BAFF treatment induced activation of nuclear factor-κB2 following elevation of the expression level of *Bcl-2*, which may be involved in the molecular mechanism of BAFF-mediated inhibition of apoptosis. Interestingly, BAFF treatment was also found to induce the expression of a series of genes, such as that for CD40, related to cell survival, suggesting the involvement of a multiple mechanism in the BAFF-mediated anti-apoptotic effect. MLMA cells should provide a model for investigating the molecular basis of the effect of BAFF on B cells *in vitro* and will help to elucidate how B cells survive in the immune system in which BAFF-mediated signalling is involved.

Keywords: apoptosis; B-cell-activating factor; Bcl-2; B-cell receptor; CD20

Introduction

The immune system comprises a variety of immune effector cells, including T and B lymphocytes and antigen-presenting cells, such as dendritic cells and others; it protects individuals from infections and cancer. To maintain these sophisticated mechanisms, a very subtle balance between the life and death of the immune effector cells must be maintained to eliminate, by apoptosis, potentially harmful self-reactive lymphocytes and only allow the survival, development and activation of safe and protective immune cells. For this purpose, a number of molecules are involved in this regulatory system.[1]

B-cell-activating factor (BAFF, also termed BlyS, TALL-1, THANK and zTNF4) produced by monocytes, dendritic cells and some T cells is a member of the tumour necrosis factor (TNF) superfamily and is a type 2 transmembrane-bound protein that can also be expressed as a soluble ligand.[2] BAFF was first described as a factor that stimulates cell proliferation and the secretion of immunoglobulin in B cells.[3–7] Transgenic mice that overexpress BAFF in lymphoid tissues exhibited hyperplasia of the mature B-cell compartment.[8–10] In contrast, mice deficient in BAFF showed a deficit in peripheral B lymphocytes[10,11] and an almost complete loss of follicular and marginal zone B lymphocytes in secondary lymphoid organs. This suggests an absolute requirement for BAFF in normal B-cell development.[10] In contrast, a later examination of immunized BAFF-null mice validated the BAFF-independent nature of germinal centre formation and that antibody responses, including high-affinity responses, were attenuated, indicating that BAFF is required for maintenance, but not initiation, of the germinal centre reaction.[12] Based on the above evidence, BAFF is considered to be a survival and maturation factor for B lymphocytes and has emerged as a crucial factor that modulates B-cell tolerance and homeostasis.[2,13] However, the precise role of BAFF in B-cell development is

still controversial and it has been reported that the capacity of B lymphocytes to bind BAFF is correlated with their maturation state and that the effect of BAFF is dependent on the maturation stage of the B lymphocytes.[2-14]

Recent studies have further shown that BAFF affects not only B lymphocytes but also T lymphocytes.[15,16] The three distinct receptors for BAFF, namely the BAFF receptor (BAFF-R, also termed BR3), the B-cell maturation antigen (BCMA), and the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), have been identified and BAFF binds with a similar high affinity to these receptors.[7,17-23] Among these receptors, however, BAFF-R is thought to be responsible for the survival and differentiation of B cells,[24] whereas the molecular basis of BAFF-mediated signalling remains unclear.

A number of systems inducing apoptosis in B cells are present to eliminate inappropriate clones, such as self-acting B cells. For example, it is reported that stimulation via particular surface molecules, including B-cell receptor antigen (BCR) and CD20, induces apoptosis in cultured B cells.[25,26] The balance between apoptosis-inducing systems and survival systems, such as CD20 and BAFF-mediated signalling, would be important for the maintenance of appropriate B-cell development, though details are not known.

To elucidate the molecular basis of the interaction between apoptosis-inducing signals and BAFF-mediated cell survival signals in B cells, we have employed a B-cell line that expresses BAFF-R and is sensitive to CD20-mediated and BCR-mediated apoptosis. In this paper, we present evidence that BAFF-mediated stimulation inhibits the apoptosis induced by both CD20-mediated and BCR-mediated signalling. The possible mechanisms involved in BAFF-mediated cell responses that regulate these apoptotic stimuli are discussed.

Materials and methods

Cells and reagents

The human hairy cell leukaemia cell line MLMA was obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37° in a humidified 5% CO_2 atmosphere.

Recombinant human BAFF and a proliferation-inducing ligand (APRIL) were obtained from R&D Systems, Inc. (Minneapolis, MN), and used at a concentration of 400 ng/ml for cell stimulation unless otherwise described. The mouse monoclonal antibodies (mAbs) used for the immunofluorescence analysis were anti-CD10, anti-CD20, anti-CD21, anti-CD22, anti-CD24, anti-CD40, anti-human leucocyte antigen DR (HLA-DR; Beckman Coulter, Inc., Fullerton, CA); anti-CD19 (Becton Dickinson and Company, BD, Franklin Lakes, NJ); anti-κ, anti-λ, anti-μ, anti-δ, anti-γ (Dako, Denmark A/S); anti-BAFF-R (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-CD45 (American Type Culture Collection, ATCC, Manassas, VA). The rat mAbs against BCMA (Vicky-1) and TACI (1A1) were purchased from Santa Cruz Biotechnology. The mouse mAbs used for the immunochemical analysis were anti-caspase-2, anti-caspase-3 and anti-glycogen synthase kinase-3β (GSK-3β; Becton Dickinson); anti-caspase-9 (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan); anti-nuclear factor-κB (NF-κB) p52 (C-5), anti-Bcl-2 (100) from Santa Cruz; and anti-β-actin (AC-15) from Sigma-Aldrich Co. (St Louis, MO). The rabbit polyclonal antibodies used were anti-cleaved poly ADP-ribose polymerase (PARP), anti-cleaved caspase-3, anti-phospho-GSK-3β (Ser9) and anti-phospho-GSK-3α/β (Ser9, 21) from Cell Signaling Technology, Inc. (Danvers, MA). A goat anti-NF-κB p50 (C-19) from Santa Cruz was also used. Secondary antibodies, including fluorescein isothiocyanate- (FITC) and enzyme-conjugated antibodies, were purchased from either Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) or Dako. To cross-link BCR, purified anti-μ rabbit polyclonal antibody (10 μg/ml) from Jackson ImmunoResearch Laboratories, Inc. was used. To cross-link CD20, a mouse anti-CD20 mAb from Beckman Coulter and a secondary anti-mouse immunoglobulin antibody from Jackson ImmunoResearch Laboratories, Inc. were used each at a concentration of 5 μg/ml.

Immunofluorescence analysis and detection of apoptosis

Cells were stained with FITC-labelled mAbs and analysed by flow cytometry (EPICS-XL, Beckman Coulter) as described previously.[27] To quantify the incidence of apoptosis, cells were incubated with FITC-labelled annexin V using a MEBCYTO-Apoptosis kit (Medical & Biological Laboratories Co., Ltd) and then analysed by flow cytometry according to the manufacturer's directions. Apoptotic cells were also detected by nuclear-staining with DAPI and examined by confocal microscopy as described previously.[28] The enzymatic activity of caspases -2, -3, -9 was assessed by using a colorimetric protease assay kit for each caspase (Medical & Biological Laboratories Co., Ltd) according to the manufacturer's protocol.

Immunoblotting

Immunoblotting was performed as described previously.[29] 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, 1 mM phenylmethylsulphonylfluoride, 100 mM NaF, and 2 mM Na_3VO_4), and the total protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). For each cell lysate, 20 μg was separated by

sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using a semidry Transblot system (Bio-Rad). After blocking with 3% skimmed milk in phosphate-buffered saline, the membrane was incubated with the appropriate combination of primary and secondary antibodies as indicated, washed intensively, and examined using the enhanced chemiluminescence reagent system (ECL plus; GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

DNA microarray analysis

The DNA microarray analysis was performed using GeneChip (Affymetrix, Santa Clara, CA). Total RNA isolated from MLMA cells treated with and without BAFF for 12 hr was reverse transcribed and labelled using One-Cycle Target Labeling and Control Reagents as instructed by the manufacturer (Affymetrix). The labelled probes were hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix). The arrays were analysed using GeneChip Operating Software 1.2 (Affymetrix). Background subtraction and normalization were performed with GeneSpring GX 7.3 software (Agilent Technologies, Santa Clara, CA). Signal intensities were prenormalized based on the median of all measurements on that chip. To account for the difference in detection efficiency between the spots, prenormalized signal intensities on each gene were normalized to the median of prenormalized measurements for that gene. The data were filtered with the following steps. (1) Genes that were scored as absent in both samples were eliminated. (2) Genes with a signal intensity lower than 90 in both samples were eliminated. (3) Performing cluster analysis using filtering genes, genes were selected that exhibited increased expression or decreased expression in BAFF-treated cells.

Results

Immunophenotypic characterization of MLMA cells

While screening to identify human cell lines expressing BAFF-R, we found that MLMA cells expressed higher levels of BAFF-R than other human B-cell lines. Although the MLMA cell line is known to have been established from a patient with hairy-cell leukaemia, details were not reported. Therefore, we first examined the immunophenotypic characteristics of MLMA cells. Consistent with the JCRB records, flow cytometric analysis revealed that MLMA cells expressed high levels of μ heavy chain and low levels of δ heavy chain with expression of κ light chain (Fig. 1a). In addition to the CD19 and HLA-DR, MLMA cells were found to express mature B-cell

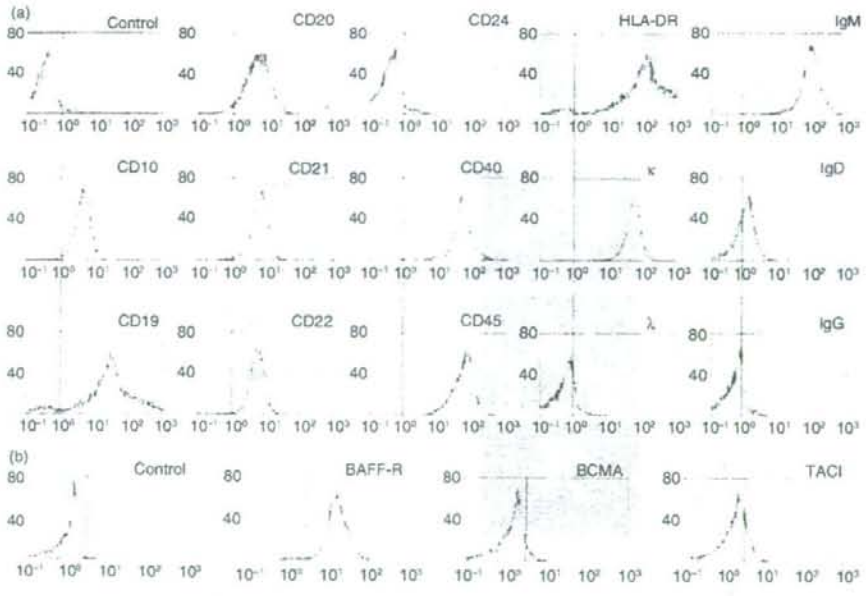

Figure 1. Immunophenotypic characterization of MLMA cells. (a) MLMA cells were stained with specific fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies (mAbs) against B-cell differentiation antigens and analysed by flow cytometry. The *x*-axis represents fluorescence intensity and the *y*-axis the relative cell number; control was isotype matched mouse immunoglobulin. (b) The expression of B-cell-activating factor receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and B-cell maturation antigen (BCMA) on MLMA cells was also examined as in (a).

antigens, including CD20, CD21, CD22 and CD40, but not CD24. Notably, MLMA cells showed the expression of CD10. When the expression of three types of receptors for BAFF was similarly examined, MLMA cells exhibited apparent expression of BAFF-R, while the levels of BCMA and TACI were found to be quite low (Fig. 1b). The data indicate that MLMA cells exhibit immunophenotypic characteristics of mature B cells expressing BAFF-R.

Cross-linking of BCR and CD20 induces apoptosis in MLMA cells

It has been well documented that cross-linking of BCR using anti-μ heavy chain antibodies induces apoptosis in some B cells *in vitro*.[25] Recent studies including our own have also shown that CD20 cross-linking mediates apoptosis in human B-cell lines in a manner involving raft-mediated signalling.[26,30] Therefore, we next examined whether cross-linking of either BCR or CD20 mediated apoptosis in MLMA cells. As shown in Fig. 2(a), when anti-μ antibodies were added to the culture, a time-dependent increase in the number of cells bound to annexin V was observed, suggesting the occurrence of apoptosis in MLMA cells after BCR cross-linking. The apoptosis was confirmed by the morphological appearance of nuclear fragmentation, a typical feature of apoptosis, detected by either Giemsa-staining or nuclear-staining with DAPI (Fig. 2b). Immunoblotting revealed the cleavage of caspases -9, -3 and -2 and of PARP after treatment with anti-μ antibodies (Fig. 2c), indicating that caspase activation was involved in the apoptosis. In the case of caspase-3, we also detected a 17 000 molecular weight cleaved fragment by using a specific antibody (Fig. 2c). In addition, elevation of the enzymatic activity of each caspase after cross-linking of BCR was detected by a colorimetric protease assay (Fig. 2d). We also examined the effect of anti-CD20 antibodies and found that CD20 cross-linking signalling induced apoptosis in MLMA cells (Fig. 2).

BAFF inhibits CD20-mediated and BCR-mediated apoptosis in MLMA cells

Next, we examined whether BAFF was able to inhibit apoptosis mediated by cross-linking of CD20 and BCR. As shown in Fig. 3(a), when BAFF was added to the

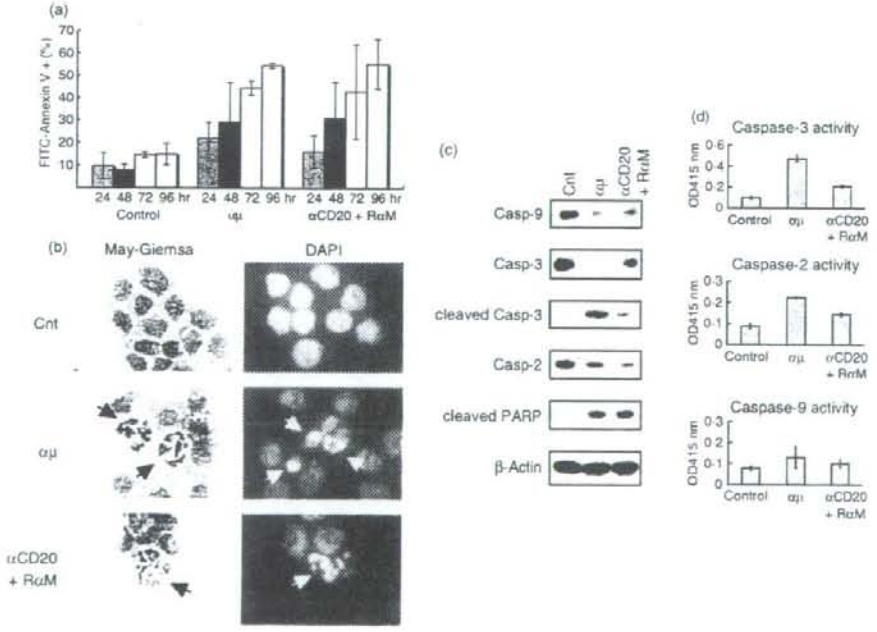

Figure 2. Induction of apoptosis in MLMA cells mediated by CD20 and B-cell antigen receptor. (a) MLMA cells were treated with either rabbit anti μ heavy chain polyclonal antibody (αμ, 10 μg/ml) or a combination of anti CD20 monoclonal antibody (mAb; αCD20, 5 μg/ml) and secondary rabbit anti-mouse immunoglobulin antibody (RαM, 5 μg/ml) for 48 hr and binding with fluorescein isothiocyanate (FITC)-conjugated annexin V was examined by flow cytometry. Each experiment was performed in triplicate and the means + SD are indicated. (b) The same sample preparations as in (a) were cytocentrifuged and morphological appearance was examined by Giemsa staining and nuclear staining with DAPI, using light microscopy and confocal microscopy, respectively. (c) Cell lysates were obtained from the same sample preparation as in (a) and the proforms of each caspase, cleaved caspase 3 and cleaved PARP were detected by immunoblotting.

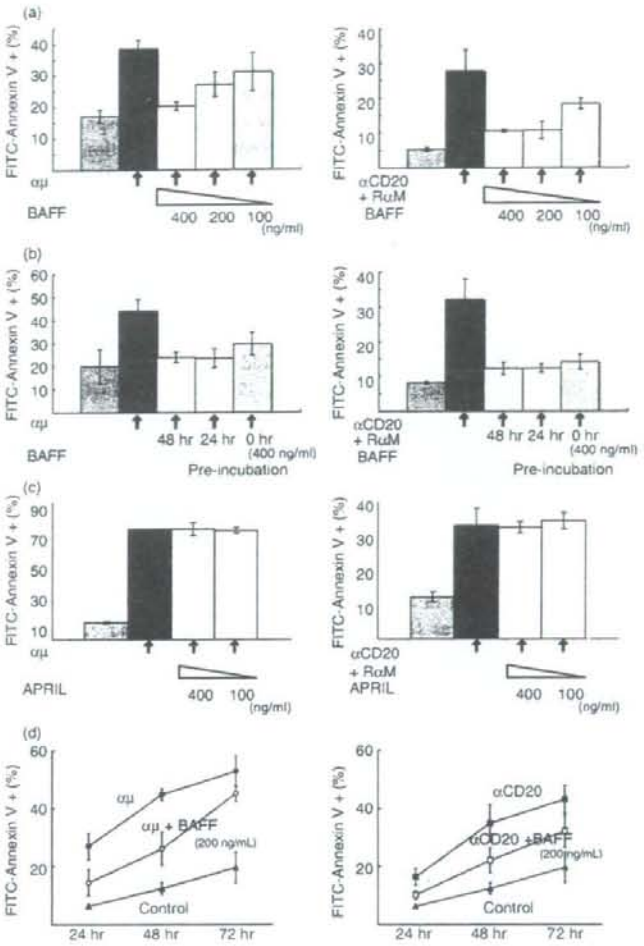

Figure 3. Effect of B cell-activating factor (BAFF) on B cell receptor (BCR) induced and CD20 induced apoptosis in MLMA cells. (a) MLMA cells were treated with either rabbit anti μ heavy chain polyclonal antibody (mAb) (αμ, 10 μg/ml, left panel) or a combination of anti CD20 mAb (αCD20, 5 μg/ml) and secondary rabbit anti mouse immunoglobulin antibody (RαM, 5 μg/ml) (right panel) for 48 hr in the presence or absence of different concentrations of BAFF as indicated and binding with fluorescein isothiocyanate (FITC) conjugated annexin V was examined as in Fig. 2(a). (b) MLMA cells preincubated with or without 400 ng/ml of BAFF for the indicated periods were treated with either αμ (left panel) or a combination of αCD20 and RαM (right panel) and examined as in (a). (c) The effect of APRIL on apoptosis induction was also examined as in (a). (d) MLMA cells were treated as in (a) and apoptosis was induced. The inhibitory effect of simultaneous addition of BAFF (200 ng/ml) against apoptosis was examined at different time points as in (a).

culture, the incidence of apoptosis induced by both BCR-mediated and CD20-mediated stimuli was reduced as assessed by annexin V-binding. Although inhibition tended to be more effective with a higher dose of BAFF, the effect was not significant. We also examined the effect of pretreatment with BAFF on the inhibition of apoptosis but found none (Fig. 3b). In contrast, APRIL, another ligand for BCMA and TACI, did not affect apoptosis induced by the BCR-mediated and CD20-mediated stimuli, indicating the specificity of BAFF's effect (Fig. 3c). Therefore, we concluded that BAFF-mediated stimuli are able to inhibit apoptosis mediated by the cross-linking of either CD20 or BCR and simultaneous treatment with apoptosis-inducing stimuli is almost sufficient to achieve maximum BAFF-mediated inhibition of apoptosis, at least in these cases. However, the inhibitory effect of BAFF against apoptosis mediated by the cross-linking of either CD20 or BCR was only partial and it was more obvious when the inhibition of apoptosis was examined at several different time-points.

Cellular effect of BAFF involved in the inhibition of apoptosis in MLMA cells

We further examined the molecular basis of the BAFF-mediated inhibition of apoptosis in MLMA cells. First, we tested the effect of BAFF on the growth of MLMA cells. As shown in Fig. 4, when BAFF was added to the culture, the cell proliferation was slightly enhanced, as assessed by cell counting, suggesting that BAFF promotes the growth of MLMA cells.

Next, we examined the intracellular signalling induced in MLMA cells by BAFF treatment. As shown in Fig. 5(a), immunoblot analysis revealed cleavage of p100, the precursor of NF-κB2, and a increase in p52, the active form of NF-κB2 after BAFF treatment, suggesting that the

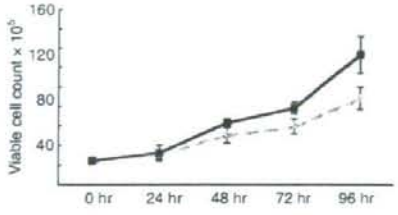

Figure 4. Effect of B cell activating factor (BAFF) on MLMA cell proliferation. Starting from a cell concentration at 5×10^5/ml, MLMA cells were cultured in the presence (solid line) and absence (dotted line) of 400 ng/ml of BAFF and cell numbers were counted at the time points indicated. Each experiment was performed in triplicate and the means + SD are indicated.

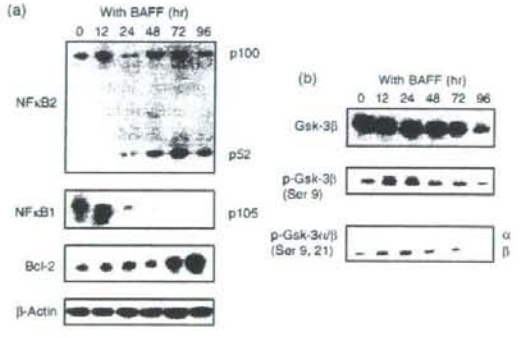

Figure 5. Intracellular signalling events and induction of Bcl-2 protein by B-cell-activating factor (BAFF). Cell lysates were prepared from MLMA cells treated with 400 ng/ml of BAFF for the periods indicated and an immunoblot analysis was performed using the antibodies indicated.

activation of NF-κB2 occurred after the treatment. We also observed the cleavage of the precursor of NF-κB1 after BAFF treatment (Fig. 5a). We further examined the activation of other molecules after treatment with BAFF and found that GSK-3β was transiently phosphorylated (Fig. 5b). In addition, we observed an elevation in the level of Bcl-2, an anti-apoptotic protein, after BAFF treatment.

To investigate the early responses to BAFF in MLMA cells, global screening of candidate genes whose expression is regulated by BAFF was performed by employing a microarray system. First, we selected up-regulated genes that are expressed in MLMA cells treated with BAFF for 12 hr at a level at least 1·5-fold higher than in untreated cells. Under these conditions, 178 probes were selected as up-regulated genes (Table 1). Consistent with the results of the immunoblot analysis presented in Fig. 5(a), the gene expression of Bcl-2 was found to be up-regulated by BAFF treatment (Table 1). Interestingly, the gene expression of CD40, a member of the TNF-receptor family involved in B-cell survival, was also increased after treatment with BAFF. The genes that are known to be involved in anti-apoptotic effect, including *Myb*, Epstein–Barr virus (EBV)-induced gene 3 (*EBI3*), and caspase 8 and FADD-like apoptosis regulator (*CFLAR*), were also up-regulated by BAFF treatment.

We further confirmed the increased CD40 protein expression by flow cytometry (Fig. 6a). Similarly, down-regulated genes that were expressed in BAFF-treated cells at a level at least 0·75-fold lower than in untreated cells were selected. As shown in Table 2, 517 probes were selected as down-regulated genes. The above results of global gene expression profiling suggest that the expression of various types of genes was influenced by BAFF stimulation in MLMA cells.

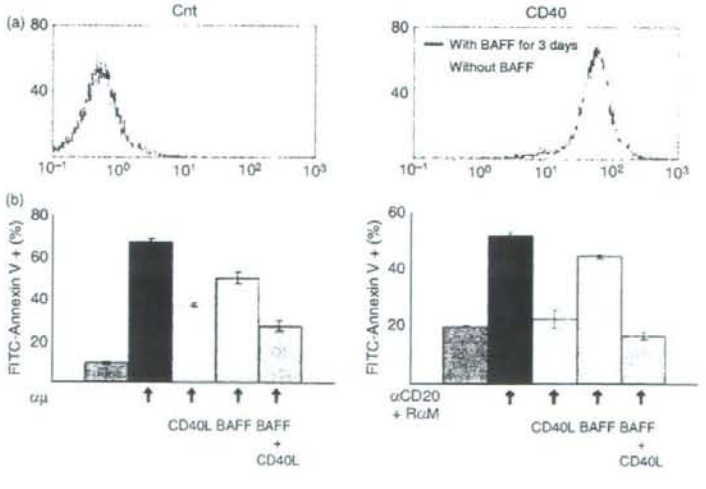

Figure 6. Effect of B cell activating factor (BAFF) on CD40 expression in MLMA cells. (a) MLMA cells cultured with or without BAFF for 3 days were stained with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (mAb) against CD40 and analysed by flow cytometry as in Fig. 1. (b) The inhibitory effect of CD40 stimulation on apoptosis induction was examined. MLMA cells were treated with 500 ng/ml of CD40-ligand in the presence of 2·5 ng/ml of interleukin-4 to stimulate CD40. The effects of either stimulation of CD40 alone or simultaneous stimulation of CD40 and BAFF receptor on apoptosis similarly induced as in Fig. 2 were examined.