Heterogeneous expression of CD32 and CD32-mediated growth suppression in human myeloma cells

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Background and Objectives. An increased level of serum M-protein IgG may affect the growth or survival of myeloma cells through the Fcγ receptor (FcγR) in human myelomas. We examined the expression of FcγR (CD32, CD16 and CD64) and compared the effect of anti-CD32 antibody on the viability of myeloma cells to that on the viability of normal plasma cells.

Design and Methods. Surface antigen and gene expressions were examined by flow cytometry and reverse transcription polymerase chain reaction, respectively. We examined the effect of anti-CD32 antibody on the viability of $CD19^-$ myeloma cells (including immature and mature myeloma cells) and $CD19^+$ normal plasma cells. In order to confirm the involvement of CD19 in the anti-CD32-mediated growth suppression, we used CD19 transfectants of myeloma, B-cell and erythroleukemia cell lines that we have already established.

Results. CD32 was significantly expessed on primary myeloma cells, but immature, $MPC\text{-}1^-$ myeloma cells expressed CD32 more weakly than mature, $MPC\text{-}1^+$ cells. Treatment with anti-CD32 antibody decreased the viability of normal plasma cells ($CD38^{++}$ $CD19^+$) more than that of myeloma cells ($CD38^{++}$ $CD19^-$); CD32-mediated growth suppression was greater in mature $MPC\text{-}1^+$ cells than in immature $MPC\text{-}1^-$ cells. The introduction of CD19 into $CD19^-$ cell lines significantly increased the sensitivity of the cells to treatment with anti-CD32 antibody as well as addition of IgG complex; furthermore, increased phosphorylation of CD32 and SHIP was detected in CD19-transfected cell lines.

Interpretation and Conclusions. Myeloma cells lacking CD19 expression are less sensitive to CD32-mediated growth suppression than are $CD19^+$ normal plasma cells.

Key words: myeloma, CD32, CD19, myeloma cell growth, immature myeloma cells.

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Human myeloma cells are heterogeneous with regard to the expression of surface antigens. Based on a recent analysis, immature $MPC\text{-}1^-$ cells and mature $MPC\text{-}1^+$ myeloma cells were identified. Immature myeloma cells are biologically different from mature myeloma cells: for example, immature myeloma cells respond well to interleukin-6 (IL-6) to proliferate.[1,2] However, in human myeloma, there is usually a markedly increased level of serum M protein (usually IgG), which may affect the growth and survival of myeloma and plasma cells. The IgG molecule is a multifunctional glycoprotein that binds antigen to form immune complexes that activate effector mechanisms resulting in their clearance and destruction of the immune complexes.[3] Due to their specificity for the Fc region of the γ heavy chain of IgG, these receptors were named Fcγ receptor (FcγR).[4] FcγR are members of the Ig gene superfamily and are expressed on various types of hematopoietic cell (e.g. platelets, monocytes, macrophages, eosinophils, basophils, neutrophils, natural killer cells and lymphocytes) and link cellular and humoral immunity by serving as a bridge between antibody specificity and effector cell function.[5] Stimulation of cells through FcγR results in a wide variety of effector functions, including antibody-dependent cellular cytotoxicity,[6] phagocytosis,[7] oxidative burst[8] and the release of mediators of inflammation.[9] Perturbations of the immune complex-mediated inflammatory responses may, however, result in chronic inflammation and tissue damage. FcγR have been classified into three types, high affinity FcγRI (CD64), low affinity FcγRII (CD32) and FcγRIII (CD16) and multiple isoforms: FcγRIa, FcγRIb, FcγRIc, FcγRIIa1, FcγRIIa2, FcγRIIb1, FcγRIIb2, FcγRIIc, FcγRIIIa and FcγRIIIb.[3,10–11] A majority of FcγR possess the immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic domain; however, FcγRIIb has an immunoreceptor tyrosine-based inhibitory motif (ITIM), necessary for its inhibitory effects.[4] The inhibitory function of FcγRIIb is best characterized in the B cell, although it has been demonstrated in other cells, such as myeloid and monocytic cells.[12–14] CD19, a 95-kD cell surface glycoprotein, is a B-cell-specific membrane protein belonging to the Ig superfamily[15] and is expressed on B cells from the pre-B stage to terminally differentiated plasma cells. CD19 is considered to function to define signaling thresholds for cell surface receptors that regu-

late B lymphocyte selection, activation and differentiation.[16,17] Myeloma cells from patients with multiple myeloma (MM), as well as myeloma cell lines, specifically lose CD19 expression through the loss of *Pax-5* gene expression.[18] The enforced expression of CD19 into myeloma cell lines induced the marked suppression of proliferation in serum-containing medium.[19] However, growth suppression of these CD19 transfectants of myeloma cell lines was somewhat weaker in serum-free or synthetic medium than in the serum-containing medium. It has been speculated that the serum components can affect the growth of CD19 transfectants of myeloma cells *in vitro*. Koncz *et al.* have already reported that co-clustering of CD19 with CD32 (FcγRIIb) inhibited CD19-induced Ca^{2+} influx in human B-cell lines.[20] We speculated that a high concentration of IgG affects the growth of plasma and myeloma cells of myeloma patients. In order to test this hypothesis, we examined the expression of FcγR (CD32, CD16 and CD64) and the effect of anti-CD32 antibody on the viability of primary myeloma cells and various myeloma cell lines. Furthermore, we analyzed the possible role of CD19 on the CD32 (FcγRII)-mediated suppression of myeloma cell growth, comparing the results of $CD19^-$ and $CD19^+$-transfected cells. Finally, we discuss the significance of expression of CD32 and CD19 on human myeloma cells.

Design and Methods

Primary myeloma cells

Bone marrow mononuclear cells (BMMNC) from the bone marrow aspirates used in this study were obtained from 20 patients with IgG MM and 12 patients with monoclonal gammopathy of undetermined significance (MGUS) (IgG type). The diagnosis of MM or MGUS was made according to the criteria proposed by the South Western Oncology Group. BMMNC were obtained from the patients with informed consent according to the Helsinki protocol and this study was approved by the Internal Review Board of Hiroshima Red Cross Hospital.

Cell lines and CD19 transfectants

Two human B-cell lines, Raji and C110 (Epstein Barr virus-transformed cells), two human myeloma cell lines, U266 and KMS5, and one human erythroleukemia cell line, K562, were cultured in RPMI1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (M. A. Bioproducts, Walkersville, MD, USA) at 37°C with 5% CO_2. The synthetic medium was composed of RPMI1640 with 200 μg/mL bovine serum albumin (AlbuMax, GibcoBRL, Grand Island, NY, USA) and 2 μg/mL transferrin (GibcoBRL), and used as the serum-free culture medium as described previously.[21] Cloned full-length CD19 cDNA was directly ligated into the pCR-TOPO vector (Invitrogen, San Diego, CA, USA) to give pCR-CD19 as already reported.[19] Human myeloma cell lines, U266 and KMS5 cells, and the human erythroleukemia cell line, K562, were transfected with pCI-neo vector (neo-control, mock) or pCI-CD19 (U266-19, KMS5-19 or K562-19) by lipofection. The transfected cells were then maintained in the selection medium, and cloned as previously reported by our group.[19]

Cell surface antigen expression and flow cytometry

Cells (1×10^5) were harvested, stained with phycoerythrin (PE)-labeled anti-CD16 (FcγRIII) (3G8, Beckman Coulter, Hialeah, FL, USA), anti-CD32 (FcγRII) (2E1, Beckman Coulter) or anti-CD64 (FcγRI) (22, Beckman Coulter) antibody, and then subjected to flow cytometry (Epics Elite ESP, Beckman Coulter). BMMNC from patients were also stained with fluorescein isothiocyanate (FITC)-labeled anti-CD38 (T16, Beckman Coulter) and PE-labeled anti-CD19 (IOB4, Beckman Coulter) or anti-MPC-1 (Japan Immunoresearch Laboratories (JIMRO), Takasaki, Japan) antibodies as described previously.[1]

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis for FcγRII gene expression

Total cellular RNA extracted from cells with Trizol (Invitrogen Carlsbad, CA, USA) was reverse transcribed to cDNA using random hexamers with Superscript II, as recommended by the manufacturer (GIBCO-BRL, Gaithersburg, USA). All genes, including a housekeeping standard (*G3PDH*), were amplified within the logarithmic phase after 25 to 35 cycles. The oligonuceotide primers used, as described previously, were:[22]

G3PDH sense: 5'-ACCACAGTCCATGCCATCAC-3',
antisense: 5'-TCCACCACCCTGTT GCTGTA-3';
FcγRIIa1 sense: 5'-TGCATCTGACTGTGCTTTCC- 3',
antisense: 5'-CTGATGGCAATCATTTGACG-3';
FcγRIIa2 sense: 5'-TCTGGGATGGCTATGGAGAC-3',
antisense: 5'-CTGATGGCAATCATTTGACG-3';
FcγRIIb1 sense: 5'-GCCTCTGGTCAAGGTCACAT-3',
antisense: 5'-ATTGTGTTCTCAGCCCCAAC-3';
FcγRIIb2 sense: 5'-GCCTCTGGTCAAGGTCACAT-3',
antisense: 5'-TCAAATCCCAATGCAAGACA-3';
FcγRIIc sense: 5'-GGAATCCTGTCATTTTTACCT-3',
antisense: 5'-CTCAAATTGGGCAGCCTTCAC-3'.

In vitro assay of viability of primary myeloma cells

BMMNC from bone marrow aspirates were obtained from myeloma patients and cultured for 3 or 6 days with or without anti-CD32 antibody (2E1,Beckman Coulter) (1 or 20μg/mL) in the synthetic medium. After that, the cells were harvested and stained with FITC-labeled-CD38, PE-CD19 or PE-MPC-1 antibody, and subjected to flow cytometry. Cell viability was examined with forward (FS) and side scatter (SS); the results of this evaluation were completely compatible with the data from staining with FITC-annexin V (IM-3546, Beckman Coulter) and propidium iodide (PI; Sigma, St Louis, MO, USA) as already reported.[18] In each case, the strongly positive CD38 fraction ($CD38^{++}$) was evaluated as the fraction of plasma cells (myeloma cells), and in this $CD38^{++}$ fraction, $CD38^{++}CD19^-$ and $CD38^{++}CD19^+$ were evaluated as myeloma cells and normal plasma cells, respectively. Finally, $CD38^{++}MPC\text{-}1^+$ and $CD38^{++}MPC\text{-}1^-$cells were considered to be mature and immature myeloma cells, respectively.[1,2]

The effect of anti-CD32 antibody on the growth of mock or CD19-transfected cell lines

Cell lines (mock or CD19-transfectants)[19] were cultured with anti-CD32 antibody (2E1, Beckman Coulter) or control IgG (mouse IgG2a, Coulter) in the synthetic

medium for 2 or 4 days. After that, viable cells were evaluated every 2 days by the FS/SS pattern from the flow cytometer.

The effect of a high concentration of IgG and of IgG-anti-IgG complex on the growth of mock-transfected or CD19-transfected cell lines

Cell lines (mock-transfected or CD19-transfectants)[19] were cultured with a high concentration of monoclonal IgG (0, 0.3 or 3 mg/mL), purified from a MM patient, in the synthetic medium for 2 or 4 days. After that, viable cells were evaluated by the FS/SS pattern every 2 days. The cell lines were also incubated with or without a high concentration of monoclonal IgG (10 mg/mL) in the synthetic medium at 4 °C for 1 hr, and then washed with the synthetic medium. The cells were cultured with or without the F(ab')$_2$ fragment of anti-human IgG F(ab')$_2$ (Rockland, Gilbertsville, PA, USA) (IgG-anti-IgG complex formation) for 2 or 4 days.

Immunoprecipitation and western blot analysis

Cells (2×10^8) were pre-incubated in synthetic medium at 37 °C for 2 hr, followed by incubation with anti-CD32 antibody (2E1, Beckman Coulter) (20 μg/mL) or with control IgG (10 μg/mL) for 10 minutes. Cell lysates were incubated with anti-CD32 antibody (AT10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-SH2 domain-containing inositol 5-phosphatase (SHIP) antibody (Upstate Biotechnology, Charlottesville, VA, USA) or anti-SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1) antibody (C-19, Santa Cruz, CA, USA) and immune complexes were then precipitated with protein A/G-agarose (Sigma, St Louis, MO, USA) following overnight incubation at 4°C. For western blotting, cell lysates or immunoprecipitates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to electrophoretic transfer onto Hybond C super (Amersham, Arlington Heights, IL, USA). The blots were probed with anti-phospho-tyrosine (P-Tyr-100, Cell Signaling Technology, Beverly, MA, USA) antibody. After stripping, the blots were reprobed with anti-CD32B (N-17, Santa Cruz, CA, USA), anti-SHIP antibody or anti-SHP-1 antibody.

Statistical analysis

Statistical analysis was conducted using Student's *t* test in the Statistical Package for the Social Sciences software (SPSS Japan, Inc., Tokyo, Japan). Levels of statistical significance were defined as $p<0.05$ and $p<0.01$.

Results

Expression of FcγR (CD32, CD16 and CD64) on primary myeloma cells and myeloma cell lines

Since there are at least three different types of FcγR, CD16, CD32 and CD64, we examined the expression of CD16, CD32 and CD64 on human plasma and myeloma cells, including plasma cells from patients with MGUS. As shown in Table 1, primary myeloma cells from 20 cases with IgG MM showed heterogeneous expression of CD32, and plasma cells from 12 cases of MGUS showed a high percentage of CD32 expression, except for one case (patient n. 8). Furthermore, the percentage of CD32$^+$ cells in the CD38^{++} fraction correlated well with the percentage of MPC-1$^+$ cells in the CD38^{++} fraction (Table 1, Figure 1A and B). Figure 1A shows four representative cases. In patients MM10 and MM8, in whom MPC-1$^+$ mature cells were dominantly present, the percentage expressions of CD32 in the CD38^{++} fraction were quite high (96.6% and 94.0%, respectively), and the mean fluorescent intensities of CD32 expression on these cells were also high (157.1 and 57.7, respectively). However, in patients MM5 and MM12, who had few MPC-1$^+$ cells and a predominance of MPC-1$^-$ immature cells, the percentage expressions of CD32 in the CD38^{++} fraction were low (14.0% and 12.9%,

Table 1. Expression of CD32 on human myeloma cells.

	% in the CD38^{++} cells				mean fluorescence
MM patients	CD38^{++}	MPC-1$^+$	CD16$^+$	CD32$^+$	of CD32$^+$ cells
MM 1	11.6	68.0	2.6	65.4	49.5
MM 2	80.0	84.9	0.6	86.4	61.6
MM 3	42.6	25.8	10.9	43.2	15.0
MM 4	35.3	6.4	4.2	9.9	27.5
MM 5	58.0	9.8	0.3	14.0	23.8
MM 6	48.5	42.8	1.4	68.7	26.1
MM 7	12.1	57.3	4.9	78.5	73.0
MM 8	60.4	69.4	7.6	94.0	57.7
MM 9	10.8	50.0	2.2	67.9	52.5
MM 10	15.4	96.1	1.3	96.6	157.1
MM 11	53.6	85.1	1.0	88.8	51.8
MM 12	40.2	6.2	0.8	12.9	15.1
MM 13	20.5	72.6	0.7	88.1	41.4
MM 14	25.8	56.9	1.9	75.3	37.0
MM 15	42.0	7.3	0.3	18.7	15.8
MM 16	15.1	97.3	3.1	97.9	124.8
MM 17	16.6	69.8	3.1	83.8	62.0
MM 18	22.1	94.5	1.2	86.2	66.1
MM 19	27.0	3.8	0.8	7.2	31.8
MM 20	93.0	46.7	1.2	62.4	70.3

	% in the CD38^{++} cells				mean fluorescence
MGUS	CD38^{++}	MPC-1$^+$	CD16$^+$	CD32$^+$	of CD32$^+$ cells
MGUS 1	3.8	97.1	3.5	90.3	93.5
MGUS 2	2.6	62.1	2.1	68.2	23.1
MGUS 3	3.2	92.9	26.8	97.5	117.0
MGUS 4	1.5	45.4	8.5	75.6	38.5
MGUS 5	4.0	92.7	7.0	87.7	44.9
MGUS 6	0.7	87.5	2.4	88.3	46.6
MGUS 7	2.5	88.5	4.4	93.4	72.6
MGUS 8	7.0	5.3	0.8	7.2	31.8
MGUS 9	3.2	96.2	1.99	98.3	113.6
MGUS 10	2.0	75.0	1.9	92.3	64.4
MGUS 11	1.8	50.0	1.1	58.4	24.9
MGUS 12	5.8	87.9	4.5	90.5	86.2

		% of positive cells			mean fluorescence
Cell lines		MPC-1$^+$	CD16$^+$	CD32$^+$	of CD32$^+$ cells
U266	–	2.2	1.3	3.5	13.1
KMS5	–	83.4	0.3	92.5	68.8
K562	–	1.2	0.4	99.9	487.3
KU S	–	65.8	8.1	25.8	18.2
C110	–	78.9	2.0	74.7	25.4
Raji	–	96.6	0.8	97.0	48.6

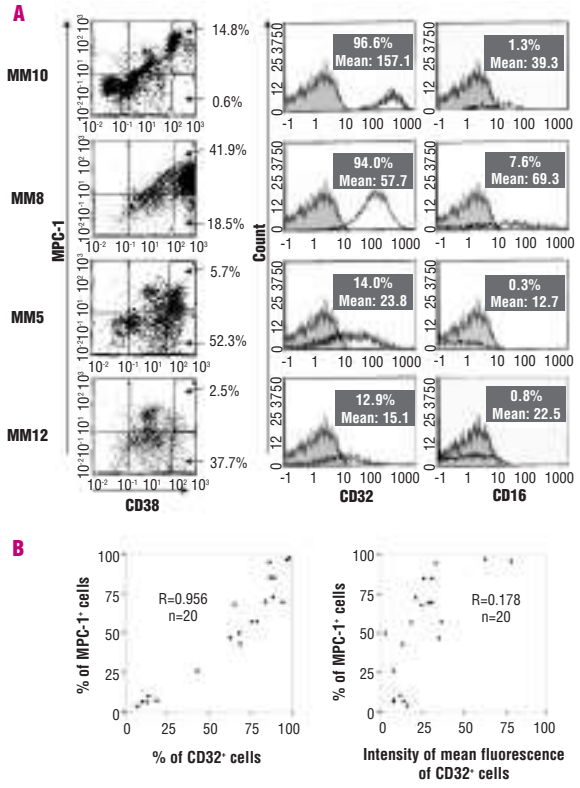

Figure 1. Expression of CD32 (FcγRII) on primary myeloma cells and its correlation with the expression of MPC-1 **A.** Levels of expression of CD32 on primary myeloma cells from bone marrow aspirates of four representative myeloma patients (MM10, MM8, MM5 and MM12) were analyzed by flow cytometry. $CD38^{++}$ cell fractions are plasma (myeloma) cells, and the arrow (↓) shows the percentage of $CD38^{++}$ $MPC\text{-}1^{+}$ or $CD38^{++}$ $MPC\text{-}1^{-}$ cells in the bone marrow mononuclear cells. **B.** Correlation between the percentage of CD32 and MPC-1 in bone marrow mononuclear cells obtained from 20 patients with IgG MM. Correlation coefficients (R) are shown.

respectively), and the mean fluorescent intensities of CD32 expression on these cells were also low (23.8 and 15.1, respectively). As shown in Figure 1B, the correlation ratio (R) between the percentage of $MPC\text{-}1^{+}$ cells and the percentage of $CD32^{+}$ cells in the $CD38^{++}$ fraction was quite high (R=0.956). The correlation ratio between the percentage of $MPC\text{-}1^{+}$ cells in the $CD38^{++}$ fraction and the intensity of the mean fluorescence of $CD32^{+}$ cells was high (R=0.718). As for the expression of CD16, both primary myeloma cells and plasma cells from MGUS showed almost no significant expression of CD16 except for one case of MM (MM3) and one case of MGUS (MGUS3). CD64 expression was not significant on primary myeloma, plasma cells (*data not shown*) or myeloma cell lines (Figure 2A). In conclusion, mature, $MPC\text{-}1^{+}$ myeloma cells expressed more CD32 than did immature, $MPC\text{-}1^{-}$ myeloma cells.

No change in the expression of CD32 between mock-transfected and CD19-transfected human myeloma cell lines

In order to clarify the involvement of the CD19 molecule in the CD32-mediated suppression of myeloma cell growth, we used mock-transfectants and CD19-transfectants of two myeloma cell lines (U266 and KMS5) and one erythroleukemia cell line (K562) as already reported.[19] As shown in Figure 2A, we confirmed that there was no apparent change in the expression of CD32 between the mock-transfectants and the CD19-transfectants of U266, KMS5 and K562 cells. We also confirmed that U266, KMS5 and K562 cells did not express either CD16 or CD64 significantly, although the B-cell lines, C110 and Raji, expressed CD32 and CD64.

Expression of FcγRIIa and FcγRIIb genes in myeloma cells

Since the anti-CD32 antibody recognizes multiple isoforms of FcγRII, we examined the expression of

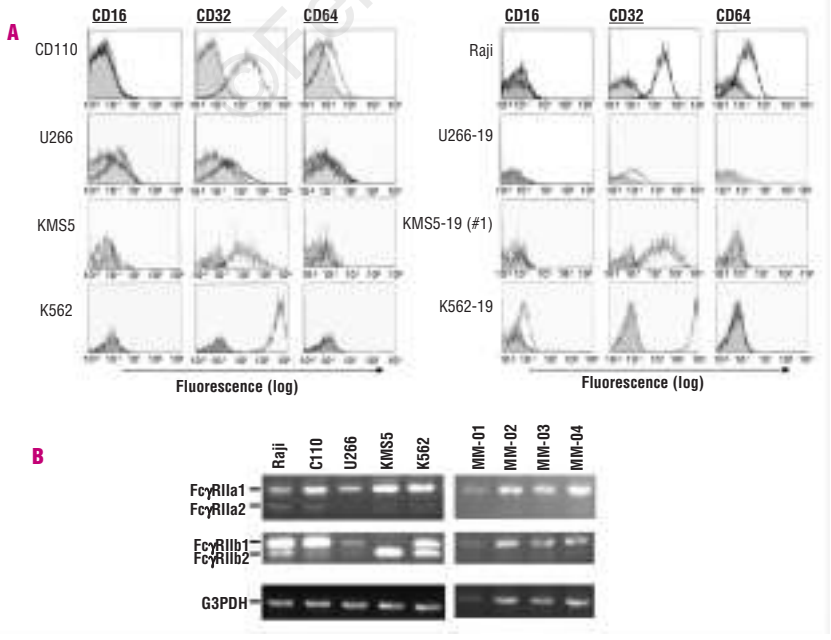

Figure 2. Expression of FcγRII (CD32), FcγRI (CD64) and *FcγRIII* (CD16) on myeloma cell lines, and FcγRII gene expression. **A.** The cell surface expression of FcγRI (CD64), FcγRII (CD32) or FcγRIII (CD16) on B-cell lines (C110 and Raji), myeloma cell lines (U266 and KMS5) and their CD19 transfectants (U266-19 and KMS5-19), an erythroleukemia cell line (K562) and its CD19-transfectant (K562-19). The gray area indicates staining with isotype control antibody in each panel. **B.** RT-PCR anslysis of *FcγRII* expression in the human B-cell lines, myeloma cell lines, erythrolekemia cell line and four cases of primary myeloma cells (MM-01-MM-04). Two distinct *FcgRIIa* (*FcγRIIa1*, *FcγRIIa2*) and two distinct *FcgRIIb* (*FcγRIIb1*, *FcγRIIb2*) RT-PCR products were detectable.

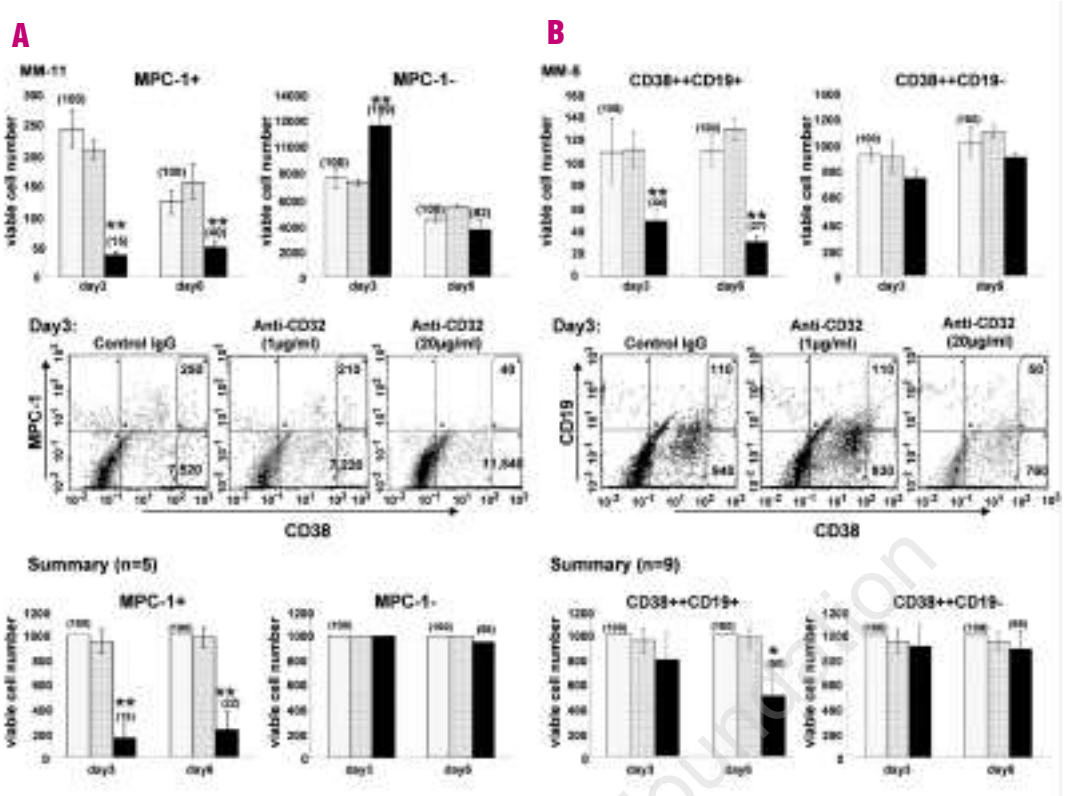

Figure 3. CD32-mediated growth suppression on primary myeloma cells *in vitro*. Columns show viable cell numbers of representative cases (MM-11 and MM-5) in IgG myeloma after culture with anti-CD32 antibody (2E1, Beckman Coulter) (0, 1, ■20 μg/mL) for 3 or 6 days. A. $CD38^{++}MPC\text{-}1^{+}$, $CD38^{++}MPC\text{-}1^{-}$. B. $CD38^{++}CD19^{+}$, $CD38^{++}CD19^{-}$ cells were analyzed by flow cytometry, respectively. The means±SD of triplicate culture are presented, and statistically analyzed using Student's t test; *p<0.05; **; p<0.01.

FcγRIIa1, *FcγRIIa2*, *FcγRIIb1*, *FcγRIIb2* and *FcγRIIc* using RT-PCR. As shown in Figure 2B, RT-PCR products corresponding to *FcγRIIa1* were predominantly detected in the B-cell lines (Raji and C110), myeloma cell lines (U266 and KMS5), K562 cells and primary myeloma cells (MM1 to MM4). The *FcγRIIb1* transcript was predominantly detected in the two B-cell lines, U266 and primary myeloma cells, while KMS5 cells predominantly expressed *FcγRIIb2*, and K562 cells expressed *FcγRIIb* and *FcγRIIb2* equally. Thus, we confirmed that primary myeloma cells, as well as myeloma cell lines, express *FcγRIIb*, which contains the inhibitory ITIM motif.

FcγRII (CD32)-mediated growth effects on primary myeloma cells in vitro

Since we confirmed the expression of CD32, especially FcγRIIb, on primary myeloma cells, we examined whether treatment with anti-CD32 antibody could induce growth suppression of these primary myeloma cells *in vitro*. As shown in Figure 3A, after 3 and 6 days culture with anti-CD32 antibody (2E1, Beckman Coulter) (20 μg/mL), viable cell numbers of $CD38^{++}$ fractions were decreased; in particular, the $MPC\text{-}1^{+}$ cells in $CD38^{++}$ fractions were decreased, while $MPC\text{-}1^{-}$ cells appeared to experience no suppression. These findings were confirmed in five cases of IgG myelomas. Furthermore, treatment with anti-CD32 antibody (20 μg/mL) induced a stronger suppression of viable $CD19^{+}$ cells (normal plasma cells) in the $CD38^{++}$ fractions than of $CD19^{-}$ myeloma cells. Summary data of nine cases of IgG myelomas are shown in Figure 3B (lower figure). These data suggest that CD32-mediated growth suppression is correlated with the level of CD32 expression on cell surfaces and may somehow depend on the presence of CD19.

Increased sensitivity of CD32-mediated growth suppression by the induction of CD19 in myeloma cell lines

We found that the growth-suppressing effect of treatment with anti-CD32 antibody was greater on $CD19^{+}$ normal plasma cells than on $CD19^{-}$ myeloma cells *in vitro*. In order to clarify the possible involvement of the CD19 molecule in the CD32-mediated growth suppression, we compared the effect of treatment with anti-CD32 antibody on cell viability in mock-transfectants with that of treatment of CD19-transfectants of two myeloma cell lines (U266 and KMS5) and one erythroleukemia cell line (K562) as reported previously.[19] First, we confirmed that two B-cell lines, C110 and Raji cells, both expressed CD32 (as shown in Figure 2A) and CD19 (*data not shown*), and that both cell lines were sensitive to treatment with anti-CD32 antibody (as shown in Figure 4). CD19-transfected U266 and KMS5 cells showed greater suppression of viability than did mock-transfected cells 4 days after treatment with anti-CD32 antibody, while the significant difference between mock transfected and CD19-transfected of U266 cells was observed at the highest concentration of anti-CD32 antibody. Also, CD19-transfectants of KMS5

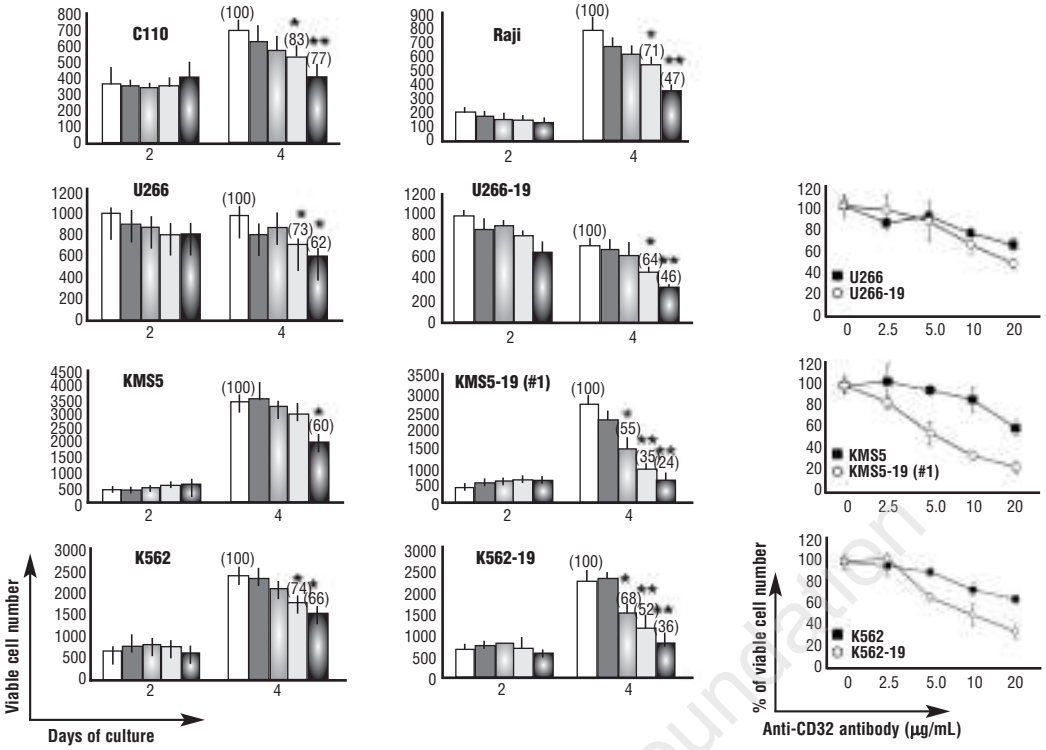

Figure 4. CD32-mediated growth suppression in CD19-transfectants of myeloma cell lines. Number of viable cells after treatment with anti-CD32 antibody (2E1, Beckman Coulter) (2.5 (□), 5 (■), 10 (▢), 20 (□) μg/mL) or control (■) in the serum-free synthetic medium for 2 or 4 days. Growth curves of CD19-transfectants (○) or mock-transfected (■) cells (as already reported)[19] are also shown. The means±SD of three independent experiments are presented, and statistically analyzed using Student's t test; *$p<0.05$; **$p<0.01$.

cells were more sensitive to CD32-mediated growth suppression than were CD19-transfectants of U266 cells. The same effect was also observed in CD19-transfectants of the non-B-cell line, K562 cells. Therefore, we confirmed that CD19 molecules contributed to the increased growth suppressive effect of treatment with anti-CD32 antibody in myeloma cell lines.

Contribution of CD19 to the increased growth suppressive effect of the high concentration of IgG and of IgG-anti-IgG complex on the growth of myeloma cell lines

First, we confirmed that the B-cell lines, C110 and Raji cells, were sensitive to a high concentration of monoclonal IgG and the formation of an IgG-anti-IgG complex, as shown in Figure 5A and B. The addition of a high concentration of monoclonal IgG (3 mg/mL) significantly reduced the number of viable CD19-transfected KMS5 cells, but not that of CD19-transfected U266 cells. Furthermore, the formation of IgG-anti-IgG complex increased the growth suppression of CD19-transfectants of both U266 and KMS5 cells more than that of mock-transfected cells. We also confirmed that treatment with a high concentration of IgG and with IgG-anti-IgG complex induced a marked suppression of growth of CD19-transfectants of a non-B-cell line, K562. We were, therefore, able to confirm that CD19 molecules also contribute to the increased suppressive effect of a high concentration of IgG and of IgG-anti-IgG complex formation on cell viability in myeloma cell lines.

Increased phosphorylation of FcγRII (CD32) and SHIP after treatment with anti-CD32 antibody in CD19-transfectants

Based on the findings mentioned above, we attempted to investigate how anti-CD32 antibody treatment suppressed growth of CD19-transfectants of myeloma cell lines more than that of mock-transfected cells. First, we confirmed that in a B-cell line, Raji cells, treatment with anti-CD32 antibody (2E1, Beckman Coulter) induced tyrosine phosphorylation of a protein of approximately 40 KDa, immunoprecipitated by the anti-pan CD32 (AT10) antibody, as shown in Figure 6 A. This tyrosine phosphorylation of CD32 was significantly detected in CD19-transfectants of KMS5 (KMS5-19) cells, but not in mock transfected KMS5 cells after treatment with anti-CD32 antibody (Figure 6A). Since myeloma cell lines, as well as B-cell lines, express FcγRIIb with an inhibitory ITIM motif, we examined how inhibitory molecules, such as SHIP or SHP-1, were phosphorylated after the ligation of CD32 molecules with anti-CD32 antibody (2E1, Beckman Coulter). As shown in Figure 6B, in B-cell lines such as Raji cells, SHIP, but not SHP-1, was phosphorylated strongly after treatment with anti-CD32 antibody, and increased

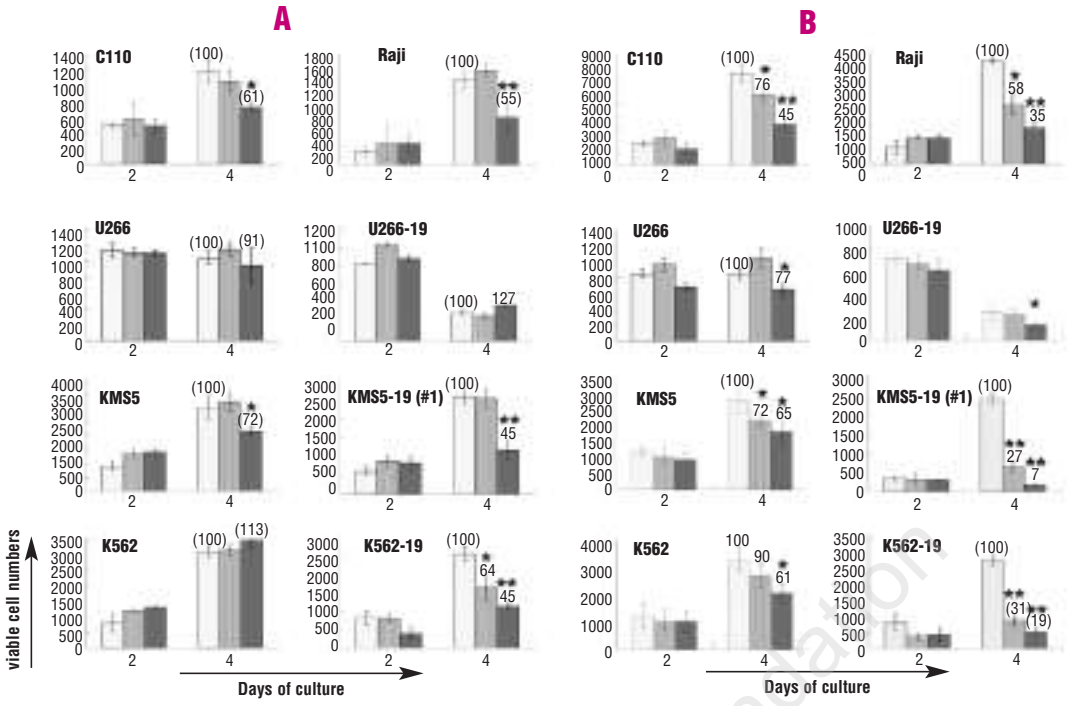

Figure 5. Suppressive effect of high concentrations of IgG and of IgG-anti-IgG complex on the growth of myeloma cell lines **A.** Numbers of viable cells are shown after culture with or without a high concentration of monoclonal IgG (0; 0.3; ▒3 mg/mL) in the serum-free synthetic medium for 2 or 4 days. The values in parentheses are percentages of the control compared with that of cells with no added IgG. **B.** The cells were incubated with or without monoclonal human IgG (10 mg/mL) at 4 °C for 1 hr. After washing, the cells were cultured with control IgG (10 μg/mL) or anti-human IgG (10 μg/mL) for 2 or 4 days. (medium alone; human IgG + control IgG ; ▒ human IgG + anti human-IgG). The means±SD of three independent experiments are shown. *$p<0.05$; **$p<0.01$.

phosphorylation of SHIP, but not of SHP-1 (Figure 6C) was also detected in CD19-transfectants of KMS5 (KMS5-19) cells but not in mock-transfected (KMS5) cells. Thus, we confirmed that anti-CD32 antibody treatment increased phosphorylation of FcγRII (CD32) and SHIP, but not of SHP-1, in CD19-transfectants more than in mock-transfected cells.

Discussion

In this study, we first showed that primary myeloma cells from IgG myeloma patients had heterogeneous expression of CD32 on their surface, and that mature, MPC-1$^+$ myeloma cells expressed more CD32 than the immature, MPC-1$^-$ myeloma cells. Treatment with anti-CD32 antibody induced greater growth suppression in mature, MPC-1$^+$ myeloma cells than in immature, MPC-1$^-$ myeloma cells. This result is simply due to the level of expression of CD32 on the surfaces of the two types of cells; immature, MPC-1$^-$ myeloma cells express less CD32

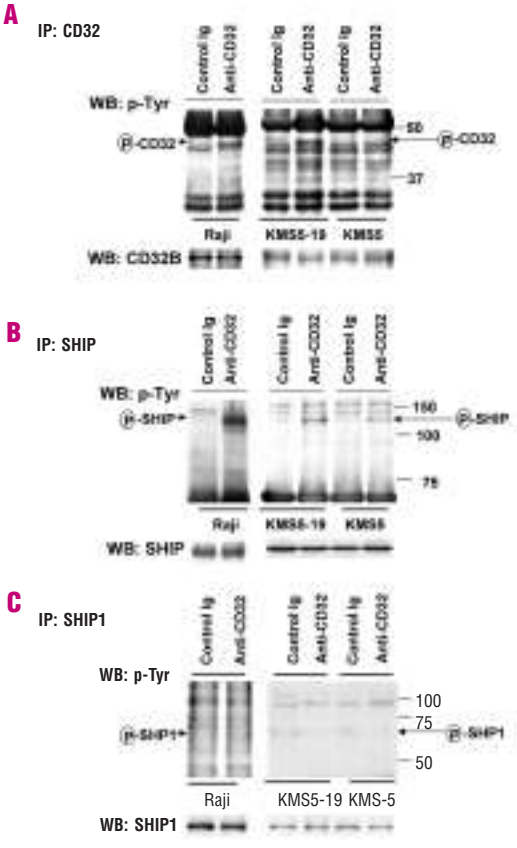

Figure 6 (Right). Phosphorylation of CD32 and SHIP after treatment with anti-CD32 antibody. Cells (Raji, KMS5 or KMS5-19) were cultured with anti-CD32 antibody (2E1, Beckman Coulter) for 10 min, and cell lysates were immunoprecipitated with anti-CD32 antibody (AT10, Santa Cruz) (**A**), anti-SHIP antibody (**B**) or anti-SHP-1 antibody (**C**). Western blotting (WB) was then performed with anti-phospho-tyrosine (PY100) antibody.

and are less sensitive to CD32-mediated growth suppression than are mature, MPC-1$^+$ myeloma cells. This phenotypic finding was also found in patients with IgA, IgD and Bence Jone type myeloma (*data not shown*). Most of the plasma cells from MGUS patients are mature MPC-1$^+$cells, and these plasma cells show high expression of CD32. As regards the myeloma cell lines, U266 cells are representative of immature MPC-1$^-$ myeloma cells, with low expression of CD32. KMS5 cells, on the other hand, represent mature, MPC-1$^+$ myeloma cells with a high expression of CD32. It was clear that KMS5 cells are more sensitive than U266 cells to treatment with anti-CD32 antibody, a high concentration of IgG or IgG-anti-IgG complex. Another interesting finding was that treatment with anti-CD32 antibody induced more growth suppression of CD19$^+$ normal plasma cells than of CD19$^-$ myeloma cells, and the enforced expression of CD19 into CD19$^-$ myeloma cell lines increased the sensitivity to treatment with anti-CD32 antibody. Thus, it is possible that CD19 is required for the increased growth suppression of FcγRII b (CD32)-expressing cells through the ligation of CD32 with anti-CD32 antibody. These findings that normal plasma cells are more sensitive to CD32-mediated growth suppression than are myeloma cells could be one of the mechanisms explaining why serum normal Ig is suppressed in MM patients.

The anti-CD32 antibody recognizes multiple isoforms of FcγRII, and primary myeloma cells, as well as myeloma cell lines, express FcγRIIa1 and FcγRIIb1 (Figure 2B). FcγRII has two roles in the regulation of cell signaling: activation signaling by ITAM-containing FcγRIIa and inhibitory signaling by ITIM-containing FcγRIIb.[23] Van Den Herik-Oudijk *et al*. reported that the *FcγRIIa* gene encoded a transmembrane receptor with 76 intracellular amino acids (FcγRIIal) and a receptor that lacked its transmembrane domain (FcγRIIa2).[24] Alternative splicing of FcγRIIb transcripts gives rise to two isoforms (IIbl and IIb2), which are identical except for a 19 amino acid insert in FcγRIIb1, different from IIb2 due to its lack of information encoded in the second signal peptide (S2) exon.[25] The transfection of FcγRIIbl and IIb2 in B cells showed that both isoforms were capable of downregulating increases in Ca^{2+} on co-cross-linking with surface IgG (B-cell receptor), and that FcγRIIb2 was capable of internalizing immune complexes.[24] This suggests that FcγRIIb1 and FcγRIIb2 might play similar functional roles. The inhibitory function of FcγRIIb is dependent on the phosphorylation of tyrosine residues in the cytoplasmic domain.[26–28] SH2 domain-containing inositol 5-phosphatase (SHIP) and SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1) activation has been implicated mostly in the negative signaling mediated by the inhibitory receptor FcγRIIb.[29] FcγRIIb (CD32) acts as a negative regulator in B cells and mast cells by recruiting inhibitory molecules, such as SHIP and SHP-1.[6,7] In this study, we found that treatment with anti-CD32 antibody induced increased tyrosine phosphorylation of CD32 (FcγRIIb) and SHIP in the CD19$^+$ B-cell lines and CD19-transfectants of myeloma cell lines more than in mock-transfected cells. However, we could not detect any significant tyrosine phosphorylation of SHP-1 following treatment with anti-CD32 antibody, although both SHIP and SHP-1 were reported to be involved in the FcγRIIb-mediated suppression in B cells.[29,30] On the other hand, it has been reported that CD32-mediated inhibition of Ca^{2+} mobilization in human B cell lines was induced by cross-linking CD19, and by co-clustering CD19 and B-cell receptor.[20] In order to test the possibility of a physical interaction between CD32 with CD19, we performed immunoprecipitation experiments with anti-CD32 antibody followed by western blotting with anti-CD19 antibody in anti-CD32 antibody-treated myeloma cell lines, but we could not detect any significant interaction between the two antigens (*data not shown*). Thus, it remains to be clarified why CD19 is required for CD32 (FcγRIIb)-mediated growth suppression and what molecules interact with CD32 and SHIP to transduce the inhibitory signals.

Changes in the level of expression of CD32 during human B-cell development have also been reported. During B-cell development, CD32 expression is not found until the pre-B-cell stage.[24] A profound down-regulation of CD32 is observed in the germinal center, and expression of CD32 is up-regulated on mature B-cell lines and plasma cells.[24,31] In this study, we found that CD32 expression was correlated with MPC-1 expression on plasma (myeloma) cells. Mature myeloma cells (CD38^{++}MPC-1$^+$) expressed much more CD32 than did immature myeloma cells (CD38^{++}MPC-1$^-$) (Figure 1B and Table 1). Recently, the *FcγRIIb* gene was reported to be regulated by the transcription factors GATA4 and Yin-Yang1 (YY1).[32] In human myeloma cells, the up-regulation of FcγRIIb expression would contribute to the increased sensitivity of CD32-mediated growth suppression although the exact mechanism of *FcγRIIb* gene regulation remains to be clarified.

In conclusion, we provide the first evidence that ligation of CD32 with anti-CD32 antibody can suppress the growth of normal CD19$^+$ plasma cells more than CD19$^-$ myeloma cells and that immature MPC-1$^-$ myeloma cells are less sensitive to this CD32-mediated suppression because they express less CD32. This FcγRIIb-mediated growth suppression of normal plasma cells may explain why serum normal Ig is suppressed in MM. On the other hand, the identification of surface molecules and cytoplasmic molecules capable of eliciting inhibitory signals in myeloma cells can contribute to the search for potential targets for novel therapies in human myeloma.

XZ: performed the research, wrote the manuscript; SA: assisted in the research, analyzed data; K-iO: assisted in the research, analyzed data; HA: patient care, performed BM aspiration; MMK: designed the research.

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