Blockade of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab

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Recently, anti-CD20 (rituximab) and anti-Her2/neu (trastuzumab) antibodies have been developed and applied to the treatment of malignant lymphoma and breast cancer, respectively. However, bulky lymphoma is known to be resistant to rituximab therapy, and this needs to be overcome. Fresh lymphoma cells were collected from 30 patients with non-Hodgkin's lymphoma, the expression of CD20 and CD55 was examined by flow cytometry, and complement-dependent cytotoxicity (CDC) assays were carried out. Susceptibility to CDC with rituximab was decreased in a tumor size-dependent manner ($r = -0.895$, $P < 0.0001$), but not in a CD20-dependent manner ($r = -0.076$, $P = 0.6807$) using clinical samples. One complement-inhibitory protein, CD55, contributed to bulky lymphoma-related resistance to CDC with rituximab. A decrease in susceptibility to CDC with rituximab was statistically dependent on CD55 expression ($r = -0.927$, $P < 0.0001$) and the relationship between tumor size and CD55 expression showed a significant positive correlation ($r = 0.921$, $P < 0.0001$) using clinical samples. To overcome the resistance to rituximab by high expression of CD55 in bulky lymphoma masses, small interfering RNA (siRNA) was designed from the DNA sequence corresponding to nucleic acids 1–380 of the CD55 cDNA. Introduction of this siRNA decreased CD55 expression in the breast cancer cell line SK-BR3 and in CD20-positive cells of patients with recurrent lymphoma; resistance to CDC was also inhibited. This observation gives us a novel strategy to suppress bulky disease-related resistance to monoclonal antibody treatment. (*Cancer Sci* 2006; 97: 72–79)

In recent years, monoclonal antibodies have been used increasingly to treat patients with malignancies such as lymphoma and breast cancer.[1–3] In particular, the anti-CD20 antibody, also called rituximab, is usually very effective for treatment of malignant lymphoma, and most patients can receive rituximab as monotherapy or combination chemotherapy.[4,5] However, in some cases with bulky mass and at stage IV, lymphoma cells become resistant to rituximab treatment.[6,7] Apart from the number of tumor cells being greater in these cases, how this resistance occurs has not yet been clarified.

Recently, some researchers have reported four mechanisms for the action of rituximab: (i) inhibition of proliferation; (ii) induction of apoptosis; (iii) complement-dependent cytotoxicity (CDC); and (iv) antibody-dependent cellular cytotoxicity (ADCC).[7,8] Because CDC could more rapidly and efficiently act on the target cells attacked by rituximab, CDC may be the most important of the mechanisms of rituximab.

The role of complementary regulatory proteins in the modulation of rituximab efficacy has been addressed, and several surface membrane proteins regulate the deposition of active complement proteins on cellular membranes to prevent cell lysis. Regulators of the complement system play an important role in CDC, and CD46, CD55 and CD59 are well known to inhibit the complement system.[9] Among these inhibitors, CD55 and CD59 seem to be the most important.[10] No differences in the expression of CD59 molecules have been reported between normal B cells and malignant B cells, whereas CD55 expression was shown to be different among individual patients with B-cell malignancy.[11] Nevertheless, *in vitro* susceptibility to rituximab-induced CDC could not be predicted by the level of these proteins in chronic lymphocytic leukemia (CLL) cells, and *in vivo* susceptibility could not be predicted in follicular lymphoma (FL) and CLL patients.[12,13] In contrast, some researchers have reported direct correlations among CDC, CD55 and CD59 using B-cell lines.[14]

CD55, also known as decay accelerating factor, is a major regulator of the alternative and classical pathways of complement activation and is expressed on all serum-exposed cells. CD55 is a 70-kDa glycoprotein, which is a glycosylphosphatidylinositol (GPI)-anchored protein.[15] CD55 can bind the complex of C3a and Bb, which is in the classical pathway, and it blocks the cascade of the complement system. A functional disorder of CD55 in blood cells causes paroxysmal nocturnal hemoglobinuria (PNH).[16] In these cases, the cascade of the complement system can not be controlled, and CDC activity is enhanced mainly against red blood cells. CD55 can enhance dissociation between C3 convertase and C4bC2/C3bBb complexes, and then inhibit the cascade of the

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complement system. While it is true that CD55 levels are low to absent in PNH, the disease is caused by phosphatidylinositol glycan-A (PIGA) gene mutations that lead to a failure to assemble GPI anchors. Hence, all GPI-anchored proteins are missing in this disease.

Previous researchers have shown that certain conditions for cancer cells, such as hypoxia, poor nutrition and bulky mass, make them chemoresistant.[17,18] When gastric cancer cells were exposed to hypoxia, hypoxia inducible factor (HIF)-1 was induced and the cells were resistant to Cis-platin (CDDP).[18] When lymphoid cells were able to resist doxorubicin (adriamycin), expression of nuclear factor (NF)-κB and its transcription activity were enhanced in doxorubicin (adriamycin)-resistant cells.[17]

Because CDC activity is especially important for rituximab therapy and CD55 may function as a mostly important inhibitor of CDC, it is possible that a decline in CDC activity by CD55 molecules may cause resistance to rituximab. CDC correlates directly with the expression of CD20 antigen in malignant B cells, and *in vitro* susceptibility to rituximab-mediated CDC depends primarily on CD20 protein expression. However, there have yet been no reports about the relationship between tumor size and sensitivity to CDC or between tumor size and CD55 expression.

More recently, small interfering RNA (siRNA) has been developed and applied to knock down target gene expression.[19] For example, the nuclear factor of activated T cells (NFAT) and NF-κB were shown to be constitutively active in large B-cell lymphoma cells, and downregulation of NFATc1 and NF-κB in malignant B-cell lymphoma with siRNA inhibited lymphoma cell growth.[20] Although many researchers tried siRNA for genes of membrane proteins such as growth factor receptors,[21] there have been no successful reports describing siRNA for complement inhibitors.

To clarify the resistance to rituximab and overcome the resistance, especially with regard to bulky mass unresponsiveness and efficacy for re-treatment, we examined the relationship between CDC activity and rituximab, and CD55 expression in our patients, using siRNA for CD55 to treat CDC with rituximab.

Materials and Methods

Cell lines

Human malignant B-cell lines as well as Daudi and Raji cells (ATCC) were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) with 10% fetal calf serum (FCS) at 37°C. The cell lines were used as sensitive and resistant controls in CDC with anti-CD20 antibody. The human breast cancer cell lines MCF7 and SK-BR3 (ATCC) were cultured in Dulbecco's minimal essential medium (DMEM; Gibco) with 10% FCS.

Complement-mediated cytotoxicity assay

Cells were washed once with fresh complete medium, and anti-CD20 antibody (rituximab; Roche, Basel, Switzerland) or anti-Her2/neu antibody (trastuzumab; Roche) was added at a concentration of 20 μg/mL. Cells were incubated at 37°C for 1 h, and then human AB blood serum from healthy volunteers with informed consent was added at 20% (v/v). After incubation at 37°C for 1 h, propidium iodide (PI; Sigma, St Louis, MO, USA) was added and CDC assays were carried out by flow cytometry with FACscan (Becton Dickinson, San Jose, CA, USA). For CDC assays using a microplate reader, Daudi, Raji and SKBR3 cells were seeded at 1×10^5 cell/mL in each well, and then rituximab or trastuzumab (20 μg/mL) and normal AB serum (20% [v/v]) were added. The reaction was incubated at 37°C for 1 h, and the cells were washed with phosphate-buffered saline (PBS) at least three times. Ten microliters of Calcein-AM (2 μg/mL) (Dojindo, Kumamoto, Japan) was added to each well and mixed thoroughly. After incubation at room temperature, fluorescence intensity was measured at 485 nm/535 nm wavelengths with a microplate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland).

Surface markers

Cells were washed once with PBS, and were then were stained with phycoerythrin (PE)-conjugated anti-CD20, and fluoresceinisothiocyanate (FITC)-conjugated anti-CD55 (Becton Dickinson). Flow cytometry was then carried out using FACscan. The intensities of C20 and CD55 expression were normalized compared with a control. For confocal laser scanning microscopy, rituximab and trastuzumab were labeled with Alexa Fluor 594 (Molecular Probes; Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. In brief, 100 μg of antibody was labeled with Alexa Fluor 594 for 20 min after alkalization with carbonate. The mixture was put into a spin column and spun down at 1500*g*, and the flow-through was collected as Alexa Fluor 594-conjugated antibody.

Laser scanning confocal microscopy and phase-contrast microscopy

To see CDC activity on living cells, pictures were taken by a CDC camera with phase-contrast microscopy after the CDC assay with rituximab or trastuzumab. The cells were also stained with Alexa Fluor 594-labeled rituximab or Alexa Fluor 594-labeled trastuzumab and FITC-labeled anti-CD55 antibody, and serum was added to the culture medium. The stained cells were observed in real time under a confocal laser scanning microscopy system (Olympus, Tokyo, Japan).

Collection of clinical samples

Fresh lymphoma cells were collected from the lymph nodes of 30 patients with non-Hodgkin's lymphoma (11 cases of diffuse large B-cell type, 10 cases of marginal zone cell type, five cases of follicular cell type, two cases of small lymphocytic type, one case of B-cell immunoblastic type, and one case of diffuse small cell type) after receiving informed consent. In brief, the lymph nodes were resected surgically and specimens were broken into small pieces with scissors and ground between two glass slides. The cells were collected after centrifugation and washed with RPMI-1640 containing 10% FCS. Cell counting and viability were assessed by toluidine-blue exclusion dye test, and CD19-positive cells were isolated using a magnetic cell sorting (MACS) system. The isolated cells were stained with FITC-conjugated anti-CD19, PE-conjugated anti-CD20, and FITC-conjugated anti-CD55 antibodies and flow cytometry was then carried out.

Vector and siRNA for CD55

CD55 cDNA in Ultimate open reading frame (ORF) clones (clone *ID*: IOH3209) was purchased from Invitrogen, and amplified by polymerase chain reaction (PCR) (forward, 5′-CGCGGATCCGCGATGACCGTCGCGCGG-3′; and reverse, 5′-TCCCCCGGGGGACTAAGTCAGCAAGCC-3′). The PCR product was subcloned into the pEGFP-C1 vector (Clontech, Mountain View, CA, USA). To generate double-stranded RNA for CD55, three parts of the DNA sequence, corresponding to nucleic acids 1–380, 381–817 and 821–1146 in the CD55 cDNA, were amplified by PCR. These sequences were named CD55-N, CD55-M and CD55-C, respectively. RNA transcription was then performed with this DNA template to generate sense and antisense single-stranded RNA. After production of double-stranded RNA, a reaction with the Dicer enzyme was carried out using a BLOCK-iT Dicer RNAi kit (Invitrogen). For siRNA for CD55, the siRNA was transfected into Raji and SK-BR3 cells using Lipofectamine 2000 (Invitrogen). In brief, 0.75 ng of siRNA and 5 μL of Lipofectamine 2000 in Optimen medium were mixed and incubated at room temperature for 20 min. The mixture was added to culture medium with SK-BR3 cells and fresh lymphoma cells, and the cells were incubated at 37°C for 72 h and 24 h, respectively. To see downregulation of CD55 expression, the CD55-transfected cells were stained with FITC-conjugated anti-CD55 antibody, and then expression of CD55 was observed without fixation of the cells at the same intensity of emission and excision as under laser scanning confocal fluorescent microscopy.

Statistical analysis

Correlation of susceptibility to CDC with tumor size, CD20 expression and CD55 expression were tested using the Spearman rank correlation coefficient. Statistical comparisons were carried out using two-sided Student's *t*-tests. All statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC, USA).

Results

Negative correlation between tumor size and susceptibility to CDC with rituximab

Rituximab is known to be effective at the early stages of indolent and aggressive lymphomas, but the effect of rituximab declines in some patients with bulky disease and a large number of lymphoma cells. According to this fact, we investigated whether susceptibility to CDC is dependent on the size of the tumor. The diameter of extirpated lymph nodes, CDC assay and CD20 expression were examined in fresh samples from 30 patients with lymphoma, as described in 'Materials and Methods'. As shown in Fig. 1a, the relationship between susceptibility to CDC and size of extirpated lymph nodes showed a significant negative correlation ($R = -0.895$, $P < 0.001$). In contrast, the relationship between susceptibility to CDC and CD20 expression, and between size of extirpated lymph nodes and CD20 expression, did not reveal significant correlations, as shown in Fig. 1b,c ($R = -0.076$, $P = 0.6807$ and 0.072, $P = 0.6979$, respectively). This suggests that susceptibility to CDC is dependent on the size of the lymphoma tumor, and that expression of CD20 does not contribute to susceptibility to CDC with rituximab in non-Hodgkin's lymphoma.

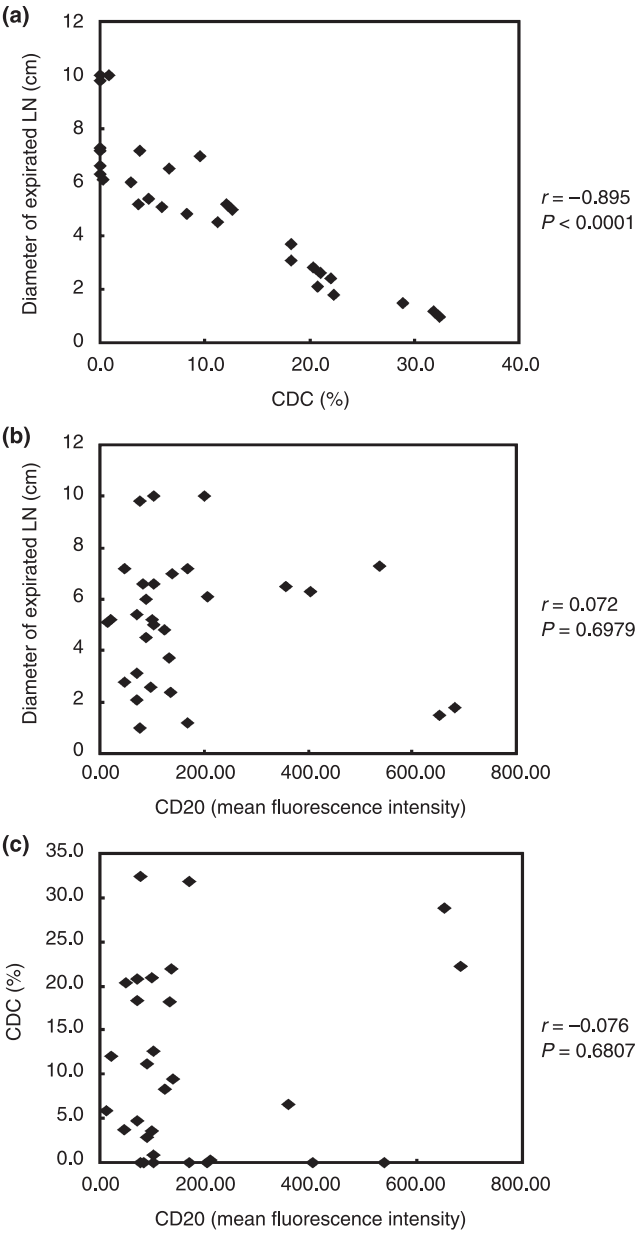

Fig. 1. Relationships between the size of extirpated tumors, susceptibility to complement-dependent cytotoxicity (CDC), and CD20 expression. The size of tumors from 30 patients with non-Hodgkin's lymphoma was measured and the cells were collected. After isolation of CD19-positive cells, FACScan analysis was carried out with anti-CD20 antibody, and CDC assay with rituximab was performed. Intensity of CD20 expression was normalized compared with a control. (a) Scatter plot and correlation analysis for size of extirpated tumor versus susceptibility to CDC. (b) Scatter plot and correlation analysis for size of extirpated tumor versus mean fluorescence intensity of CD20. (c) Scatter plot and correlation analysis for mean fluorescence intensity of CD20 versus susceptibility to CDC. All correlations were tested using the Spearman rank correlation coefficient.

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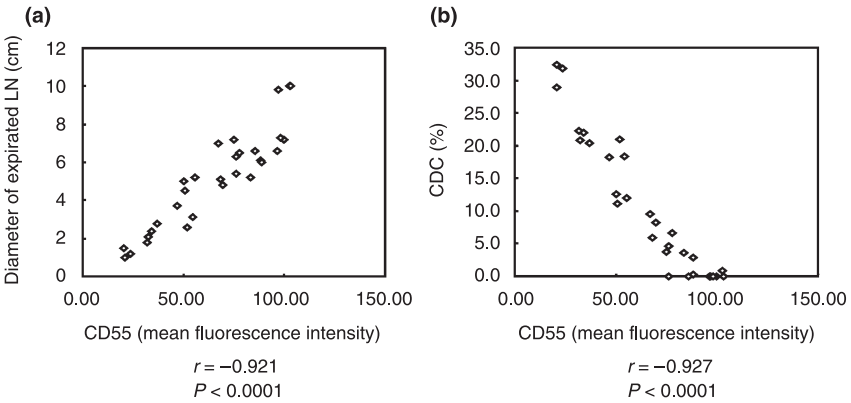

Fig. 2. Relationships between tumor size, CD55 expression and susceptibility to complement-dependent cytotoxicity (CDC). The size of tumors from 30 patients with lymphoma was measured and the cells were collected. After isolation of $CD19^+/CD20^+$ cells, FACscan analysis for CDC assay and CD55 expression were carried out. The intensity of CD55 expression was normalized compared with a control. (a) Scatter plot and correlation analysis for size of extirpated tumors versus CD55 expression. (b) Scatter plot and correlation analysis for CD55 expression versus susceptibility to CDC. All correlations were tested using the Spearman rank correlation coefficient.

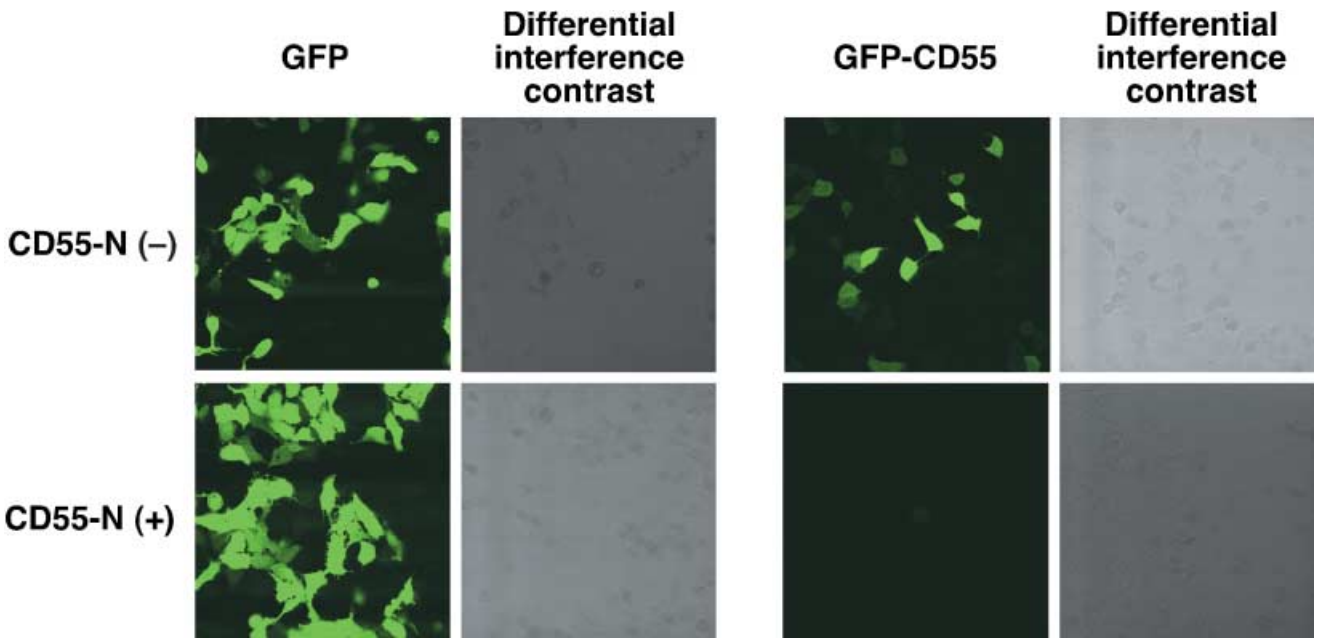

Fig. 3. Effect of small interfering RNA (siRNA) against the 5′-site of the *CD55* gene on expression of the exogenous *CD55* gene. MCF7 cells were transfected with pEGFP or pEGFP-CD55 in the presence or absence of siRNA. After 24 h, the cells were observed by laser scanning microscopy.

Size, CD55 expression and CDC in clinical samples

To investigate the relationship between the size of the extirpated tumor and CD55 expression in clinical samples, correlations between the size of extirpated tumor and fluorescence mean intensity of CD55, and between susceptibility to CDC with rituximab and fluorescence mean intensity of CD55, were analyzed statistically (Fig. 2). As shown in Fig. 2a, the level of CD55 expression on lymphoma cells was statistically correlated with the size of the lymph node ($r = 0.921$, $P < 0.001$). In contrast, the relationship between susceptibility to CDC with rituximab and fluorescence mean intensity of CD55 statistically revealed a negative correlation ($r = -0.927$, $P < 0.001$) (Fig. 2b). This suggests that increasing size of tumor contributes to higher or enhanced CD55 expression and resistance to CDC with rituximab.

Effect of siRNA for CD55 on CD55-transfected MCF7 cells

To overcome the resistance to CDC with rituximab on bulky mass, siRNA against a part of CD55 (CD55-N for 1–380 nucleotides) was designed and cotransfected with the pEGFP or pEGFP-CD55 plasmid into MCF7 cells (Fig. 3). When the cells were cotransfected with both pEGFP and siRNA for CD55, the expression of green fluorescent protein (GFP) did not change compared with transfection with only pEGFP vector (Fig. 3, upper panels). On the other hand, when the cells were cotransfected with both pEGFP-CD55 and siRNA for CD55, the expression of GFP-CD55 disappeared compared with transfection with only the pEGFP-CD55 vector (Fig. 3, lower panels). This suggests that CD55-N, siRNA against 1–380 nucleotides in the CD55 gene, is effective for blocking the expression of CD55.

Decrease in CD55 expression by siRNA overcomes resistance to CDC in breast cancer cell line SK-BR3

We investigated the use of a monoclonal antibody against the Her2/neu molecule for breast cancer, named trastuzumab.

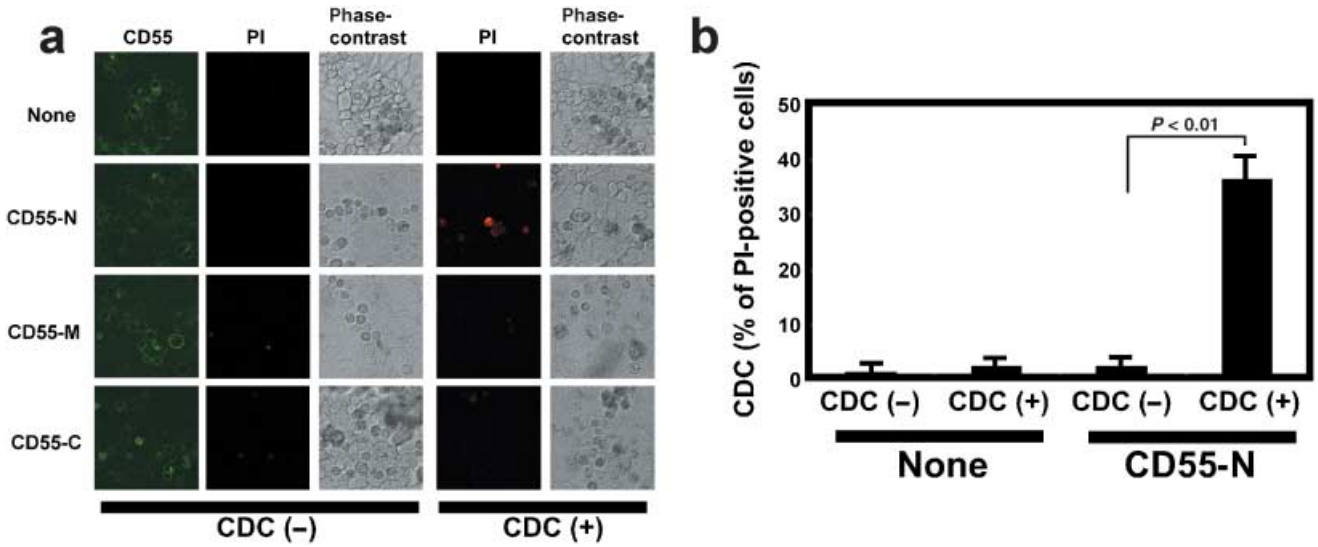

Fig. 4. Blockade of endogenous CD55 on breast cancer cells by small interfering RNA (siRNA). (a,b) SK-BR3 cells were transfected with siRNA against three parts of CD55, namely CD55-N, CD55-M and CD55-C, for 72 h. After transfection, the cells were stained with the anti-CD55 antibody and DAPI, and then the complement-dependent cytotoxicity (CDC) assay with trastuzumab was carried out with or without adding fresh human AB serum (a, left and right panels). (b) The percentage of propidium iodide-positive cells was calculated by counting 100 cells. Data are the mean ± SD (error bars) from experiments with triplicate samples. All statistical tests were two-sided Student's *t*-tests.

Because the breast cancer cell line SK-BR3 expresses Her2/neu and CD55 on its cell surface, siRNAs against three parts of CD55 (CD55-N for 1–380 nucleotides; CD55-M for 381–817 nucleotides; and CD55-C for 821–1146 nucleotides) were designed and introduced into SK-BR3 cells (Fig. 4). To detect dying cells, PI staining was used for the CDC assay with trastuzumab, and then the percentage of PI-positive cells was evaluated under laser scanning confocal microscopy. Most SK-BR3 cells expressed CD55 molecules without transfection of siRNA against CD55 (Fig. 4a, left). In contrast, expression of CD55 on SK-BR3 cells transfected with CD55-N disappeared 72 h after transfection, or became much weaker than without transfection of siRNA against CD55 (Fig. 4a, right). SK-BR3 cells transfected with CD55-M or CD55-C did not reveal knock down of CD55 expression to the level seen with CD55-N (Fig. 4a). Only 3.0 ± 1.0% of SK-BR3 cells without transfection of siRNA (mock transfection) against CD55 became PI-positive by CDC with trastuzumab, whereas 36.0 ± 6.0% of cells were PI-positive by CDC with trastuzumab after the transfection of siRNA (Fig. 4b). This suggested that siRNA against nucleotides 1–380 of CD55 (i.e. CD55-N) was effective for decreasing CD55 expression and sensitivity to CDC on adherent cells such as SK-BR3.

Blockade of CD55 expression by siRNA overcomes resistance to CDC in fresh lymphoma cells

To investigate the effect of siRNA against CD55 on fresh lymphoma cells, lymphoma cells were isolated from the lymph nodes of five patients with recurrent lymphomas and transfected with siRNA against CD55 (Fig. 5). As shown in Fig. 5a, lymphoma cells from all five cases with recurrent lymphoma strongly expressed CD55 molecules under laser scanning confocal microscopy. When fresh lymphoma cells were transfected with CD55-N for 24 h, but not CD55-M and CD55-C, CD55 expression on fresh lymphoma cells was significantly knocked down under laser scanning confocal microscopy, compared with the control (Fig. 5a, left columns). The percentage of PI-positive cells showed no significant differences among transfections with and without CD55-N, CD55-M and CD55-C before the CDC assay (Fig. 5b). The percentage of PI-positive cells in the transfection with CD55-N significantly increased from 7.1 ± 2.8% to 67.9 ± 8.1%. This indicates that the siRNA against CD55 (CD55-N) could efficiently knock down the expression of CD55 on SK-BR3 and freshly isolated lymphoma cells from recurrent lymphomas, and that it could induce cell death in SK-BR3 and freshly isolated lymphoma cells from recurrent lymphomas by CDC. This suggests that the degree of CD55 expression can determine resistance to CDC with antibody therapy, and that the therapies, which target CD55 molecules such as siRNA and its monoclonal antibody, would be helpful in antibody therapy for bulky disease.

Discussion

Treatment of malignancies has been largely based on chemotherapy and radiotherapy. Although improvement in response rates and survival has been obtained with these therapies over the years, a significant proportion of patients do not respond to treatment, or they relapse. Moreover, conventional cytotoxic therapy is often associated with significant morbidity. Recently, molecular targeting therapy has been developed[22] and monoclonal antibodies against CD20 and HER2/neu have been used for molecular targeting therapy.[1–3] Also, in recent therapies for malignancies, monoclonal antibodies have emerged as important therapeutic agents.

In the preset study, we have shown a negative correlation between the size of extirpated lymph nodes and susceptibility to CDC with rituximab, but the level of CD20 expression did

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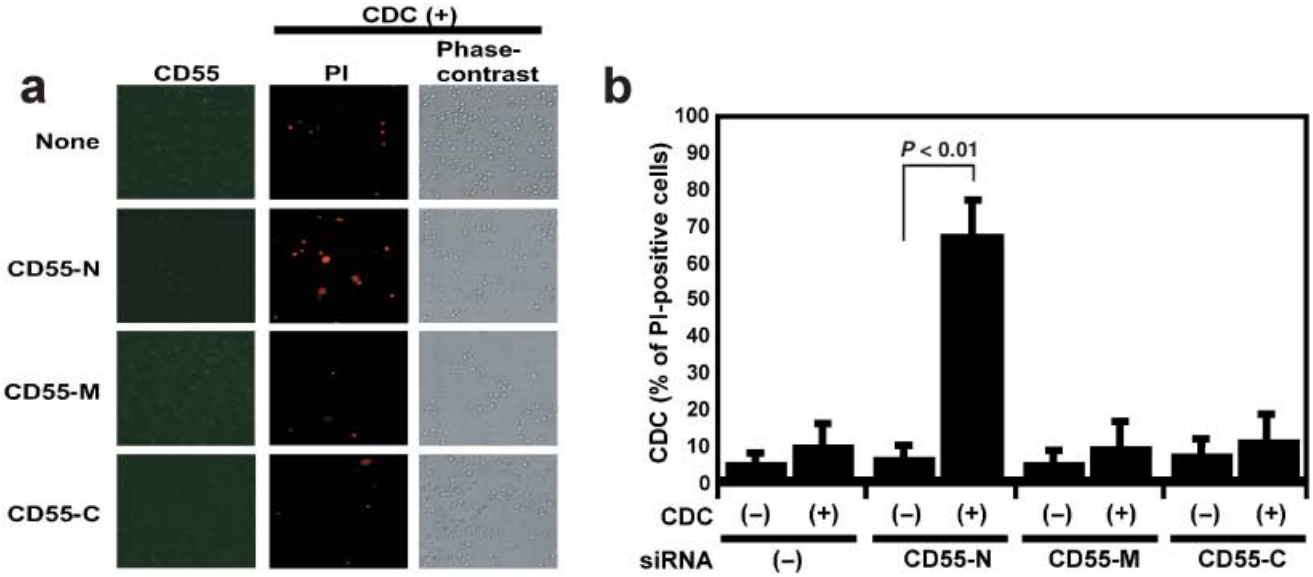

Fig. 5. Blockade of CD55 on primary lymphoma cells by small interfering RNA (siRNA). (a,b) Lymphoma cells from the lymph nodes of five patients with chemotherapy refractory and resistant lymphoma were transfected with siRNA against three parts of CD55, namely CD55-N, CD55-M and CD55-C, for 24 h. (a) After transfection, the cells were stained with anti-CD55 antibody and propidium iodide (PI), and then the complement-dependent cytotoxicity (CDC) assay with rituximab was carried out with or without adding fresh human AB serum. (b) The percentage of PI-positive cells was calculated by counting 100 cells. Data are the mean ± SD (error bars) from experiments with triplicate samples. All statistical tests were two-sided Student's *t*-tests.

not correlate with the size of the lymph node or susceptibility to CDC with rituximab. To date, no other studies have analyzed the relationship between size of lymph node and susceptibility to CDC with rituximab. It has been shown previously that CDC is directly correlated with CD20 expression.[11,23] In contrast, Manches *et al*.[24] have reported in detail that there is no direct correlation between lysis and expression of CD20 in global lymphoma such as FL, mantle cell lymphoma (MCL), small lymphocytic lymphoma (SLL), diffuse large B cell lymphoma (DLCL), and non-tumor B cells, as we showed in the current study. They also suggested that other regulators such as C-reactive protein (CRP) might play important roles in this complement system.

Although antibody therapy is a good tool, resistance sometimes occurs due to unknown mechanisms.[8,25] Patients with bulky mass, especially more than 7 cm of lymphoma mass, often show resistance to rituximab and are not curable.[26] We have demonstrated that CDC activity negatively correlates with the size of extirpated lymph nodes, and that the formula's intercept is 7.447 cm. This suggests that CDC is ineffective to tumors greater than 7.447 cm in size, and that our observation is consistent with the report of Coiffier *et al*.[26] Additionally, CD55 expression significantly correlates with the size of extirpated lymph nodes, suggesting that CD55 expression may play an important role in CDC resistance with antibody therapy. High densities of Daudi and Raji cells, associated with bulky mass, also became resistant to CDC with rituximab, and expression of CD55 increased during cell culture (Terui *et al*., unpublished data). The relationship between cell density and size of tumors, resistance to CDC and CD55 expression are the same in not only extirpated lymph nodes from patients but also in experimental cell lines. Although previous reports have discussed whether CD55 can be an indicator of prognosis, no one has reported the relationship between cell density and tumor size, resistance to CDC and CD55 expression. Low or high CD55 expression has been reported in CLL cells.[11] However, some researchers have reported that *in vitro* susceptibility to rituximab-induced CDC could not be predicted by the levels of CD55 protein in CLL cells, nor *in vivo* in FL and CLL patients.[12,13] On the other hand, Golay *et al*.[27] have reported that relative levels of CD55 and CD59 may become useful markers to predict clinical responses. Overexpression of CD55 on some tumor cell lines and in colorectal carcinomas has been shown to be an indicator of poor prognosis. This result is consistent with the present study, as we found that CD55 expression in bulky disease may be a useful indicator of this prognosis. Recently, Madjd *et al*.[28] reported that loss of CD55 is related to poor prognosis in breast cancer. High expression of CD55 was significantly associated with low-grade lymph node negativity and with good prognosis. Survival analysis showed that CD55 overexpression was associated with a more favorable outcome. On the other hand, loss of CD55 is associated with poor survival. They established a novel anti-CD55 antibody for use in immunohistochemistry. Although they classified weak to strong intensity of CD55, it is possible that the antibody recognized the non-glycosylated SCR3 domain of CD55 molecule, but not the glycosylated CD55 molecule. The authors pointed out that loss of CD55 is associated with poor prognosis, but not with monoclonal antibody resistance. In the present study, we demonstrated that blockage of CD55 overcomes resistance to antibody therapy and that CDC plays an important role in tumor attack in antibody therapy. As the mechanism that we refer to is different from their study, it may depend on the type of cancer investigated.

Malignant progression has been reported to be associated with tumor hypoxia, and the inside of the bulky mass showed low oxygen partial pressure (PO_2) (<10 mmHg).[29] Because hypoxia induces COX-2 expression and prostaglandin E_2 (PGE_2) production in not only human vascular endothelial cells[30] but also tumor cells,[31,32] PGE_2 may be produced more in bulky tumors with hypoxia. Recently, it has been reported that PGE_2 upregulates expression of the complement inhibitor CD55 in colorectal cancer.[33] This suggests that bulky mass of lymphoma and other cancers may express CD55 to high levels via PGE_2 production.

It has been reported that the protective activity of rituximab or the 1F5 antibody is completely abolished in syngeneic knockout animals lacking C1q, the first component of the classical complement pathway C ($C1qa^{-/-}$).[34] This indicates that complement activation is fundamental for rituximab therapeutic activity *in vivo*. As CDC is more rapidly and efficiently triggered by monoclonal antibodies in cells with higher expression of their target molecules, we focused on how sensitivity to CDC can be recovered in the resistance to monoclonal antibody therapy. In antibody therapy, blockage of CD55 may be useful for recovery of sensitivity to CDC. It has been reported that anti-CD55 and anti-CD59 antibodies can enhance CDC sensitivity with rituximab, and that CD55 and CD59 may become useful markers to predict the clinical response.[24] Although they did not mention the therapy against resistance to antibody therapy using anti-CD55 and anti-CD59 antibodies,[24] there are three ways to block the function of CD55: (i) blocking the antibody against CD55; (ii) siRNA[35] for CD55; and (iii) small molecules as CD55 inhibitors. We have demonstrated that siRNA for CD55 successfully inhibited functional CD55 protein, and that CDC activity was enhanced in the CD55-knock down breast cancer cell line SK-BR3 and in clinical samples from lymphoma patients. In particular, siRNA is a better tool for blocking CD55, as siRNA can inhibit not only expression of CD55 but also the function of CD55. Nagajothi *et al*. also showed genetic and biochemical methods to decrease CD55 expression and other GPI-anchored proteins.[36] This suggests that a decline in CD55 levels could be enough to make the tumor sensitive to CDC with rituximab and trastuzumab.

In conclusion, we have shown that CD55 blockade by siRNA enhances rituximab-mediated cytotoxicity. This observation gives us a novel strategy to suppress bulky disease-related resistance to monoclonal antibody treatment.

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