Role for Low-Affinity Receptor for IgE (CD23) in Normal and Leukemic B-Cell Proliferation

By Sylvie Fournier, Manuel Rubio, Guy Delespesse, and Marika Sarfati

CD23 gene is overexpressed and abnormally regulated in the most frequent adult leukemic disorder, B chronic lymphocytic leukemia (B-CLL). Switch on and off in the upregulation of surface CD23 expression consistently occurs in the early stage of normal B-cell activation, suggesting a key role for CD23 in this process. We show here that, after ligation of mlg in the presence of interleukin-4, the increase of CD23 protein precedes B-cell DNA synthesis and mainly results from the strong induction of CD23 type-B isofrom. Exposure of normal B cells to conventional or phosphorothioate-derivatized CD23 antisense oligonucleotides (predominantly type B) significantly augments B-cell proliferation induced by antigen receptor stimulation or direct contact with activated T cells. Unexpectedly, CD23 antisense, but not sense, oligonucleotides specifically enhance rather than suppress CD23 expression on B cells. Finally, a selective increase in CD23 type-B expression provokes the entry of resting (Go) CLL B cells into G1 and S phase of the cell cycle in the absence of any other stimulus, whereas it synergizes with tumor necrosis factor-α to increase the number of activated B cells. These results provide compelling evidence that CD23 represents an important molecule directly involved in the process of normal or leukemic B-cell activation and growth.

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MATERIALS AND METHODS

Reagents. Recombinant IL-4 was obtained from Immunex Corp (Seattle, WA) and tumor necrosis factor-α (TNF-α) was obtained from Roche (Ghent, Belgium). F(ab)2 fragment of goat anti-human IgM (Ab) was purchased from Cappel Lab (Organov Teknika, Ontario, Canada) and anti-CD23 (clone 135) was produced in our laboratory. Conventional oligonucleotides (AS and S) were synthesized on an automated DNA synthesizer using standard phosphoramidate chemistry and purified by precipitation three times in 0.3 mol/L of Na Acetate and 3 vol of ethanol. Phosphorothioate-derivatized oligonucleotides (oligo-S; AS-S and S-S) were purchased from Genosys Biotechnologies Inc (Woodland, TX). The sequences of CD23 type A antisense and sense oligonucleotides are 5'-CTTCCTCCATGGCGGTTC-3' and 5'-GACGGCCAATGGAGGAAG-3', respectively. They correspond to the region 179 to 195 of the type A CD23 cDNA. The sequences of CD23 type-B antisense and sense oligonucleotides are 5'-CTTATTTGCCCGCGC-3' and 5'-GGCGGGGACCCGAATAG-3', respectively. They encompass the region 1 to 17 of the type-B CD23 cDNA.

Cell preparations and culture conditions. B-tonsillar lymphocytes or B cells from chronic lymphocytic patients (B-CLL cells)
were purified by centrifugation over Ficoll-metrizoate, treatment with 5 mmol/L leucine methyl ester in RPMI 1640 for 40 minutes at 37°C, two rounds of rosetting with aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes, and centrifugation over Ficoll-metrizoate. Cells were then positively selected using CD19-coated magnetic beads and detachabead (Dynal AS, Oslo, Norway). The resulting population was found to be greater than 98% CD20<sup>+</sup> by rosetting with AET-treated sheep erythrocytes and recentrifugation over Ficoll-metrizoate. Purified CD4<sup>+</sup> T cells were obtained by negative selection using lymphokwik TH (One Lambda, Los Angeles, CA). T cells were activated by overnight incubation on anti-CD3 MoAb (64.1)-coated culture plates (24 wells); they were then washed and irradiated with 5,000 rads. For T-contact--induced B-cell proliferation, 1 × 10<sup>5</sup> irradiated anti-CD3 activated CD4<sup>+</sup> T cells were cocultured for 5 days in HB101 medium containing rIL-2 (25 U/mL) with 5 × 10<sup>4</sup> CD19<sup>+</sup> B cells that have been preincubated for 4 hours with 1 μmol/L oligo-S (ASB-S, SB-S, ASA-S, and SA-S).

**Measurement of cell proliferation.** For the measurement of DNA synthesis, cell cultures were pulsed with 0.5 μCi of <sup>3</sup>H-thymidine (Amersham Corp. Arlington Heights, IL) for the last 6 hours of the culture period and radioactivity was measured in a liquid scintillation counter. For cell cycle analysis, B cells were stained with acridine orange according to Darzykiewicz<sup>21</sup> and analyzed by flow cytometry (FACSCAN software; Becton Dickinson, Mountain View, CA).

**Northern blot analysis.** Total RNA was isolated, electrophoresed (10 μg), and transferred onto a nylon membrane (Biotrans; ICN, Irvine, CA) as described.<sup>22</sup> The blot was successively hybridized at 45°C with a (γ<sup>32</sup>P)-labeled 60-base synthetic oligonucleotide that recognizes the sequence 176-235 of the CD23 type-A cDNA and the sequence 25-84 of the CD23 type-B cDNA.<sup>23</sup> Hybridization buffer was 5× SSC (0.75 mol/L NaCl, 75 mmol/L Na citrate, pH 7), 15% formamide, 5% PEG-8000, 0.5% Denhart’s reagent, 50 μg/mL yeast tRNA, and 100 μg/mL salmon sperm DNA. Dehybridization was performed at 70°C in 1 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, and 0.5× Denhart’s reagent. Equal transfer of the RNA was assessed by methylene blue staining of the blot.<sup>21</sup>

**Immunofluorescence.** B cells were incubated with biotinylated anti-CD23 MoAb (clone 135) for 1 hour at 4°C, washed, and stained with phycoerythrin-conjugated streptavidin.<sup>20</sup> Cells were analyzed by flow cytometry using FACSCAN (Becton Dickinson).

**RESULTS AND DISCUSSION**

**Induction of CD23 expression (type B) precedes B-cell DNA synthesis.** To establish a possible role of CD23 in B-cell proliferation, we have selected mlg ligation in the presence of IL-4 as a model of B-cell activation. Kinetic studies using positively selected B cells indicate that anti-IgM and IL-4 costimulation induces a strong peak of surface CD23 expression (48 hours) that precedes that of B-cell DNA synthesis (72 hours; Fig 1A). In the absence of a costimulatory signal, IL-4 weakly upregulates CD23 and has no B-cell growth activity (Fig 1A).<sup>3</sup> To determine which isoform of
Fig 2. CD23 antisense oligonucleotides augment B-cell DNA synthesis. Tonsillar B cells were cultured with or without oligonucleotides in the presence of anti-IgM (3 μg/mL) and IL-4 (0.3 ng/mL) as described in Materials and Methods. B cells were pulsed with 0.5 μCi 3H-thymidine for the last 6 hours of the culture period. (A) Three days of culture with or without 15 μmol/L AS and S. Stimulation indices (CPM in stimulated cells/CPM in unstimulated cells) are mean values from 9 independent experiments (Student’s paired t-test). The mean ± SD of unstimulated cells is 430 ± 276 CPM. (B) Three days of culture with increasing concentrations of AS and S (1.5, 10, 15, and 25 μmol/L). (C) B cells were cultured for 2 to 4 days with 15 μmol/L AS or S. (D) Three days of culture with 15 μmol/L AS or SB in the presence of various concentrations of anti-IgM Ab. (B), (C), and (D) are one representative of two experiments. (E) Anti-IgM and IL-4–stimulated B cells were cultured for 3 days and stained for CD23 expression. Results are expressed in a three-dimensional plot (Lysys II software; Becton Dickinson). X and Y axis represent FL2 (log of orange fluorescence) and FSC (Linear forward scatter), respectively. (F) Three days of culture with 15 μmol/L conventional oligo or 1 μmol/L oligo-S. Stimulation indices (CPM in cultures with AS/CPM in cultures with SI are mean values from seven independent experiments (Student’s unpaired t-test; **P < .01). The mean ± SD of anti-IgM and IL-4–stimulated B cells is 4,282 ± 2,173 CPM.

CD23 is involved, the same B cells were analyzed by Northern blot using 32P-labeled oligonucleotide probes specifically directed to CD23 mRNA type A or B. The data (Fig 1B) indicate that the two CD23 mRNA are induced under these conditions; however, some differences may be noted between the two isoforms. First, at 48 hours, the steady-state level of CD23 mRNA type B in response to anti-IgM and IL-4 is much higher than that obtained in response to IL-4 alone, whereas such a difference is not observed for CD23 mRNA type A. Second, kinetic studies indicate that the level of CD23 mRNA type B peaks at 24 hours and decreases at 96 hours, whereas CD23 mRNA type A, when induced, is maintained throughout the culture. It is tempting to speculate that the peak of surface CD23 expression and B-cell proliferation in response to anti-IgM and IL-4 costimulation is directly related to the superinduction of CD23 type B, whereas the IL-4–induced CD23 expression correlates mainly with that of type A. A link between expression of CD23 type B and B-cell proliferation is further supported by our observations that suppression of anti-IgM and IL-4–induced B-cell DNA synthesis by anti-CD23 MoAb is concurrent to a significant reduction of CD23 mRNA type B but not A (M. Sarfati, personal data). We therefore postulated that the CD23 B isoform is predominantly involved in B-cell proliferation.

CD23 oligonucleotides (type B) enhances B-cell DNA synthesis and CD23 expression. Although CD23 type A is reported to be involved in endocytosis, no function has been
was extracted from 15 the presence of anti-lgM (3 pglmLl and IL-4 (0.3 ng/mL). Total RNA ascribed to CD23 type B in B lymphocytes.22 To directly enhance rather than suppresses anti-IgM and IL-4-induced proliferation. Surprisingly, ASB but not ASA significantly augments CD23 mRNA type B (Fig 3). Taken together, because antisense to CD23 A and B enhance to the same extent CD23 expression, whereas B-cell DNA synthesis is preferentially augmented by antisense to CD23 B, it is proposed that membrane form of CD23 type B is predominantly involved in anti-IgM and IL-4-induced B-cell proliferation.

We next induced B-cell activation by contact with irradiated anti-CD3 preactivated T cells in the presence of IL-2 and tested the regulatory effect of CD23 oligo-S. Similarly to Ag receptor stimulation, ASB-S displays significant potentiating activity on T-cell contact-dependent B-cell proliferation (Table 1). The mean ± SD of stimulation indices obtained from three independent experiments is 3.2 ± 0.4 for ASB-S versus 1.8 ± 0.05 for ASA-S (data not shown). The oligo-s have no activity in the absence of IL-2. From these data, it is hypothesized that CD23 Ag and its counterstructure may represent a pair of molecules important for B-cell activation. In that regard, CD21 has been proposed to serve as a ligand for CD2318 and anti-CD21 MoAb was shown to modestly enhance B-cell DNA synthesis.25 However, our recent observations indicate that sCD23 does not bind to CR2/CD21 Ag but reacts with a novel ligand other than IgE.26 Finally, it is interesting to mention that oligo-S do not influence anti-CD40--induced B-cell proliferation in the presence or absence of IL-4 (M. Sarfati et al, personal observations, September 1992 and May 1993). In this particular system, anti-CD40 MoAb immobilized on L cells transfected with CDw32 is a potent inducer of CD23 expression in the absence of IL-4.17

CD23 type-B oligos provokes entry of resting B-CLL cells

Table 1. Effect of CD23 Oligo-S on T-Cell-Dependent B-Cell Activation

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<td></td>
<td>Expt. 1</td>
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<tr>
<td>B</td>
<td>0.07 ± 0.05</td>
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<td>8.6 ± 0.9</td>
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<tr>
<td>+ ASB-S</td>
<td>22.9 ± 2.3 (3.0)</td>
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<tr>
<td>+ SB-S</td>
<td>7.6 ± 0.5</td>
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Irradiated anti-CD3 activated CD4+ T cells (1 × 10^5) were cocultured for 5 days with 5 × 10^5 CD19 + B cells that have been preincubated for 4 hours with 1 μmol/L oligo-S. IL-2 was used at 25 U/mL. Stimulation indices (CPM of cells cultured with ASB-S/CPM of cells cultured with SB-S) are shown in parenthesis.

were designed and introduced into B lymphocytes with the sense oligonucleotides possibly bind to the noncoding strand of the gene and form a triple-stranded helix, preventing the binding of a potentially inhibitory regulator."

Because phosphorothioate-derivatized antisense oligonucleotides (oligo-S) have the characteristics to be more stable and more soluble than conventional oligonucleotides,23,24 we next tested the biologic activities of CD23 oligo-S. As shown in Fig 2F, optimal concentration (1 μmol/L) of ASB oligo-S (ASB-S) is significantly more active in promoting B-cell growth than 15 μmol/L conventional ASB (P = .008, n = 6; Student’s unpaired t-test) or than ASA-S (P = .004, n = 7). The effect of ASB-S is selectively inhibited by SB-S and not by SA-S and vice versa for ASA-S (data not shown), suggesting that their biologic effect is directly related to their ability to hybridize with CD23. This hypothesis is supported by showing that ASB-S, but not SB-S, significantly and selectively augments CD23 mRNA type B in anti-IgM and IL-4--stimulated B cells, whereas ASA-S enhances CD23 mRNA type A and not B (Fig 3). Taken together, because antisense to CD23 A and B enhance to the same extent CD23 expression, whereas B-cell DNA synthesis is preferentially augmented by antisense to CD23 B, it is proposed that membrane form of CD23 type B is predominantly involved in anti-IgM and IL-4--induced B-cell proliferation.

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into G1 phase of cell cycle. Finally, to verify that CD23 oligo-S truly enhance B-cell proliferation and do not simply interfere with 3H-thymidine incorporation, cell cycle analysis was performed on resting G0 human CLL B cells. In contrast to normal B cells that exclusively express CD23 type A, freshly isolated B-CLL cells do express CD23 type A and B. Upon in vitro incubation at 37°C, B-CLL cells largely lose CD23 and remain at a resting stage unless stimulated by exogenous cytokines. As depicted in Fig 4, 7 days of exposure to ASB-S but not to SB-S provokes the entry of the B-CLL cells into G1 and S phase of cell cycle in the absence of any other stimuli; ASB-S synergizes with TNF-α to further increase the number of activated B cells. ASB-S strongly induces 3H-thymidine uptake in the presence or absence of TNF-α (means ± 1SD of stimulation indices of 3 independent experiments are 115 ± 31 and 12 ± 2, respectively). Although the two CD23 oligo-S increase CD23 expression to the same extent, ASA-S has no significant effect on B-cell DNA synthesis (data not shown). The potential biologic significance of these in vitro observations is directly illustrated in CLL disease, characterized by the accumulation of slow-dividing monoclonal B cells arrested at the G0/G1 stage of the cell cycle. The CD23 protein is overexpressed and abnormally regulated in this frequent B-lymphoproliferative disorder and, most interestingly for the patients, their serum sCD23 level correlates with the size of the tumor burden and is of significant prognostic importance.

In conclusion, it is proposed that a selective increase in surface CD23 expression (predominantly type B) is a key component in the normal and leukemic B-cell activation process. This provocative interpretation of the data cautiously awaits confirmation from transgenic or gene targeting technology because CD23 type-B isoform is absent in rodents.

ACKNOWLEDGMENT

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