CD45 Cell Surface Antigens Are Linked to Stimulation of Early Human Myeloid Progenitor Cells by Interleukin 3 (IL-3), Granulocyte/Macrophage Colony-stimulating Factor (GM-CSF), a GM-CSF/IL-3 Fusion Protein, and Mast Cell Growth Factor (a c-kit Ligand)

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Summary

CD45 antigens are protein tyrosine phosphatases. A possible link was evaluated between expression of CD45 antigens on human myeloid progenitor cells (MPC) (colony-forming unit-granulocyte/macrophage [CFU-GM], burst-forming unit-erythroid [BFU-E], and colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte [CFU-GEMM]) and regulation of MPC by colony-stimulating factors (CSF) (interleukin 3 [IL-3], GM-CSF, G-CSF, M-CSF, and erythropoietin [Epo]), a GM-CSF/IL-3 fusion protein, and mast cell growth factor (MGF; a c-kit ligand). Treatment of cells with antisense oligodeoxynucleotides (oligos) to exons 1 and 2, but not 4, 5, or 6, of the CD45 gene, or with monoclonal anti-CD45, significantly decreased CFU-GM colony formation stimulated with GM-CSF, IL-3, a GM-CSF/IL-3 fusion protein, and GM-CSF + MGF, but not with G-CSF or M-CSF. It also decreased GM-CSF, IL-3, fusion protein, and MGF-enhanced Epo-dependent BFU-E and CFU-GEMM colony formation, but had little or no effect on BFU-E or CFU-GEMM colony formation stimulated by Epo alone. Similar results were obtained with unseparated or purified (greater than or equal to one of two cells being a MPC) bone marrow cells. Sorted populations of CD34+ HLA-DR+ marrow cells composed of 90% MPC were used to demonstrate capping of CD45 after crosslinking protocols. Also, a decreased percent of CD45+ cells and CD45 antigen density was noted after treatment of column-separated CD34+ cells with antisense oligos to exon 1 of the CD45 gene. These results demonstrate that CD45 cell surface antigens are linked to stimulation of early human MPC by IL3, GM-CSF, a GM-CSF/IL-3 fusion protein, and MGF.

Blood cells arise from rare populations of cells termed hematopoietic stem and progenitor cells (1). Proliferation/differentiation of stem/progenitor cells is regulated by an interacting network of accessory cell–produced cytokines (2). Based on our interest in myeloid progenitor cell (MPC) cytokine receptors (3), in other cell surface determinants on MPC that may be involved in cytokine-mediated myeloid cell regulation (4), and in the molecular (5, 6) and cellular immunology (7, 8) of lymphoid CD45 surface antigens, we became intrigued in a potential role for CD45 antigens in MPC regulation.

CD45 antigens are protein tyrosine phosphatases (9, 10). They have been variously termed human leukocyte common antigens, murine Ly5, T-200, and B220, and are a family of five or more glycoproteins (5, 11, 12). Various isoforms of CD45 are generated by alternative mRNA splicing of pri-
mary transcripts of a single gene (6, 13–15). The extracellular domains are different, but the transmembrane and cytoplasmic segments are the same. The human CD45 gene is encoded on chromosome 1 and has 33 exons (13, 15). Exon-1 does not encode a peptide. Exon-2 contains the initiation ATG codon and encodes the signal peptide. Exon 3 and exons 7–15 encode the entire extracellular domain sequences common to all CD45 isoforms. Differential usage of exons 4, 5, and 6 generate the five, and possibly eight, distinct isoforms (13, 15). CD45 antigens have been implicated in signal transduction and lymphoid activation by specific association with receptor molecules on T and B lymphocytes (7, 8, 16, 17). This led us to investigate whether CD45 antigens might be linked to growth factor regulation of MPC.

Long-term bone marrow myeloid-initiating cells in vitro express CD45 antigens (18), but these antigens had not been implicated in regulatory myeloid interactions. We used molecular, cellular, and immunological approaches to determine if CD45 surface determinants were linked to regulation of cytokine stimulation of human multipotential (colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte [CFU-GEMM]), erythroid (blast-forming unit-erythroid [BFU-E]), and granulocyte/macrophage (colony-forming unit-granulocyte/macrophage [CFU-GM]) progenitor cell proliferation. This included use of antisense oligodeoxynucleotides (oligos) to exons of the CD45 gene to modulate gene expression, mAbs to framework CD45 antigens for "blocking" and crosslinking experiments, and highly enriched populations of MPC in cell-sorted CD34+ HLA-DR− human bone marrow to verify direct effects on the rare populations of MPC. The results suggest that CD45 surface determinants on MPC are involved in the regulation of stimulation of these cells by early acting cytokines: mast cell growth factor (MGF; a c-kit ligand; also termed stem cell factor [SCF] and kit ligand [KL]) (19–23), IL-3 (2), granulocyte/macrophage-CSF (GM-CSF) (2), and a newly engineered GM-CSF/IL-3 fusion protein (24), but not by the more lineage-restricted cytokines: erythropoietin (Epo), G-CSF, and M-CSF each alone (2).

Materials and Methods

Cells

Human bone marrow cells were aspirated from the posterior iliac crest after receiving informed consent from healthy donors. Cells were separated into a low density (LD, <1.077 gm/cm3) fraction containing all or most of the MPC. LD cells were further depleted of monocytes, by adherence to plastic, and of T lymphocytes, by E-rosetting, but still contained all or most MPC (25). The nonadherent low density T lymphocyte-depleted fraction (NALDT−) contained ≤2% monocytes, as determined by non-specific esterase staining, and T lymphocytes, as determined by analysis using CD3 antibodies and a flow cytometry system (753; Coulter Electronics, Hialeah, FL). Progenitor cells were further isolated by cell sorting into a population of CD34+ HLA-DR− cells (25, 26) using a flow cytometry system (753; Coulter Electronics). This results in a fraction with a high purity (>98%) of CD34 cells and a high MPC cloning efficiency (>95%) but recovery of few cells. In some experiments, unseparated marrow cells were enriched for CD34+ cells using a column separation with anti-CD34 that involves avidin-biotin linkage (27), kindly provided by Dr. R. Berenson (Cell Pro Incorp., Bothell, WA). This results in a fraction with a lower purity of CD34+ cells (45–80%) and a lower MPC cloning efficiency (10–22%) than by flow sorting, but in recovery of a greater number of cells.

Treatment of Cells

Cells were preincubated with oligos and/or antibodies before plating in semi-solid culture medium for assessment of MPC.

Antisense and Sense Oligos. These were prepared by the phosphoramid method with no modifications made to the oligos and are listed in Table 1. Cells were incubated with 20-mer oligos (7–14 μM each) essentially as described by others for evaluation of a different gene expression (28). Oligos or control (McCoy's) medium were added to cells in suspension without serum. 2 h later, heat-inactivated (56°C for 0.5 h) FCS (HyClone Laboratories, Logan, UT) was added at 10% (vol/vol), and the cells were left in suspension culture. After another 15–17 h (or 6–8 h for the CD34+ HLA-DR− or CD34+ column-separated cells), oligos or control medium were again added and cells plated in semi-solid culture medium, without washing, as described below, or cells left in suspension and oligos or control medium added at 2, 4, and 6 d, and then plated in semi-solid medium after the seventh day with or without additional oligos or conditioned medium.

"Blocking" and Crosslinking Experiments. These were done with monocolonal anti-CD45 (9.4, IgG2a) (8, 29). For "blocking," cells were incubated with 1 μg mAb/10^6 cells at 4°C for 30 min and then plated without washing. As a control for 9.4, an isotype-matched control mAb (μG2a; Southern Biotechnology Associates, Birmingham, AL) was used. For crosslinking, cells were incubated with 1 μg mAb/10^6 cells at 4°C for 30 min, washed twice, incubated with 1 μg affinity-purified sheep anti-mouse IgG (heavy and light chains; Cappel Laboratories, West Chester, PA)/10^6 cells at 37°C for 1 h, washed twice, and plated with or without oligos. To assess whether crosslinking resulted in capping, cells were exposed to FITC-conjugated anti-CD45 and then to FITC-conjugated sheep anti-mouse IgG. To determine fluorescence intensity and percent CD45+ cells after oligo treatment, cells were reacted with anti-CD45 conjugated to FITC (0.5 μg) or monoclonal anti-IgG-FITC (4.0 μg; Coulter Immunology, Hialeah, FL). Flow cytometric analysis was performed using a FACScan® (Becton Dickinson & Co., Sunnyvale, CA). Light scatter gates were set on the populations known from previous experiments to contain MPC. Data analysis was performed using a Consort 30 (Becton Dickinson & Co.) FACSD® data analysis program.

Myeloid Progenitor Cell Assays

Colony-forming Unit-Granulocyte/Macrophage. Cells were plated at designated cell concentrations in 0.3% agar (Difco Laboratories, Inc., Detroit, MI) culture (25) or 0.4% Sea Plaque Agarose (FMC Bioproducts, Rockland, ME) culture (30) medium as previously reported. Cells were stimulated by purified recombinant preparations of human (hu) IL-3 and GM-CSF (>4 × 10^9 U/mg each) (Immunex, Corp., Seattle, WA), huG-CSF (>10^9 U/mg, Immunocty, Corp.), huM-CSF (>2 × 10^9 U/mg, lot DCP-006 from the long form; a gift from Dr. Peter Ralph, Cetus Corp., Emeryville, CA), a purified huGM-CSF/IL-3 fusion protein (PIXY321; Immunex Corp.) (24), and purified murine (mu) MGF (Immunex Corp.) or by medium conditioned by the human 5637 cell line (30). Cultures were incubated at lowered (5%) O2 tension (30) in an ESPEC N2:O2:CO2 incubator BNP-210 (Tibbi ESPEC Corp., South Plainfield, NJ), and colonies (>40 cells/ag-
aggregate) and clusters (3-40/cells aggregate) scored after 7 or 14 d. Colony and cluster morphology was assessed as before (25, 30).

**Burst-forming Unit-Erythroid and Colony-forming Unit-Germ line/ Erythroid/Macrophage/Megakaryocyte.** Cells were plated as above but in 1.3% methylcellulose with recombinant Epo (1 U/ml) (Amgen Corp., Thousand Oaks, CA) with or without IL-3, GM-CSF, GM-CSF/IL-3 fusion proteins, or MGF (26). Cultures were incubated for 14 d.

**Statistics.** Results are expressed as mean ± 1 SEM of three plates/point for CFU-GM and four plates/point for BFU-E/CFU-GEMM assays. Levels of significance were determined using student's t distribution.

**Results**

**Influence of Antisense Oligos to CD45 Exons on CSF-dependent Colony Formation**

The aim of our study was to evaluate a role for CD45 antigens on growth factor-dependent proliferation of human bone marrow MPC by first using an antisense oligo approach to decrease CD45 antigens on these cells. Our prior studies with spleen cells from C57Bl/6-Ly5' congenic (Ly5.1+) mice found that the sense oligo to the human equivalent of exon 2 of the mouse CD45 gene had no effect on Ly5 antigen expression, but antisense oligo to this region reduced cell surface Ly5 antigen expression by 45-48% (p < 0.001). This suggested the feasibility of such an approach using human cells. Thus, normal human bone marrow cells separated into a LD or NALDT- fraction, but containing >98% of the MPC, were incubated in suspension with either of the oligos listed in Table 1. Oligos were added at 0 and 19 h, or 0, 19, 48, 96, 124, and 168 h before plating in semi-solid agar culture medium containing maximal amounts of 5637CM (10% [vol/vol], as a source of GM-CSF and G-CSF [2]) or rhuGM-CSF (200 U/ml), rhuIL-3 (200 U/ml), rhuG-CSF (200 U/ml), or rhuM-CSF (2,000 U/ml). As shown in Table 2, none of the oligos influenced CFU-GM colonies formed after 7 d in semi-solid medium in response to 5637CM, but 41-48% significant inhibition (p < 0.001) of these colonies was seen when cells were incubated with antisense oligos to exons 1 or 2 and scored after a 14-d incubation. No inhibition was seen with the sense oligos to exons 1 or 2, or the antisense oligos to exons 4, 5, or 6, after 14 d in semi-solid medium with 5637CM. Treatment with the combination of antisense oligos to exons 1 and 2 did not further decrease colony formation. Two additions of oligos (0 and 19 h) were as effective as inhibiting MPC growth as the six additions (0, 19, 48, 96, 124, and 168 h) (data not shown). Since colony formation after 7 vs. 14 d in the presence of 5637CM is due mainly to the presence of G-CSF vs. GM-CSF, respectively, in the 5637CM (2), cells were also grown in the presence of rhuGM-CSF, with day 14 colony results equivalent to those using 5637CM (Table 2). The results were similar whether LD or NALDT- cells were used. To rule out the possibility that the differences were due only to the day of scoring (GM-CSF being able to stimulate few or no colonies after only 7 d of incubation in semi-solid medium), NALDT- bone marrow cells were incubated in suspension with two additions of control medium, sense oligos to exon 1 or antisense oligos to exons 1 and 2, before plating in semi-solid agarose medium and incubation for 14 d with plateau concentrations of either rhuGM-CSF (200 U/ml), rhuIL-3 (200 U/ml), rhuG-CSF (200 U/ml), or rhuM-CSF (2,000 U/ml). As seen in Fig. 1, antisense oligos to exons 1 and 2, but not sense oligos to exon 1, greatly decreased (p < 0.001) day 14 colony and cluster formation stimulated with rhuGM-CSF and rhuIL-3, but had no effect on colony and cluster formation stimulated with rhuG-CSF or rhuM-CSF. Colonies and clusters formed in the presence of GM-CSF or IL-3 were ~60% granulocyte/macrophage (GM), 30% macrophage (M), and 10% granulocyte (G), while those formed in the presence of G-CSF or M-CSF were, respectively, >97% G or M. No colonies or clusters formed in the absence of CSF.

Antisense oligos were also evaluated for effects on Epo (1 U/ml)-dependent BFU-E colony formation and on rhuGM-CSF- or rhuIL3-enhanced Epo-dependent BFU-E colony formation in semi-solid methylcellulose medium (average of two to six experiments with LD or NALDT- cells). Only antisense oligos to exons 1 and 2 slightly (~26 ± 6 and ~16 ± 7%), but significantly (p < 0.05) decreased Epo-stimulated BFU-E colony formation. RhuGM-CSF and rhuIL3 significantly enhanced (+70 ± 12 and +57 ± 3; p < 0.001)

| Oligonucleotides were prepared by the phosphoramidite method. No modifications were made to Oligos. Hu, human; mu, murine. | 449 | Broxmeyer et al. |

**Table 1. Antisense and Sense Oligodeoxynucleotides to Exons of the Human and Mouse CD45 Gene**

| 5' end | 3' end |
|--------|--------|
| TCA    | GAC    | GAG    | GAA    | CAA    | TTT    | CC     |
| AAC    | CAC    | TTG    | CTA    | GCT    | GCA    | TG     |
| TCT    | GTC    | TCC    | AGA    | GCA    | GA     | AA     |
| GGA    | AAT    | TGT    | TCC    | TCG    | TCT    | GA     |
| TTT    | GCC    | TTT    | CTG    | GAC    | ACA    | GA     |
| GAG    | TAG    | GTA    | AGG    | GGT    | CAC    | TT     |
| GCA    | GAG    | GGC    | GTC    | TGC    | GAG    | TC     |
| AGG    | TAA    | GGC    | AGC    | AGA    | GCT    | GT     |
Table 2. Influence of Antisense and Sense Oligodeoxynucleotides to Exons of CD45 Genes on Colony Formation by CFU-GM

| Oligodeoxynucleotides | Day 7 CFU-GM (10% [vol/vol] 5637 CM) | Day 14 CFU-GM (10% [vol/vol] 5637 CM) | Day 14 CFU-GM (200 U rhuGM-CSF) |
|------------------------|--------------------------------------|--------------------------------------|----------------------------------|
| Antisense Ex-1         | +0.3 ± 4                             | -41 ± 4*                             | -55 ± 6*                         |
| Antisense Ex-2         | -0.3 ± 2                             | -46 ± 7*                             | -53 ± 6*                         |
| Antisense Ex-1 and -2  | ND                                  | -48 ± 2*                             | -51 ± 5*                         |
| Sense Ex-1             | ND                                  | +1 ± 3                               | 0 ± 4                            |
| Sense Ex-2             | -6 ± 3                               | -6 ± 2                               | -3 ± 2                            |
| Antisense Ex-4         | -5 ± 3                               | -5 ± 4                               | -0.2 ± 4                         |
| Antisense Ex-5         | +6 ± 3                               | -1 ± 3                               | +5 ± 4                            |
| Antisense Ex-6         | -0.3 ± 3                             | 0 ± 3                                | +1 ± 3                            |

Results shown averages for a total of three to six separate experiments each using LD or NALDT- normal human bone marrow cells. Control numbers of day 7 colonies stimulated with 5637 CM, day 14 colonies stimulated with 5637 CM, and day 14 colonies stimulated with GM-CSF, respectively, ranged from 33 to 105, 32 to 125, and 26 to 135. The results were similar regardless of which antisense oligo to exon 1 was used (see Table 1).

*p < 0.001.

Epo-dependent BFU-E colony formation, and this increase was completely suppressed (−26 ± 4 and −20 ± 5 with IL3, and −39 ± 2 and −39 ± 6% with GM-CSF; p < 0.001) by preincubation of cells with antisense oligos to exons 1 and 2, but not with antisense oligos to exons 4, 5, and 6, or with sense oligos to exons 1 and 2. The combination of antisense oligos to exons 1 and 2 did not decrease colony formation to a greater extent than that seen with antisense oligos to either exon 1 or exon 2. Results were similar whether LD or NALDT- cells were used.

Influence of CD45 mAbs on CSF-dependent Colony Formation

"Blocking" Experiments. As another approach, NALDT- bone marrow cells were preincubated with either control medium, anti-CD45 (9.4) or an isotype IgG2a)-matched control antibody (each at 1 μg/10^6 cells) at 4°C for 30 min before plating, without washing, in semi-solid agarose medium with different CSFs. As shown in Fig. 2, preincubation of cells with anti-CD45 monoclonal, but not with isotype-matched control, significantly (p < 0.001) suppressed colony and cluster formation by CFU-GM stimulated with rhuGM-CSF or rhuIL-3, but not with rhuG-CSF or rhuM-CSF. Similar experiments evaluated CD45 mAb on colony formation by BFU-E and CFU-GEMM (Fig. 3). CD45 antibody pretreatment had no effect on colonies formed in the presence of Epo alone, but it completely ablated Epo-dependent BFU-E and CFU-GEMM colony formation enhanced by rhuIL-3 or rhuGM-CSF.

Crosslinking Experiments with and without Subsequent Oligo Treatment. As a third approach to linking CD45 antigens with regulation of MPC proliferation, NALDT- marrow cells were treated with nonblocking concentrations of monoclonal anti-CD45 (9.4; 1 μg/10^6 cells) under crosslinking conditions for CD45 antigens. Cells were preincubated with CD45 antibody for 30 min at 4°C, washed, and incubated at 37°C for 1 h, with sheep anti-mouse IgG (1 μg/10^6 cells). Preliminary experiments had demonstrated >65% capping of CD45 on a mouse T cell line using FITC-labeled monoclonal anti-Ly-5 (CD45) under these conditions. In the present experiment (Fig. 4), treated cells were cultured in semi-solid agarose medium in the presence of different CSFs and assessed for CFU-GM colony formation. When cells were treated with anti-CD45 and a second antibody (designated capping), there was a significant decrease (p < 0.001) in
Comparative influences of rhuGM-CSF, rhuIL3, rhuG-CSF, and rhuM-CSF on inhibition of CFU-GM colony and cluster formation by "blocking" concentrations of CD45 mAb (9.4; 1 μg/10^5 cells). NALDT^- cells (2.5 x 10^4) were plated in semi-solid agarose culture medium. Results show one representative of three similar experiments. (*) Day colonies and clusters were scored. Scoring G-CSF colonies on day 14 did not change results: no inhibition was seen (data not shown).

Colony formation stimulated with rhuIL3 or rhuGM-CSF (Fig. 4, A and B), but not with rhuG-CSF or rhuM-CSF (Fig. 4, C and D). Treatment with anti-CD45 only, the isotype-matched control, or second antibody only did not decrease CSF-stimulated colony formation (data not shown). Similar results were noted for BFU-E and CFU-GEMM (data not shown), in that conditions of crosslinking, but not other conditions (anti-CD45 alone, or second antibody alone), completely suppressed rhuGM-CSF (200 U/ml)- or rhuIL3 (200 U/ml)-enhanced colony formation by Epo (1 U/ml)-dependent BFU-E- and CFU-GEMM. That CD45 capping actually occurred on MPC under crosslinking conditions was assessed on NALDT^- CD34^+HLA-DR^- normal human bone marrow cells. In this experiment, 49 ± 1 CFU-GM^+, 27 ± 3 BFU-E^-, and 14 ± 3 CFU-GEMM colonies formed per 100 cells plated in the presence of a combination of Epo (2 U/ml), rmuMGF (50 ng/ml), rhuGM-CSF (100 U/ml), and rhuIL3 (100 u/ml). In this population, capping of CD45 was apparent on ~50% of the cells 1 h after addition of FITC-labeled sheep anti-mouse IgG at 37°C. No capping was apparent with only the second antibody. Capping was also apparent on 35–50% of CD34^+ column-separated human bone marrow cells (two experiments with MPC cloning efficiencies of 10–22%).

The above results suggested involvement of CD45 antigens on rhuGM-CSF- or rhuIL3-stimulated CFU-GM, and IL3- and GM-CSF-enhanced Epo-dependent BFU-E and CFU-GEMM colony formation, but since the oligo, "blocking", and crosslinking treatments only resulted in partial decrease in colonies, subpopulations of MPC were implicated in this association. To determine if CSF-stimulated colony formation could be further reduced, NALDT^- bone marrow cells were first treated with anti-CD45 plus second antibody and then immediately incubated in suspension culture with control oligos (antisense to exon 5 or sense to exon 1) or active oligos (antisense to exons 1 or 2), added twice within 19 h, before plating cells in agarose culture medium with different CSFs (Fig. 4). Treatment for crosslinking (designated "capping" in the Fig. 4) or with antisense oligos to exon 1 or 2 significantly (p < 0.001) reduced rhuIL3 (Fig. 4 A) and rhuGM-CSF (Fig. 4 B), but not rhuG-CSF (Fig. 4 C) or rhuM-CSF (Fig. 4 D) stimulated CFU-GM colonies and clusters. Additionally, combination pretreatment (crosslinking and antisense oligos to exons 1 or 2) did not result in any further inhibition of rhuIL3- or rhuGM-CSF-stimulated colonies and clusters (Fig. 4, A and B). Combination treatment had no effect on rhuG-CSF- or rhuM-CSF-stimulated colonies and clusters (Fig. 4, C and D). As shown in Fig. 5, similar results were noted with BFU-E and CFU-GEMM.

| Treatment | BFU-E | CFU-GEMM |
|-----------|-------|----------|
| Control medium | | |
| Isotype control | | |
| Anti CD45 | | |
| EPO (1 U) | | |
| EPO (1 U) + IL-3 (200 U) | | |
| EPO (1 U) + GM-CSF (200 U) | | |

Figure 3. Comparative influences of rhuIL-3 and rhuGM-CSF on inhibition of Epo-stimulated BFU-E and CFU-GEMM colony formation by "blocking" concentrations of CD45 mAb (9.4; 1 μg/10^5 cells). NALDT^- cells (2.5 x 10^4) were plated in semi-solid methylcellulose culture medium. Results show one representative of three similar experiments.
suggest that CD45 antibody and CD45 antisense to exons 1 and 2 act on the same population of cells.

**Comparative Influences of a GM-CSF/IL3 Fusion Protein or MGF on Colony Formation of Oligo- or Anti-CD45-pretreated Bone Marrow Cells**

GM-CSF and IL-3 are early acting CSFs compared to the more lineage-restricted effects of G-CSF and M-CSF (2, 26). To further evaluate the possibility that CD45 involvement of MPC regulation was restricted to early populations of MPC, other early acting cytokines were used. As seen in Fig. 6, the GM-CSF/IL-3 fusion protein stimulated more CFU-GM colonies than GM-CSF, IL-3, or the combination of GM-CSF plus IL-3. Preincubation of cells with antisense oligo to exons 1 and 2, but not with sense oligos to exon 1 or antisense oligos to exon 4, decreased colony formation stimulated with GM-CSF, IL-3, GM-CSF plus IL-3, and the GM-CSF/IL-3 fusion protein, but did not influence colony formation stimulated by G-CSF. Inhibition was greatest with cells stimulated with the fusion protein. Both MGF and the fusion protein were compared, at maximal concentrations, for effects on BFU-E (Fig. 7 A) and CFU-GEMM (Fig. 7 B) colony formation by NALDT- marrow cells pretreated with anti-CD45, or an isotype-matched antibody control. Pretreatment of cells with anti-CD45, but not with the isotype control, inhibited enhanced Epo-dependent colony formation by cells stimulated with IL-3, the fusion protein, or MGF; the more colonies that formed in response to cytokine, the greater the degree of suppression with anti-CD45. Similar effects were seen when CFU-GM were evaluated (data not shown). MGF enhances size, as well as number of colonies formed (19-23). Colonies formed in the presence of MGF were much larger than those formed in the absence of MGF. Pretreatment of cells with antisense oligo to exons 1 or 2 or with anti-CD45 greatly reduced the size of colonies formed in the presence of MGF, in most cases to that seen in the

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**Figure 4.** Comparative influence of CSF on inhibition of CFU-GM colony and cluster formation by treatment of cells with CD45 mAb (1 μg/10⁶ cells) plus a second antibody under conditions conducive for crosslinking of CD45 antigens and then with antisense oligos to exons 1 and 2 of the human CD45 gene. Treated NALDT- cells were then plated at 2.5 x 10⁴/ml in semi-solid agarose culture medium with (A) rhulIr3; (B) rhuGM-CSF; (C) rhuG-CSF; and (D) rhuM-CSF and cultures scored after 14 d of incubation. Results show one representative of two complete experiments with similar results.
Figure 5. Comparative influences of rhuIL-3 and rhuGM-CSF on inhibition of BFU-E colony formation by treatment of cells with CD45 mAb (1 μg/10^6 cells) plus a second antibody under conditions conducive for crosslinking of CD45 antigens and then with antisense oligos to exons 1 and 2 of the human CD45 gene. NALDT- cells (2.5 x 10^4/ml) were plated in semi-solid methylcellulose culture medium. Results show one of two similar experiments.

The antigenic density profile for CD45, shown in Fig. 8, demonstrates that treating CD34+ column-enriched human bone marrow MPC with antisense oligos to exon 1 of the CD45 gene decreases within 6 h the density expression of CD45 antigens and then with antisense oligos to exons 1 and 2 of the human CD45 gene. NALDT- cells (2.5 x 10^4/ml) were plated in semi-solid methylcellulose culture medium. Results show one of two similar experiments.

The optimal cloning efficiency in these experiments was 50% for CFU-GM plus BFU-E when 125 cells were plated in the presence of Epo (1 U/ml) and 10% (vol/vol) 5637CM. In this population, treatment of cells with antisense oligos to exons 1 and 2 (Fig. 9 A) or anti-CD45 (Fig. 9 B) suppressed colony formation stimulated by IL-3, GM-CSF, or MGF. This substantiated that the effects of antisense oligos and anti-CD45 were directly on the MPC and not mediated by accessory cells.

Discussion

CD45 antigenic determinants have previously been implicated in lymphocyte activation and signal transduction (7, 8, 16, 17). The results presented herein now link these antigens with myelopoietic regulatory events. A number of approaches lead us to conclude that CD45 antigens on human MPC are involved in the regulation of stimulation of these cells by early acting cytokines such as GM-CSF, IL-3, a GM-CSF/IL-3 fusion protein, and MGF, but not by the more lineage-restricted growth factors (Epo, G-CSF, or M-CSF). The results also suggest that CD45 involvement reflects effects primarily in earlier, more immature progenitors (CFU-GEMM and subpopulations of BFU-E and CFU-GM), rather than in more mature and lineage-restricted progenitors (CFU-G and CFU-M). The approaches used to elucidate a role for CD45 in myeloid cell regulation included use of: (a) antisense oligos to exons of the CD45 gene; (b) "blocking" experiments with mAb to CD45 antigenic determinants; (c) crosslinking experiments with anti-CD45 and sheep anti-mouse IgG; and (d) combined crosslinking and antisense oligo experiments. Results with LD and NALDT- cells were confirmed using enriched populations of MPC in CD34+ column-separatated and highly enriched populations of MPC in NALDT- sorted CD34+HLADR+ cells.

In the first case, antisense oligos to exons 1 and 2 of the CD45 gene, but not sense oligos to exons 1 and 2, or antisense oligos to the alternative splicing exons 4, 5, and 6, decreased colony formation of human marrow CFU-GM stimulated by GM-CSF alone and in combination, by a potent GM-CSF/IL-3 fusion protein, and by GM-CSF plus MGF, but not by G-CSF or M-CSF. The greater the stimulation, the more apparent the inhibition. Also, antisense oligos to exons 1 and 2, but not sense oligos to exon 1 and 2, or antisense oligos to exons 4, 5, and 6, completely suppressed the enhanced Epo-dependent BFU-E, and most of the Epo-dependent CFU-GEMM colony formation noted in the presence of IL-3, GM-CSF, the GM-CSF/IL-3 fusion protein, and MGF. Oligo pretreatment had little or no effect on the more mature populations of BFU-E stimulated only in the presence of Epo. An enriched population of CD34+ column-separated cells gated in the MPC-containing region was used to directly demonstrate that the antisense to exon 1
Figure 6. Comparative influences of rhuGM-CSF, rhuIL-3, rhuGM-CSF plus rhuIL-3, rhuGM-CSF/IL-3 fusion protein, and rhuG-CSF on inhibition of CFU-GM colony formation by antisense oligodeoxynucleotides to exons 1 and 2 of the human CD45 gene. Cells were plated in semi-solid agarose culture medium. Results show one of three similar experiments.

decreased numbers of CD45 cells as well as density distribution of CD45 antigens. Incomplete suppression of CD45 surface antigens in this case probably reflects the turnover rate of CD45 antigens (31). The possibility that the antisense oligos made to exons 1 and 2 of the CD45 gene were recognizing non-CD45 genetic material and decreasing production of non-CD45 proteins involved in myeloid cell regulation is considered highly unlikely. Suppression of MPC proliferation was attained with three different and nonoverlapping complementary oligos (20 mers to exons 1 and 2 of the CD45 gene). Furthermore, the oligo results were substantiated by antibody “blocking” and crosslinking experiments.

In the second case, mAb to framework CD45 antigens, when used at relatively high concentrations (1 μg/10^6 cells), but not isotype-matched controls, decreased colony formation of CFU-GM stimulated by GM-CSF, IL-3, the GM-CSF/IL-3 fusion protein, and GM-CSF plus MGF, but not by G-CSF or M-CSF. Antibody to CD45 also completely blocked IL-3, GM-CSF, GM-CSF/IL-3 fusion protein, and MGF enhancement of Epo-dependent BFU-E and CFU-GEMM colony formation, but it had no effect on colonies stimulated by Epo only. These results thus duplicate those seen with antisense oligos to exons 1 and 2 of the CD45 gene, although it is unclear at this time how the CD45 antibody inhibition is mediated. Two possibilities include blocking an interaction of a cytokine-receptor complex with CD45 antigens on the surface, or a downmodulating proliferative response signaled through a CD45 antibody–CD45 surface antigen complex.

In the third situation, monoclonal anti-CD45, when used at lower, non-“blocking” concentrations (1 μg/10^6 cells), but in combination with a second antibody under crosslinking conditions, resulted in comparable inhibition of GM-CSF, IL-3, GM-CSF/IL-3 fusion protein, and MGF stimulated colony formation of CFU-GM, BFU-E, and CFU-GEMM to that noted when cells were pretreated with antisense oligos to exons 1 or 2 of the CD45 gene or with “blocking” concentrations of anti-CD45. Neither the CD45 antibody itself
Figure 7. Comparative influences of rhuIL-3, rhuGM-CSF/IL3 fusion protein, and rmuMGF on inhibition of Epo-stimulated BFU-E (A) and CFU-GEMM (B) colony formation by monoclonal anti-CD45. NALDT- cells were plated in methylcellulose. Two different experiments are shown each in A and B.

(at 1 μg/10^6 cells), nor the second antibody itself resulted in reduced colony formation, and the isotype control plus second antibody did not suppress colony formation. That crosslinking resulted in actual capping was demonstrated with a population of sorted CD34^+ HLA-DR^+ that contained 90% MPC.

To test the possibility that lack of complete suppression of colony formation of MPC was due to incomplete treatment to decrease both CD45 surface antigens and CD45 antigen expression, cells were first treated with CD45 antibody plus a second antibody to crosslink CD45 antigens, and the cells then pretreated with antisense oligos to exons 1 or 2 of the CD45 gene to decrease expression of this gene and thus subsequent CD45 protein production. This combination of procedures did not result in further inhibition beyond that seen with either procedure itself.

The use of highly enriched populations of MPC in the CD34^+ HLA-DR^+ fraction of sorted NALDT^- cells, where at least one of two cells was a MPC, confirms that the oligo and antibody approaches had a direct effect on MPC, since the results were equivalent to that seen with unseparated bone marrow. Receptor genes for GM-CSF (32), IL-3 (33), MGF (c-kit ligand) (19–21), and Epo (34) are known, making it clear that CD45 antigens are not acting as the specific receptor for this diverse group of cytokines. However, CD45 molecules do appear to be associated with the action of these cytokines, suggesting that proliferative signals of these cytokines may share common transducing pathways. In this context, the following information may be of relevance. In lymphocytes, CD45 expression is required for antigen-induced proliferation of T cell clones or TCR complex (CD3/Ti)-induced calcium mobilization (16, 35). CD45 crosslinking causes inhibition of phosphatidyl-inositol specific phospholipase C (PI-PLC) activation in T cells (8, 17, 36). The PI-PLC-activated CD3/Ti stimulation requires tyrosine phosphorylation (37), and CD45 ligation prevents tyrosine phosphorylation of specific substrates during either CD2- or CD3-mediated activation (17). Stimulation of some of the receptors studied in the present report, including those for IL-3, GM-CSF, and MGF, induces rapid tyrosine phosphorylation of cellular substrates (38). Phospholipase C γ can be activated as one of these substrates by epidermal growth factor and
platelet-derived growth factor receptors (39, 40). The data presented here suggest that CD45 and its associated tyrosine phosphatase activity may interact with the tyrosine phosphorylation cascade induced after growth factor binding to receptors on MPC. Since T cell, CD2, CD4, and CD8 antigens have been linked with CD45 antigens and associated with internal membrane tyrosine-protein kinase p56^ck, and CD45 phototyrosine phosphatase activates T cell tyrosine protein kinase p56^ck (16, 37, 41-43), it is possible that this phosphoprotein (p56^ck) or other src family kinases may also be involved in the regulation of MPC proliferation by early acting cytokines. Testing these possibilities will probably require use of established factor-dependent cell lines whose proliferative response can be shown to be linked to CD45 antigens.
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