FEBS Letters 348 (1994) 197–200

FEBS 14228

Antisense oligonucleotide complementary to endogenous retroviral MCF *env* gene inhibits both BFU-E and CFU-S colony formation in mice

Igor V. Chernukhin*, Sophia K. Khaldoyanidi, Dina V. Dikovskaya, Feodor P. Svinarchuk, Valentin V. Vlasov, Konstantin V. Gaidul

Institute of Clinical Immunology, Novosibirsk 630091, Russian Federation
Institute of Bioorganic Chemistry, Novosibirsk 630090, Russian Federation

Received 15 April 1994; revised version received 1 June 1994

Abstract

A possible biologic activity of endogenously expressed *env* sequence of retroviral mink cell focus-forming virus (MCF) genome for hematopoietic colony formation was studied in mice. Antisense 20-mer complementary to MCF *env* sequence was used to detect the result of blockage of this gene translation on the potency of marrow cells to form colonies of erythroid (BFU-E), myeloid granulocyte-macrophage (CFU-GM), and stem cell (day 11 CFU-S) hematopoietic compartments. A large relative decrease in BFU-E number was found in bone marrow cell cultures preincubated with antisense oligonucleotide during 4 h, whereas CFU-GM colonies remained unaffected. A marked reduction of CFU-S colony formation was also registered under antisense oligomer influence. Following a decreased proliferation of erythroid progenitors, we suggest the mechanism by which antisense oligonucleotide could cause the loss of colony formation. Taken together, these data allow to propose that the expression of this gene is naturally significant for hematopoietic progenitor activity exerting some property of *env* gene products to regulate the growth of erythroid and multilineage hematopoietic precursors.

Key words: Endogenous retroviral sequences; MCF gene; Antisense oligonucleotide; BFU-E; CFU-GM; CFU-S

1. Introduction

The murine genome contains a certain number of copies of type C-related retroviral sequences which are expressed in many host tissues mostly by *env* gene products: surface glycoprotein gp70 (SU) and transmembrane protein p15E (TM) [1]. They are closely related to those of infectious retroviruses and divided into ecotropic, xenotropic and mink cell focus-forming viruses (MCF) *env* sequences [1–3]. Although numerous studies exhibit their great potential in question to be involved in leukemia genesis [1], the biologic function of these retrovirus-related sequences is rather unclear as yet. The increased expression of cell retroviral sequences which appears in immune cells in response to polyclonal mitogen activation [4–6], hormone influence [7] indicates, however, that the endogenous provirus expression is closely related to forming the active cell status.

In view of their role in the host resistance to retroviral leukemias, the study of expression of endogenous retroviral genes demonstrated that immature hematopoietic erythroid and multilineage precursors are the most frequent cells effectively expressing endogenous MCF-related SU [8]. Moreover, this expression was shut down as a consequence of differentiation of these cells to more mature forms [8]. Therefore, it may be proposed that the expression of this gene in certain hematopoietic lineage cells also relates to activity of these cells.

In the present study we have analyzed the influence of MCF *env* antisense oligonucleotide on hematopoietic progenitor colony formation using an in vitro culture system that allows colony formation by the unipotent erythroid progenitors, BFU-E, and granulocyte-macrophage progenitors, CFU-GM. An in vivo CFU-S colony formation was considered to determine a possible role of MCF *env* gene expression for stem cell activity. In this study we have used the antisense strategy, a widely used approach allowing to elucidate the function of a certain target gene. This approach was successfully applied to investigate the action of regulatory genes both for lymphocyte activation [9–11] and hematopoietic cell activity [12,13] in several culture systems.

*Corresponding author. *Present address:* Siberian Branch of the Russian Academy of Medical Sciences, 2 Timakov Street, rm. 152, 630117 Novosibirsk, Russia. Fax: (7) (383) 232-4339.

Abbreviations: SU, surface glycoprotein; TM, transmembrane protein; MCF, mink cell focus-forming virus; BFU-E, erythroid burst-forming unit; CFU-GM, granulocyte-macrophage colony-forming unit; CFU-S, spleen colony-forming unit; Epo, erythropoietin.

2. Materials and methods

2.1. Oligonucleotides

Antisense 20-mer was synthesized by a solid-phase technique using phosphoramidite chemistry and was > 99% pure after purification by HPLC. The sequence of antisense is: 5'GAGAACGCTGGACCTTCCAT3' and sense (as a control): 5'ATGGAAGGTCCAGCGTTCTC3' [14].

SSDI 0014-5793(94)00614-2

2.2. Mice

4–6-week-old female C57Bl/6 mice were used.

2.3. Cell treatment

10^7 cells were exposed to 20 μM antisense or control (sense) oligonucleotide in 1 ml of McCoy medium containing 0.3% BSA (without nuclease activity; V fraction, Sigma, USA) at 37°C. Six hours later the oligonucleotides were removed by centrifugation and the cells were plated for an in vitro colony formation assay or were used for exogenous CFU-S colony formation assay.

2.4. Colony formation assays

5×10^4 nucleated bone marrow cells per well were incubated in 0.33% semisolid agar culture containing McCoy medium supplemented with 10% fetal calf serum (FCS), 10% horse serum, 1% BSA, 10% Wehi-3 cell conditioned medium, 2 mM glutamine, 5×10^{-4} M 2-mercaptoethanol in 24-well plates at 37°C in 5% CO_2/air. CFU-GM cultures were supplemented with 5% L929 cell conditioned medium and colonies of at least 50 cells were counted after 7 days. BFU-E colonies were maintained in the presence of 5 U/ml of erythropoietin (Epo) and bursts containing at least 500 cells were counted after 10 days.

2.5. Spleen colony formation assay

CFU-S were assayed by the method of Till and McCulloch [15]. Day 11 CFU-S were counted and results were expressed as the number of spleen colonies per 10^5 cultured marrow cells.

2.6. [^{3}H]Thymidine suicide assay

Cell suspension (10^7/ml) was incubated with 250 μCi [^{3}H]thymidine (Td) in 1 ml of complete medium at 37°C for 30 min. After washing 3 times, the cells were resuspended in the medium and used as indicated above for in vitro colony formation assays.

2.7. Immunoprecipitation

After 2 h incubation in culture medium containing either sense or antisense oligomer, or none of them, the cells were metabolically labeled during 1 h with 100 μCi/ml of [^{14}C]leucine in leucine-free medium containing 5% dialysed FCS. After an additional 2 h-incubation in normal culture medium, the labeled cells were washed and then lysed in extraction buffer (50 mM Tris, pH 7.9, 0.2 M NaCl, 2% NP-40, 0.5% deoxycholate, 1 mM EDTA). A first precipitation was done with 250 μl of a 10% protein A-Sepharose. Immunoprecipitations were carried out by incubating each cell extract with 5 μl of anti-TM p15E rabbit antisera. These antisera were generated against synthetic 10-amino acid peptide (LQNRRGLDLL), conjugated to BSA, which represents an immunosuppressive domain of p15E of infectious [16] and endogenous MCF retroviruses both [14]. The immunocomplexes were precipitated with the protein A-Sepharose and then resolved by 10% SDS-PAGE.

3. Results and discussion

To gain more information on biologic function of endogenous MCF *env* gene expression for hematopoietic cell activity, we attempted to block the translation using synthetic 20-mer complementary to translation start codons.

Production of TM p15E peptide encoded by MCF genome [14] was assessed to control the influence of antisense oligonucleotide. Immunoprecipitation of ^{14}C-labeled cell proteins with anti-p15E polyclonal rabbit antisera revealed that the cells incubated with antisense oligonucleotide featured the loss of radioactive band of 15K size in contrast to the cells incubated with sense oligonucleotide (Fig. 1). It may be suggested that inhibition of endogenous env gene expression using antisense strand oligonucleotide resulted in decrease in corresponding protein synthesis.

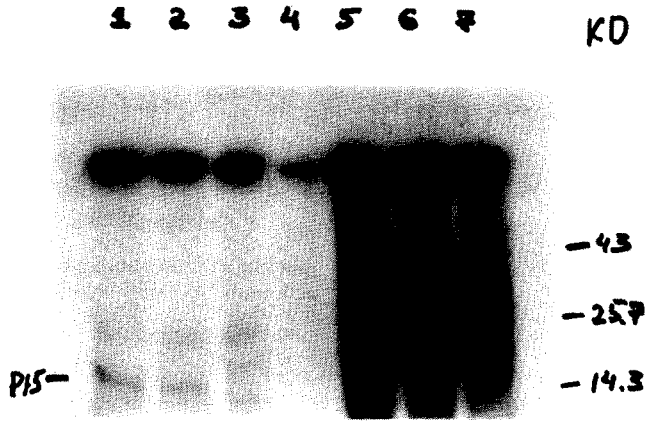


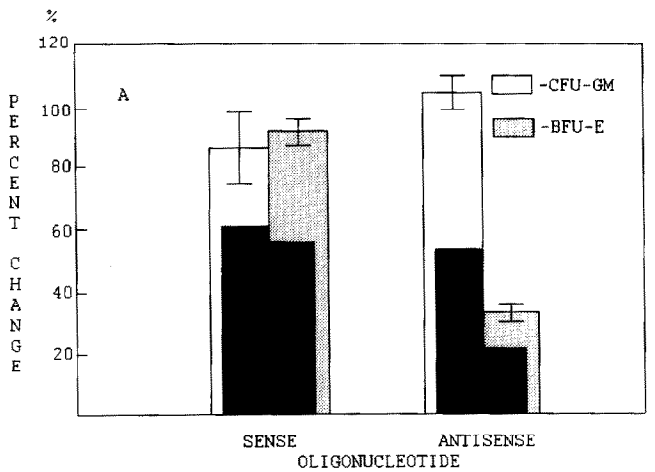
Fig. 1. Precipitation of ^{14}C-labeled cell proteins with anti-p15E antisera after incubation with either sense (line 2) or antisense oligonucleotide (line 3), or without any (line 1). Line 4 = precipitation of labeled cell proteins with preimmune rabbit sera. Equal numbers of trichloroacetic acid-precipitable counts were loaded directly onto the gel as a control of total protein synthesis: line 5 = untreated cells; line 6 and 7 = the cells treated with sense and antisense oligomer, respectively.

Such effect occurred as a result of specific cell association with antisense oligomer. When radiographied, [32P] 5′-end-labeled antisense oligonucleotide could enter only viable marrow cells. Fixation of the cells with glutaraldehyde resulted in a loss of cell-binding radioactivity suggesting that the association does not mean only nonspecific membrane-binding interaction.

Bone marrow cells were incubated with 20 μM either of antisense or sense oligonucleotide for 6 h prior to plating in semisolid culture for colony formation assays. The results of these experiments are illustrated in Fig. 2. As shown, antisense oligonucleotide caused 61% decrease in BFU-E colony number whereas CFU-GM colonies were unaffected (Fig. 2A). In order to find out whether the reduction in BFU-E colony formation occured due to an actual loss of proliferation of erythroid progenitor cells, in vitro [^{3}H]thymidine suicide assay was carried out. In contrast to controls, antisense oligonucleotide treatment was followed by 2.5-fold decrease in the number of cells in S phase (Fig. 2A). However, no significant change in the growth of GM hematopoietic progenitors was noted in any case. Therefore, the effect of blockage of MCF gene expression by antisense strand in hematopoietic progenitor cells resulted in inhibition of burst-forming cells, while myeloid cell compartment was not affected.

As shown in Fig. 2B, the growth of bursts was blocked by the antisense oligonucleotide in a dose-dependent manner. The decline in burst growth had a peak at from 10 to 20 μM concentrations of antisense oligomer. The decline was not due to non-specific toxicity because the viability of cells treated with the oligonucleotide remained similar to that of untreated cells, as determined by staining with Trypan blue.

Next we wished to study whether MCF *env* gene expression is also relevant to stem cell activity. CFU-S colony formation was applied to assess a possible change in activity of most earlier hematopoietic progenitors. As follows, the antisense oligonucleotide caused 48% inhibition in CFU-S colony formation (Fig. 3) in case bone marrow cells were preincubated 6hr prior to transplantation into recipient mice, whereas the control (sense) oligonucleotide did not change the CFU-S activity significantly.



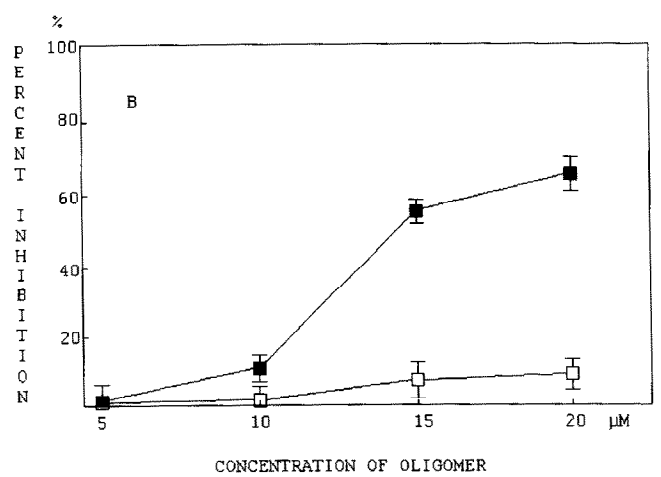


Fig. 2. (A) Inhibitory effect of antisense oligonucleotide on BFU-E colony formation. Bone marrow cells were 6-h-preincubated with either 20 μM of antisense, sense, or no oligonucleotide prior to plate in semisolid culture for colony formation assays. Colonies from quadruplicates were scored and averaged together and percent changes relatively to control (no oligonucleotides) were calculated and presented ± S.EM.. Percentages of cells entering S-phase determined by [^{3}H]Td suicide assay are shown as solid bars within each group. The percentage of killing with [^{3}H]Td was calculated as follows: $x = a - b/a \times 100$; where a = number of colonies with medium alone, b = number of colonies with [^{3}H]Td. (B) Dose-dependent inhibition of erythroid progenitor growth under the antisense oligonucleotide influence (■) and lack of inhibition of a control sense strand oligonucleotide (□). Bone marrow cells were preincubated for 6 h with indicated doses of oligonucleotide and assayed for BFU-E colony formation. Results are shown as percent of inhibition as compared with non-treated cells.

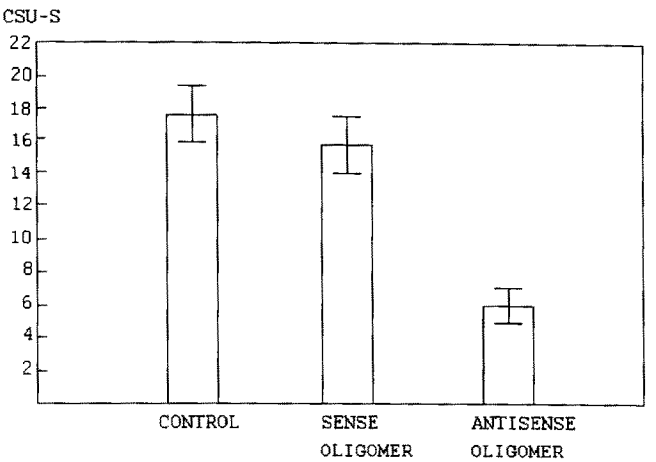


Fig. 3. Effect of antisense oligonucleotide on day 11 CFU-S colony formation. Bone marrow cells were incubated with oligonucleotides (20 μM) for 6 h, washed and exposed to lethally irradiated mice for exogenous spleen colony formation. Number of CFU-S per 10^5 cultured marrow cells was calculated. The data represent the pooled results of three separate experiments.

Summarizing, an inhibition of MCF *env* gene expression using antisense oligomer resulted in a relative loss of erythroid and CFU-S-forming cell colony growth. The decreased colony formation could be caused by restrained cells entering active cell cycle status, in part, for erythroid lineage progenitors. Since this type of hematopoietic cells also expresses most products of this gene [8], it may be then proposed that MCF env peptides exert some autocrine growth-specific activity for these cells. It is now well established that *env* gp55 peptide of Friend spleen cell focus-forming viruses exerts transforming activity toward erythroid precursor cells promoting erythroleukemia generation in mice [17–19]. This recombinant peptide is known to contain several class-specific sequences including those MCF-related [20,21]. The target provided by this peptide was shown to be Epo receptor on erythroid precursors [22–24]. After infection of mice with ecotropic Friend murine leukemia virus, recombinant viruses emerge which also include sequences derived from endogenous MCF genomes [25,26]. SU gp70 of such recombinant has an N-terminus closely related to gp55 and can bind to the Epo receptor to induce growth factor independence in an IL-3-dependent cell line [27]. Therefore,it is suggested that endogenously acquired MCF-related sequences within these peptides are involved into erythroid-promoting activity as oncogene-like derived sequences. Normally, endogenous MCF *env* gene expresses proteins which probably exert growth-regulating activity specific for erythroid and early multilineage hematopoietic progenitors.

References

[1] Zijlstra, M. and Melief, C.J.M. (1986) Biochem. Biophys. Acta 856, 197–231.
[2] Stoye, J.P. and Coffin, J.M. (1988) J. Virol. 62, 168.
[3] Frankel, W.N., Stoye, J.P., Taylor, A.T. and Coffin, J.M. (1990) Genetics 124, 221–236.
[4] Greenberg, J.S., Phillips, S.M., Stephenson, J.R. and Aaronson, S.A. (1975) J. Immunol. 115, 317–320.
[5] Krieg, A.M., Khan, A.S. and Steinberg, A.D. (1988) J. Virol. 62, 3545–3550.
[6] Itoh, Y., Maruyama, N., Kitamura, M., Shirasawa, T., Shigemoto, K. and Koike, T. (1992) Clin. Exp. Immunol. 88, 356–359.
[7] Helmberg, A., Fassler, R.S., Geley, R., Johrer, K., Kroemer, G., Bock, G. and Kofler, R. (1990) J. Immunol. 145, 4332–4337.
[8] Buller, R.S., Van Zant, G., Eldridge, P.W. and Portis, J.L. (1989) J. Exp. Med. 169, 865–880.
[9] Krieg, A.M., Gause, W.C., Gourley, M.F. and Steinberg, A.D. (1989) J. Immunol. 143, 2448–2451.
[10] Harel-Bellan, A., Ferris, D.K., Vinocour, M., Holt, J.T. and Farrar, W.L. (1988) J. Immunol. 140, 2431–2435.
[11] Heikkila, R., Schwab, G., Wickstrom, E., Loke, S.L., Pluznik, D.H., Watt, R. and Neckers, L.M. (1987) Nature 328, 445–449.
[12] Gewirtz, A.M. and Calabretta, B. (1988) Science 242, 1303–1306.
[13] Holt, J.T., Redner, R.L. and Nienhuis, A.W. (1988) Mol. Cell. Biol. 8, 963–973.
[14] Levy, D.E., Lerner, R.A. and Wilson, M.C. (1985) J. Virol. 56, 691–700.
[15] Till, J.E. and McCulloch, E.A. (1961) Radiat. Res. 14, 213–222.
[16] Ruegg, C.L., Monell, C.A. and Strand, M. (1989) J. Virol. 63, 3250–3256.
[17] Linemeyer, D.L., Menke, J.G., Ruscetti, S.K., Evans, L.H. and Scolnick, E.M. (1982) J. Virol. 143, 223–233.
[18] Wolff, L. and Ruscetti, S. (1985) Science 228, 1549–1552.
[19] Li, J.-P., Bestwick, R.K., Spiro, C. and Kabat, D. (1987) J. Virol. 61, 2782–2792.
[20] Wolff, L., Scolnick, E. and Ruscetti, S. (1983) Proc. Natl. Acad. Sci. USA 80, 4718–4722.
[21] Wolff, L., Kaminchik, J., Hankins, W.D. and Ruscetti, S.K. (1985) J. Virol. 53, 570–578.
[22] Ruscetti, S.K., Janesch, N.J., Chakraborti, A., Sawyer, S.T. and Hankins, W.D. (1990) J. Virol. 63, 1057–1062.
[23] Li, J.-P., D'Andrea, A.D., Lodish, H.F. and Baltimore, D. (1990) Nature 343, 762–764.
[24] Yoshimura, A., D'Andrea, A.D. and Lodish, H.F. (1990) Proc. Natl. Acad. Sci. USA 87, 4139–4143.
[25] Ishimoto, A., Adachi, A., Sakai, K. and Tsuruta, T. (1981) Virology 113, 644–655.
[26] Ruscetti, S., Davis, L., Feild, J. and Oliff, A. (1981) J. Exp. Med. 154, 907–920.
[27] Li, J.-P. and Baltimore, D. (1991) J. Virol. 65, 2408–2414.