Antisense RNA Inhibition of Phosphoprotein p18 Expression Abrogates the Transformed Phenotype of Leukemic Cells

Sima Jeha, Xiang-Nong Luo, Miloslav Beran, Hagop Kantarjian, and George F. Atweh

ABSTRACT

Phosphoprotein p18 was identified originally on the basis of its very high level of expression in leukemic cells of different lineages. Changes in the level of p18 accumulation and phosphorylation associated with induction of differentiation of leukemic cells suggested a potential role for this phosphoprotein in cellular proliferation and differentiation and possibly in malignant transformation. Recent studies have demonstrated that p18 plays an important role in cell cycle progression by serving as a substrate for p34^cdk2 kinase. These studies showed that inhibition of p18 expression in leukemic cells results in growth retardation and accumulation of cells in G2-M. In this study, we explore the potential role of p18 in cellular transformation by investigating the effects of inhibition of p18 expression on the malignant phenotype of K562 erythroleukemia cells. These studies show that antisense inhibition of p18 expression in leukemic cells results in growth arrest at a lower saturation density, loss of serum independence, and loss of anchorage-independent growth in vitro. In addition, inhibition of p18 expression results in a marked inhibition of tumorigenicity of leukemic cells in vivo. In the severe combined immune deficiency mouse model. These studies demonstrate that the high level of p18 expression in leukemic cells is necessary for the maintenance of the transformed phenotype and suggest p18 as a potential target for antileukemic interventions.

INTRODUCTION

Phosphoprotein p18 (also known as prosolin, stathmin, metablastin, and Op18) is expressed at high levels in many cancers, including acute leukemia (1, 2), malignant lymphoma (2, 3), neuroblastoma (4), hepatocellular carcinoma, colon carcinoma, multiple myeloma (5), and probably many others. In addition, the very low level of p18 expression in quiescent, nontransformed lymphocytes increases markedly when the cells are induced to proliferate by exposure to mitogenic stimuli (6, 7). Similarly, carbon tetrachloride-induced liver regeneration is associated with the induction of p18 mRNA expression in hepatocytes (6). In contrast, when highly proliferative, undifferentiated leukemic cells are induced to undergo terminal differentiation in vitro, the level of p18 is reduced markedly in the nonproliferative, differentiated cells (3, 6, 8, 9). Major changes in the state of phosphorylation of p18 also have been identified in the transformed phenotype and suggest p18 as a potential target for antileukemic interventions.

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3 G. F. Atweh, unpublished observations.

The abbreviations used are: DHFR, dihydrofolate reductase; MTX, methotrexate; FBS, fetal bovine serum; ScID, severe combined immune deficiency; Ph'. Philadelphia chromosome-positive.

MATERIALS AND METHODS

Antisense Inhibition of p18 Expression in K562 Erythroleukemia Cells

The strategy of antisense inhibition of p18 expression has been described in detail previously (11). Briefly, antisense p18 mRNA is expressed from a human p18 cDNA cloned in the reversed transcriptional orientation downstream of the SV40 early promoter. This p18 antisense expression vector includes a DHFR* transcription unit, which allows the amplification of the transfected genes in the presence of increasing concentrations of MTX (18). Stable cell lines were established by cotransfection with a neomycin resistance (neo’) gene. K562.DHFR refers to a K562 cell line in which the parental pSV.DHFR expression vector is integrated without any p18 sequences (11). K562.DHFR.p18(-)(1 μM MTX) refers to a K562 cell line in which the integrated antisense p18 genes are amplified to about 100 copies/diploid genome following prolonged exposure to 1 μM MTX. K562.DHFR.p18(-)(50 μM MTX) refers to a K562 cell line in which the integrated antisense p18 genes are amplified to 120–200 copies/diploid genome following prolonged exposure to 50 μM MTX (11). Our previous analysis of these cell lines showed a profound decrease in p18 mRNA expression associated with the MTX-induced amplification of the antisense constructs (11).

Assessment of Saturation Density

The control cell line, K562.DHFR, and the two p18 antisense cell lines, K562.DHFR.p18(-)(1 μM MTX) and K562.DHFR.p18(-)(50 μM MTX),
were seeded in RPMI 1640 supplemented with 10% FBS at a density of 2.5 \times 10^6 cells/ml. The cells were allowed to grow in a humidified 5% CO_2 environment until saturation density was reached. The tissue culture media were replaced daily to ensure that growth arrest would be a function of cell density rather than media depletion. Viable cell counts were determined daily in a hemocytometer by trypan blue dye exclusion. Each experiment was performed in triplicate, and the data (mean ± SD) were plotted graphically to generate growth curves for every cell line at the four different serum concentrations.

Assessment of Serum Dependence

The growth rates of the control K562 cell line and the two p18 antisense K562 cell lines were measured at different concentrations of FBS. All cells were seeded initially at a density of 2.5 \times 10^6 cells/ml. Growth of each of the three cell lines was determined at four different serum concentrations (10, 2, 0.5, and 0.25%). Viable cell counts were determined daily in a hemocytometer by trypan blue dye exclusion. Each experiment was performed in triplicate, and the data (mean ± SD) were plotted to generate growth curves for every cell line at the four different serum concentrations.

Assessment of Clonogenicity in Semisolid Media

The same three cell lines described above were analyzed in a soft agar clonogenic assay as described previously (19). Briefly, a feeder layer consisting of RPMI 1640 with 30% FBS and 0.5% low-melting point agarose was plated in 100-mm tissue culture dishes. The feeder layer was overlaid with a top layer that included 10^5 cells plated in RPMI 1640 supplemented with 30% FBS and 0.35% low-melting point agarose. Colonies that formed were counted after 2 and 4 weeks. The experiment was repeated three times, and the results were expressed as mean ± SD.

Assessment of Tumorigenicity in SCID Mice

Mice Injections. The same three K562 cell lines described above were injected into 6-8-week-old female CB17-SCID/SCID mice (Taconic, Germantown, NY). The mice were kept in a pathogen-free environment in the animal facility of the University of Texas M. D. Anderson Cancer Center. They were housed in microisolator cages containing autoclaved bedding, food, and water with no added antibiotics. Each mouse was injected i.v. with 2 \times 10^7 cells in 0.1 ml PBS through the tail vein. The mice were monitored closely and were sacrificed either when they became moribund or at the completion of the study. The mice were not preconditioned prior to the injections, and no growth factors were administered.

Molecular Analysis. The presence of human DNA in mouse tissue was assessed by the PCR using primers specific for DNA sequences of the human HLA-DQα gene (20). DNA was prepared from fresh or frozen tissues as described (21). Specific positive and negative controls were used in every PCR assay. The amplified products were separated by electrophoresis in a 1.6% agarose gel. After staining with ethidium bromide, human DNA was detected by the presence of a 225-bp amplified fragment. DNAs from tumors and normal tissues were also analyzed for the presence of human sequences by Southern blotting. Ten μg of each DNA were digested with BamHI and probed with neo sequences. Autoradiography was performed for 1 week at −70°C with an intensifying screen to enhance the ability to detect very weak signals.

RESULTS

There are a number of in vitro and in vivo assays that can distinguish between the phenotypes of transformed and nontransformed cells. We have used three in vitro and one in vivo assays to analyze the effect of antisense inhibition of p18 expression on the transformed phenotype of K562 erythroleukemia cells. First, we compared the saturation density of the control K562.DHFR cells with that of the p18 antisense-inhibited cells (Fig. 1). The highest cell density that was reached by the control K562.DHFR cells was 3.3 \times 10^6 cells/ml. The K562.DHFR.p18(−) [1 μM MTX] cells reached saturation at a density of 1.2 \times 10^6 cells/ml, whereas the K562.DHFR.p18(−) [50 μM MTX] cells reached saturation at a density of 4.4 \times 10^5 cells/ml. After the cultures reached these high densities, cell death resulted in a progressive decrease in the number of viable cells in all three cell lines (Fig. 1).

The second in vitro assay that we used is an assay of serum dependence. We analyzed the growth of the p18 antisense-inhibited cell lines and the control cell line at four different serum concentrations (10, 2, 0.5, and 0.25% FBS; Fig. 2). In the K562.DHFR control cells, the growth rate decreased modestly as the serum concentration was lowered. However, these cells were still capable of vigorous growth in culture even at a serum concentration of 0.25%. A progressive decrease in the growth rate was also seen when the K562.DHFR.p18(−) [1 μM MTX] cells were placed in media with low serum concentrations (Fig. 2B). Low serum concentrations had the biggest effect on the growth of the K562.DHFR.p18(−) [50 μM MTX] cells (Fig. 2C). In this case, when the serum concentration was reduced to 0.5 or 0.25%, total suppression of cell growth was noted (Fig. 2C). These experiments demonstrate that the growth of p18 antisense-inhibited cells is dependent on relatively high serum concentrations, whereas the control K562 cells are capable of growth at very low serum concentrations.

The third in vitro assay of transformation that we used is a clonogenic assay of growth in soft agar. When compared with the K562.DHFR cells, the clonogenic potential of the p18 antisense-inhibited cells was decreased by 66% in the K562.DHFR.p18(−) [1 μM MTX] cells and by ~ 93% in the K562.DHFR.p18(−) [50 μM MTX] cells (Fig. 3). In addition to the objective decrease in the number of colonies that developed from the p18 antisense-inhibited cells, our subjective assessment of colony size suggested that the colonies that formed from the p18 antisense-inhibited cells were generally smaller than the control colonies. There was no further increase in the size or number of either type of colony after prolonged culture up to 4 weeks in semisolid medium.
We also performed experiments in mice with SCID to investigate the effect of antisense inhibition of p18 expression on the malignant behavior of leukemic cells in vivo. In a pilot experiment, three mice were injected with K562.DHFR cells, and two mice were injected with K562.DHFR.p18(−) [50 μM MTX] cells. All three mice in the first group developed abdominal tumors within 1 month after injection. They were sacrificed on day 40 when they became moribund with massive abdominal tumors. In contrast, the two mice injected with K562.DHFR.p18(−) [50 μM MTX] cells did not have any tumors when they were sacrificed on days 49 and 81.

In a more definitive experiment, 24 mice were divided into three groups, each consisting of eight mice. The mice in group 1 were injected with K562.DHFR cells, those in group 2 were injected with K562.DHFR.p18(−) [1 μM MTX] cells, and those in group 3 were injected with K562.DHFR.p18(−) [50 μM MTX] cells. In group 1, one mouse died immediately after injection. The remaining seven mice developed abdominal tumors and were sacrificed when they became moribund (Table 1). The median survival in this group was 32 days (Fig. 4). At necropsy, all seven mice had bulky abdominal tumors. The tumors from six of these seven mice were analyzed by Southern blotting and PCR. All six tumors were found to be positive for K562 DNA. Because the liver is an organ that is infiltrated frequently by leukemic cells when SCID mice are injected with K562 cells (22), we performed Southern analysis looking for neo' sequences in liver DNA from all six mice. It should be noted that because the neo' genes are amplified in the K562 cells to a copy number that ranges from 100 to 200 copies/cell (11), the sensitivity of detection of leukemic cell infiltration in this assay is 50–100-fold greater than the sensitivity of detection when the assayed gene is present at 2 copies/cell. Thus, we estimate that this assay should allow the detection of leukemic cell infiltration when the ratio of leukemic cells to nonleukemic cells is in the range of 1:1000–1:5000. Southern analysis showed the presence of weak signals in the livers of two of these six mice (Fig. 5). When the same DNA was analyzed by PCR, four of the six livers were positive for human HLA-DQA. Three of the eight mice in group 2 died from bulky abdominal disease. A fourth mouse died on day 63 with hepatosplenomegaly as the only gross abnormality at necropsy. Because molecular analysis of the spleen and liver of this mouse did not show the presence of human DNA, the cause of death may have been an unrelated infection. The remaining four mice were sacrificed at the termination of the experiment on day 105 with no evidence of bulky

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Fig. 2. Serum dependence of p18 antisense-inhibited K562 erythroleukemia cells. A, growth curves of control K562.DHFR cells at 10% FBS (■), 2% FBS (●), 0.5% FBS (▲), and 0.25% FBS (▲). Bars, SD of three separate experiments. B, growth curves of K562.DHFR.p18(−) [1 μM MTX] cells at the same FBS concentrations as in A. C, growth curves of K562.DHFR.p18(−) [50 μM MTX] cells at the same FBS concentrations as in A.

Fig. 3. Clonogenic assays of p18 antisense-inhibited K562 erythroleukemia cells. Each bar represents the average number of colonies that formed when 1 × 10³ leukemic cells were plated in soft agar. Bar 1, K562.DHFR cell line; bar 2, K562.DHFR.p18(−) [1 μM MTX] cells; bar 3, K562.DHFR.p18(−) [50 μM MTX] cells. Error bars, SD of three different experiments.
Table 1 Presence of K562 DNA in SCID mice

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<th>Southern (neo' gene)*</th>
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* BM, bone marrow; SPL, spleen; LIV, liver; TUM, tumor; ND, not done.

** Tumors were abdominal except in 3.2, in which the tumor was thymic.

† The particular organ was enlarged at necropsy.

‡意味着Animals sacrificed at day 105 without signs of disease.

Although the exact function of p18 remains to be elucidated, numerous observations have suggested the involvement of this ubiquitous phosphoprotein in a number of important cellular processes. This protein was identified originally in several different laboratories as a result of changes in its level of expression or state of phosphorylation following exposure to different cellular effectors. These included, among others, induction of differentiation of leukemic cells (9, 10), stimulation of hormone secretion in insulinoma cells (23) and pituitary cells (23-25), mitogenic stimulation of lymphocyte proliferation (7), receptor-mediated, T-cell proliferation (26), induction of muscle differentiation (27), and neuronal development (28). Although these associations suggested a role for this protein in these diverse cellular processes, it was not possible to gain real insight into its function(s) from such descriptive studies. The cloning of the human (29) and rat (30, 31) genes that encode this protein helped elucidate its complete primary structure. Unfortunately, analysis of the structure did not provide any clues about its function, because it did not reveal significant homology to other proteins of known function.

Over the last several years, important new insights into the function of p18 have been accumulating from studies that demonstrated its direct involvement in well-characterized signal transduction pathways. The first clues came from the identification of the sites of phosphorylation of native p18 purified from the bovine brain. This analysis suggested that p18 may be a substrate for proline-directed kinases such as p34<sup>cdc2</sup> kinase and mitogen-activated protein kinase.
ANTISENSE RNA INHIBITION OF p18 EXPRESSION IN LEUKEMIC CELLS

The autoradiographs in A and β are from representative Southern blotting experiments in which DNAs isolated from tissues of SCID mice injected with leukemic cells are probed with neu' sequences. A. Lanes 1-4, DNA isolated from livers of mice injected with K562.DHFR.p18(-) [1 μM MTX] cells; Lanes 5-7, DNA isolated from abdominal tumors that developed in mice injected with K562.DHFR.p18(-) [1 μM MTX] cells. * faint bands that can be seen in Lanes 1 and 2. B. Lanes 1, DNA isolated from an abdominal tumor that developed in a mouse injected with K562.DHFR.p18(-) [1 μM MTX] cells; Lane 2, DNA isolated from an abdominal tumor that developed in a mouse injected with K562.DHFR.p18(-) [50 μM MTX] cells. The differences in the sizes of the detected fragments are a reflection of differences in the sites of integration of the neu' gene in the different cell lines.

This was followed shortly by the demonstration that p18 is a suitable substrate for these two kinases in vitro, and that the sites of p18 phosphorylation in vitro matched the previously identified sites of in vivo phosphorylation (11, 13, 14). Similar studies suggested that p18 also may be a suitable substrate for protein kinase A (14, 33) but not protein kinase C, as was suspected previously (34). More recently, Marklund et al. (35) showed that p18 also can serve as a substrate for Ca2+- and calmodulin-dependent kinase-G7.

In this report, we describe our investigation of the effects of antisense inhibition of p18 expression on the malignant phenotype of K562 erythroleukemia cells. This is a highly malignant, Ph + cell line that was established from a pleural effusion of a patient in the blastic phase of chronic myelogenous leukemia (36). A well-known characteristic of transformed cells is their ability to grow in culture to very high saturation densities. Normal cells go into quiescence when they reach a high density in culture. In contrast, transformed cells continue to grow in culture, even in the presence of very low levels of nutrients and growth factors. The saturation of both transformed and nontransformed cells has been shown to be related to the serum concentration, with higher densities achieved at higher serum concentrations (37). Generally, transformed cells reach a significantly higher saturation density than their nontransformed counterparts both at low and high serum concentrations. We have evaluated the effect of antisense inhibition of p18 expression on the saturation density of K562 erythroleukemia cells in culture. To ensure that the observed growth arrest is a function of cell density rather than depletion of serum factors, we replaced the media (RPMI 1640 supplemented with 10% FBS) daily throughout the course of these experiments. These experiments demonstrate that the inhibition of p18 expression results in a dose-dependent decrease in the saturation density of K562 erythroleukemia cells (Fig. 1).

We also analyzed the effect of low serum concentrations on the growth rates of the K562 cells in the presence or absence of antisense inhibition of p18 expression. Nontransformed cells have a greater degree of dependence on serum for their growth than their transformed counterparts. For example, transformed NIH 3T3 cells grow almost equally well at serum concentrations of 1 and 10%. Nontransformed NIH 3T3 cells, on the other hand, grow extremely poorly in the presence of 1% FBS (38). The decreased serum (or growth factor) requirement of transformed cells has been attributed to autocrine growth stimulation by growth factor-like molecules that may be produced by the transformed cells themselves (39). Our experiments demonstrate that low serum concentrations have a minimal effect on the growth of the control K562 cells and a more profound effect on the growth of the p18 antisense-inhibited leukemic cells (Fig. 2). In addition, these experiments also show that the degree of growth inhibition at low serum concentrations increases with increasing amplification of the antisense constructs.

The in vitro assay of transformation that has the best correlation with tumorigenicity in vivo is that of anchorage-independent growth (40, 41). This is usually assessed by suspending cells in semisolid medium and counting the number of colonies that form within 2 weeks. Nontransformed cells usually divide once or twice in soft agar and then go into quiescence. In contrast, transformed cells continue to divide until they form colonies that range from 100–500 cells before they go into quiescence. Using this assay, we showed that inhibition of p18 expression in K562 cells results in a profound dose-dependent decrease in colony formation in soft agar (Fig. 3). It should be emphasized that this observation is not merely a reflection of the decreased growth rate of the p18 antisense-inhibited cells. The total number of colonies that formed did not increase after prolonged incubation of the cells in semisolid medium for more than 4 weeks.

SCID mice provide a very useful animal model for the study of human leukemia. When K562 erythroleukemia cells are injected into SCID mice i.p. or i.v., the mice succumb invariably to extensive disease that usually involves the bone marrow, blood, brain, liver, spleen, ovaries, and other organs (22, 42). In the experiment summarized in Table 1, all seven animals in the control group died of extensive leukemic infiltration. It is not clear why the most prominent feature of the disease that developed in these mice was abdominal tumors with microscopic and molecular evidence of invasion of other organs as opposed to the predominant central nervous system and other organ involvement that characterized the disease described in other reports (22, 42). These differences may be a reflection of differences in the strains of the K562 cells and/or differences in the strains of the SCID mice that were used. Alternatively, the differences in the patterns of disease in the different studies may be related to differences in the preparative regimens. Although in some studies in SCID mice, the animals were treated with cyclophosphamide before injection of the leukemic cells (22), in the experiments described in this report, no such treatment was administered. However, regardless of the bases for these differences in the pattern of leukemic invasion, we did observe a very marked reduction in deaths from leukemia and prolongation of survival in mice that were injected with p18 antisense-inhibited K562 cells (Fig. 4). The degree of protection from disease increased with increasing amplification of the antisense sequences. This dose-dependent effect, which was observed in vivo, is fully compatible with in vitro observations in all three transformation assays described above.

Previous studies had shown that the in vivo treatment of SCID mice with antisense oligonucleotides directed against c-myb mRNA (22) or bcr-abl mRNA (43) resulted in a significant increase in the survival of mice injected with Ph + leukemic cells. In the experiments described above, we show that antisense inhibition of p18 expression in K562 cells not only results in the prolongation of survival but can prevent the development of disease in the majority of mice totally. This is particularly significant, because each mouse in our study was injected with 2x107 leukemic cells, an inoculum that is significantly larger than what has been used in other, similar studies (22, 43). This, however, does not imply that p18 is more important for the transformed phenotype of Ph + cells than is c-myb or bcr-abl. It should be noted that the antisense inhibition of p18 expression in our K562 cells was achieved by stable integration and MTX-induced amplification of the antisense expression constructs in
vitro before the cells were injected into the SCID mice. In contrast, antisense inhibition of c-myc and bcr-abl was performed by in vivo administration of antisense oligonucleotides to mice that were injected with the leukemic cells previously. This is undoubtedly a much less efficient method of delivery of the antisense nucleic acids to the cells than the one we used. It would be of considerable interest to compare the effects of treatments with anti-p18, anti-myb, and anti-bcr-abl oligonucleotides both as single agents and as combination therapy in the SCID mouse model of human leukemia directly.

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