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REPROGRAMMING ERYTHROLEUEKMIA CELLS TO TERMINAL DIFFERENTIATION AND TERMINAL CELL DIVISION

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TABLE OF CONTENTS

1. ABSTRACT

In vitro differentiation of murine erythroleuekemia cells recapitulates many aspects of the erythroid terminal differentiation program, including hemoglobin synthesis and proliferation arrest. It also provides an opportunity to study the changes occurring during reprogramming of tumor cells into their normal differentiation program. This review is focused on the recent progress made in understanding the key events occurring during the reprogramming of erythroleukemia cells. We discuss the contributions of PU.1 to the block to terminal differentiation exhibited by the erythroleukemia cells as well as the role of GATA-1 in restoring normal differentiation. We also discuss the role of certain cell cycle regulators in the decision to resume normal differentiation and in the resulting terminal cell divisions and arrest.

2. INTRODUCTION

Terminal differentiation consists of two processes: phenotypic differentiation and terminal cell division. The former process controls the development of the phenotypically mature cell types, while the latter ensures that the number of such cells is appropriately limited. In the hematopoietic system immature cells generally have extensive proliferative capacity whereas mature cells have little to none. Accordingly, dysregulation of terminal differentiation results in cells that are arrested at an immature stage of development with an inherently large proliferative capacity. Such cells are especially sensitive to malignant transformation. Much research has been devoted to understanding molecular events occurring during oncogenic processes as well as to finding ways to reverse such events (1). A key aspect in accomplishing these goals is to develop an understanding of the relationship between differentiation and cell proliferation (14). It also may be important to determine whether the same components of the cell cycle regulatory machinery are operating at the various stages of differentiation, i.e., early in immature cells and later in cells that are phenotypically more mature.

3. REPROGRAMMING OF ERYTHROLEUKEMIA CELLS TO TERMINAL DIFFERENTIATION AND TERMINAL CELL DIVISION

We have chosen to study the mechanisms governing proliferation at the various stages of maturation in the erythroid lineage by examining differentiating red blood cells using a cell culture model consisting of murine erythroleukemia (MEL) cells. These cells are transformed erythroid precursors that are arrested at the proerythroblast stage (8). MEL cells are derived from mice that have been infected with Friend virus, which is a complex of two viruses: Friend murine leukemia virus (F-MuLV) and spleen focus forming virus (SFFV) (9). SFFV is replication-defective, thus F-MuLV serves as the helper virus. The first stage in Friend virus induced erythroleukemogenesis occurs when infected red blood cell precursors express the SFFV encoded 55kd glycoprotein which activates the erythropoietin receptor in the absence of its natural ligand, erythropoietin, resulting in polyclonal erythroid hyperplasia (13). These hyperplastic cells are not transformed and they are fully capable of differentiating. However, rare clonal transformants arise from this population of hyperplastic cells, and develop into erythroleukemias. In 95% of these transformed cells, the SFFV genome is integrated near to the gene encoding the PU.1 transcription factor, resulting in its upregulation (9). Numerous chemical agents (e.g., hexamethylene bis-acetamide (HMBA) and dimethyl sulfoxide (DMSO) have

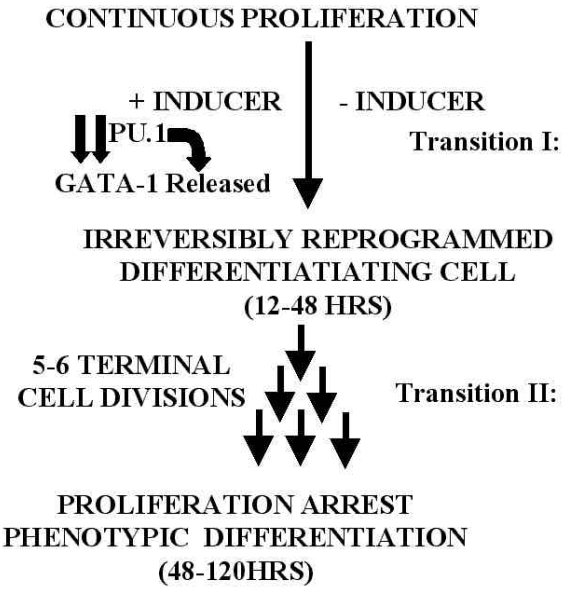

Figure 1: Profile of MEL Cell Differentiation. See text for details.

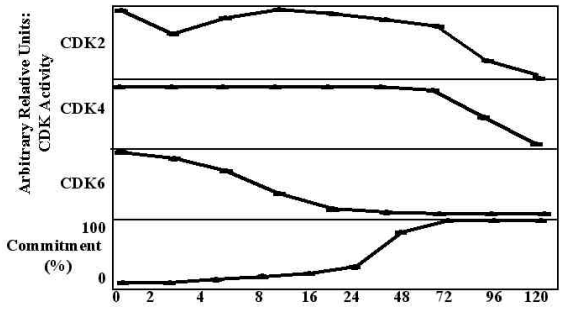

Figure 2: Representative quantitation of CDK activities during MEL cell differentiation in relation to the percentage of MEL cells committed to the differentiation program. See text for details.

been described that can reprogram the transformed MEL cells *in vitro* to reenter their terminal differentiation and terminal cell division programs. These agents also cause down-regulation of PU.1 levels. Downregulation of PU.1 is required for the reprogramming (11). We recently reported that PU.1 blocks differentiation by binding to and repressing the activity of the erythroid-specific transcription factor GATA-1, which is absolutely required for red blood cell differentiation (12). Consistent with this view we showed that inducing expression of an exogenous GATA-1 gene transfected into the MEL cells is sufficient to overcome the differentiation block imposed by PU.1 and to cause the cells to differentiate.

Our current view is that chemical agents that cause differentiation of MEL cells lead to reduced PU.1 levels which in turn releases GATA-1 from repression, resulting in differentiation. Once the levels of active GATA-1 reach a threshold, the cells are irreversibly reprogrammed to differentiate. Differentiation is accompanied by loss of the unlimited proliferative potential inherent in the MEL tumor cell. However, once committed to differentiate the cells do continue to divide 5-6 times before arresting, similar to the proliferative capacity of normal proerythroblasts (3). Thus, induction of differentiation in the erythroleukemia system is an excellent model in which to study cell cycle control during differentiation, as the cells undergo two transitions between three types of proliferative states (Figure 1): (1) continuous, uncontrolled proliferation characteristic of the tumor cells, which probably represents dysregulated proliferation control of an immature red blood cell progenitor; (2) terminal cell divisions typical of a differentiating erythroid cell; and (3) proliferation arrest characteristic of a fully mature erythroid cell. These transitions also provide the opportunity to investigate the relationship or coupling between the cell cycle and differentiation.

4. CONTROL OF THE EUKARYOTIC CELL CYCLE

The eukaryotic cell cycle is primarily regulated by a family of serine/threonine protein kinases which each consist of a catalytic subunit, a CDK, and a regulatory subunit, a cyclin (7). In mammalian cells, progression through the G1 phase is controlled by the activities of CDK4 and CDK6 which associate with one of three D-cyclins (D1, D2, and D3) and CDK2 which associates first with cyclin E, and then later in S phase with cyclin A (10). The enzymatic activities of the CDKs are controlled at several levels: by cyclin binding, CAK activation, CDK phosphorylation and dephosphorylation, and by binding of cyclin - dependent kinase inhibitors (CDKIs) (15). To date, two families of CDKIs have been identified that differ in their specificity and mechanism of inhibition. INK4 family members, $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ inhibit only CDK4 and CDK6 by interfering with cyclin D binding (5, 16). The KIP family of inhibitors, $p21^{CIP}$, $p27^{KIP1}$, and $p57^{KIP2}$ is thought to inhibit primarily CDK2 *in vivo* (4, 15).

5. TRANSITION I: FROM PROLIFERATING TUMOR CELLS TO DIFFERENTIATING CELLS UNDERGOING TERMINAL CELL DIVISIONS: THE IMPORTANCE OF CDK6

To determine the contribution of the various components of the cell cycle engine to proliferation during the three cell states discussed above (Figure 1), we assayed the activities and levels of the CDKs throughout differentiation. We observed that CDK2 activity undergoes a transient decline soon after treatment with an inducer of differentiation. CDK6 activity declines somewhat later, reaching 10% of its original activity by 24 hours of inducer treatment. Notably, CDK4 activity remains unchanged during this period (Figure 2). Since the early changes in the activities of CDK2 and CDK6 occur before the first transition (Figure 2; % Commitment), i.e., in the period leading up to the time when the decision to differentiate is made, it suggests that CDK2 and CDK6 may play specific roles in the proliferation of the tumor cells. To assess if the activities of CDK2, CDK4, and/or CDK6 were important

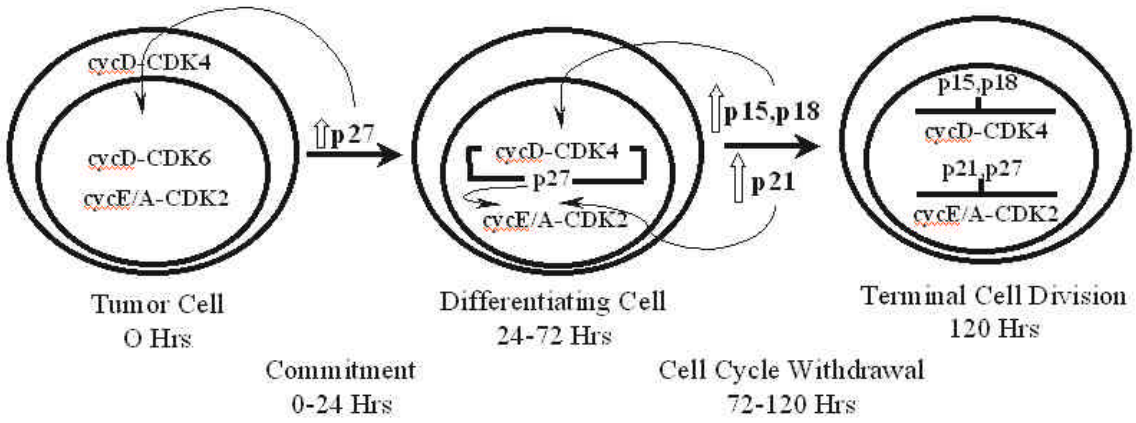

Figure 3: Schematic representation of the changes in cell cycle regulatory proteins during MEL cell differentiation. Hrs refers to time post inducer treatment. See text for details.

for maintaining proliferation in the tumor cells, we generated MEL cell transfectants expressing either human CDK2 or INK4 resistant mutants of human CDK4 (R24C) and human CDK6 (R31C) under control of a tetracycline-inducible expression vector. The levels and activities of CDK2, CDK4, and CDK6 are maintained in these transfectants even when they are treated with differentiation inducers if they are also treated with doxycyline (Dox), a tetracycline analogue. Although, these transfected cells did not proliferate any more rapidly than untransfected cells when the exogenous CDK genes were turned on, we found that only induction of exogenous CDK6 expression with Dox blocked the cell's response to DMSO-induced differentiation. In this condition the CDK6 transfected cells continue to divide in an uncontrolled manner typical of the tumor cells.

These results indicate that downregulation of CDK6 activity is necessary for DMSO-induced reprogramming of the erythroleukemia cells. On the other hand, maintenance of CDK2 activity in CDK2 transfectants did not block differentiation even though its activity does undergo a transient decline before the cells reenter the differentiation program. One way to explain the involvement of CDK2 but failure of its constitutive expression to block differentiation of the CDK transfectants is to hypothesize the existence of a substrate which is phosphorylated by both CDK2 and CDK6 (but not CDK4) sequentially at distinct sites. Sequential dephosphorylation of this substrate at both sites would be required for reentry into the terminal differentiation program. The observation that the transient decline of CDK2 occurs before the CDK6 decline adds further validity to such a sequential dephosphorylation model. The transfectants described here should prove useful in identifying the putative specific substrate(s) responsible for promoting proliferation in the tumor cells.

To date functional differences between the two cyclin D-kinases have not been reported. Thus one of the unexpected results obtained with the CDK transfectants described here is the inability of CDK4 to block DMSO-induced differentiation like CDK6. This result cannot be explained by differences in the levels or cellular location of the two types of exogenous CDKs in the transfectants. Thus we propose the existence of a specific substrate(s) which can be acted upon by CDK6 (but not by CDK4), and which is responsible for promoting proliferation in the tumor cells. In further support of this view we found that in the MEL tumor cells endogenous CDK4 is predominantly located in the cytoplasm whereas CDK6 is predominantly localized in the nucleus.

6. THE PROLIFERATION OF DIFFERENTIATING CELLS: THE IMPORTANCE OF CDK4 ACTIVITY

After the cells commit to differentiation they undergo an additional 5-6 cell divisions and then arrest. These divisions occur in the absence of CDK6 which has already declined earlier during commitment. If CDK6 is the important cyclin D-kinase in the tumor cell, could CDK4 be the important cyclin D-kinase in the differentiating cell? The observation that active cycD-CDK4 complexes undergo a rapid relocalization from the cytoplasm to the nucleus as CDK6 declines supports this view. To examine the mechanism by which the active cytoplasmically localized cycD-CDK4 complexes relocalize to the nucleus we examined the interactions of these complexes with the KIP family of CDKIs, which have previously been proposed to facilitate the nuclear localization of active cycD-CDK4 complexes (6). We observed that p27KIP1 associates with cycD-CDK4 during the relocalization of the kinase. To assess whether or not p27KIP1 is responsible for the observed relocalization we generated stable MEL cell transfectants containing tetracycline-controlled expression vectors (2) driving the synthesis of human p18INK4c and p27KIP1. While both p18 and p27 were able to bind CDK4 after Dox treatment, only p27 transfectants showed a Dox-dependent relocalization of active cycD-CDK4 complexes to the nucleus. Thus we propose that p27 facilitates the relocalization of active cycD-CDK4 complexes from the cytoplasm to the nucleus as cells become committed and begin to differentiate (Figure 3). These observations are consistent with an important role for cycD-CDK4

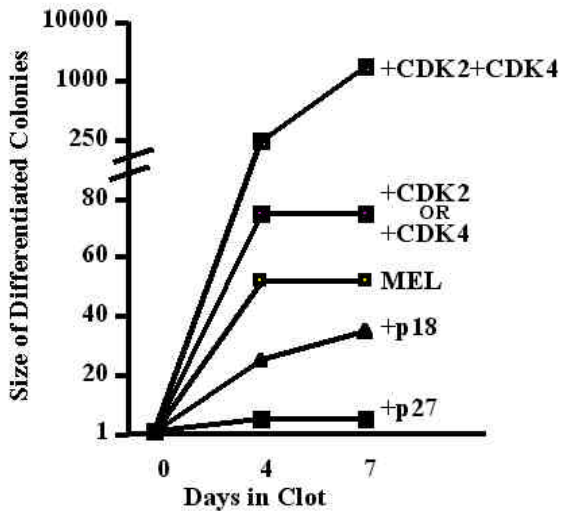

Figure 4: Representative sizes of committed colonies after expression of certain cell cycle regulators. See text for details.

complexes in the differentiating cells, as proposed above, but proof of the model requires the functional studies described below.

7. TRANSITION II: FROM PROLIFERATING DIFFERENTIATING CELLS TO TERMINAL ARREST

As mentioned above, once the MEL tumor cells reenter the erythroid differentiation program, they continue proliferating, but these cell cycles are limited to 5-6 divisions which are then followed by arrest in G1 (3). During the latter stages of this process we observed that the activities of CDK2 and CDK4 declined (Figure 2). Because their protein levels did not undergo a corresponding decline it seemed likely that the decline in their activities was due to an inhibition. The levels of the G1/S cyclins, the regulatory subunits of the CDKs were examined and found to be constant throughout differentiation. However, the levels of four cyclin-dependent kinase inhibitors (CDKIs), p15, p18, p21, and p27 were found to increase dramatically during this period, with the increase in p27 preceding the other increases. Furthermore, these CDKIs were found to be associated with the two CDKs concurrently with the inhibition of their activities. This result suggested that induction of these CDKIs leads to inhibition of CDK2 and CDK4 and proliferation arrest (Figure 2).

To assess this possibility tetracycline-inducible p18 and p27 MEL cell transfectants were used to examine the effect of premature induction of CDKIs on the proliferation capacity of individual differentiating cells. This proliferative capacity can be measured by plasma clot assays in which the size of colonies arising from individual cells is determined after several days of growth in the clot in the absence of the differentiation inducer. Cells that have not committed to differentiate produce very large colonies consisting of hundreds to thousands of undifferentiated cells. On the other hand, cells that have committed to differentiate by prior exposure to a differentiation inducer, in this case HMBA, give rise to small colonies consisting of fewer than 64 cells, all of which stain positively for hemoglobin with benzidine Thus, ordinarily, once differentiation is triggered, the differentiating cells undergo an average of 5-6 cell divisions before withdrawing permanently from the cell cycle.

Observations made with these inducible CDKI transfectants leads to two important conclusions: (1) Inhibition of both CDK2 and CDK4 by induction of exogenous p27 was sufficient to cause premature and permanent terminal cell division (Figure 4). The arrest induced by p27 in differentiating cells is not merely a simple growth arrest, it is irreversible, requiring only a brief exposure to Dox. In contrast, a similar treatment of the same transfectants prior to differentiation, causes a growth arrest which is fully reversible upon removing Dox. Thus there is a clear difference in the sensitivity of the MEL tumor cells versus differentiating MEL cells to the action of the p27 inhibitor. (2) Since inhibition of CDK2 and CDK4 by p27 resulted in 16% single cell colonies which stained positive for hemoglobin, we conclude that once committed to differentiate the cells do not need to proliferate in order to undergo phenotypic differentiation.

Since premature inhibition of CDK2 and CDK4 resulted in early and permanent terminal cell division without affecting phenotypic differentiation, we sought to determine whether preventing the decline in CDK2 and CDK4 activities could reverse terminal cell division and cause extensive proliferation of differentiating cells. Therefore we generated MEL cell transfectants inducibly expressing both CDK2 and CDK4R24C. Inducing expression of CDK2 and CDK4R24C in the differentiating cells resulted in abrogation of normal terminal cell division and extensive proliferation of differentiated cells (Figure 4). These cells were phenotypically mature as judged by both benzidine staining and western blot analysis for hemoglobin. Interestingly, a similar experiment using CDK2 and CDK6 transfectants showed that this combination could not reverse terminal cell division. These results indicate that differentiated cells can be forced to proliferate further than they normally do, giving rise to a greater number of phenotypically mature cells.

8. THE CHANGING ROLES OF CDK6 AND CDK4 AND THE COUPLING OF CELL CYCLE REGULATORS TO TERMINAL DIFFERENTIATION

Our observations suggest that certain chemical inducers of MEL cell differentiation may act by inhibiting the production or activity of key cell cycle regulators, thereby breaking the continuous uncontrolled proliferation of the tumor cells, allowing them to reenter their normal differentiation program. These observations suggest that the cell cycle and the commitment to the differentiation program are coupled. On the other hand, there appears to be complete independence of cell cycle regulation and phenotypic maturation late in the

differentiation program. This conclusion is based upon our finding that cell cycle withdrawal of differentiating MEL cells can be greatly delayed by turning on a combination of exogenous CDK2 and CDK4. Particularly significant is the observation that hemoglobin production was maintained in transfectants that were forced to undergo multiple, additional rounds of cell division. These findings could have important implications for possible cell and tissue replacement therapies and organ regeneration. Since MEL cells recapitulate erythroid differentiation, we believe that these observations are very likely applicable to normal, differentiating red blood cells. In the future it may be feasible to propagate committed red blood cell precursors by introducing into them vectors expressing CDK2 and CDK4. Then, at the desired time, their terminal cell division could be triggered by turning off the exogenous CDK2 and CDK4, thereby producing a vast supply of red cells. This approach may also be feasible for other therapeutically important cell types. Similarly, understanding the cell cycle regulators controlling the reentry of tumor cells into their normal differentiation program may lead to further advances in differentiation cancer therapy.

Surprisingly, our observations also show that CDK4 and CDK6 are not functionally equivalent in erythroid cells. Our results show that maintenance of CDK6, but not CDK4, activity in the erythroleukemia cells blocks their chemically induced differentiation. Conversely, our results in differentiating cells show that only the combined effect of CDK2 and CDK4 results in extensive proliferation of differentiating cells while expression of CDK2 and CDK6 does not have a similar effect. On the basis of these observations we hypothesize the existence of a highly regulated program of changes in the role of cyclin D-dependent kinases during erythroid cell differentiation. We suggest that CDK6 (along with CDK2) may be responsible for driving proliferation in early erythroid progenitors and that later in differentiating cells the cell cycle engine is driven by CDK4 (along with CDK2). Because CDK6 plays a very important role in proliferation of the erythroleukemia tumor cells we think it may be a target for dysregulation during oncogenesis. Furthermore, it will be interesting to ascertain whether this new concept of the changing roles of CDK6 and CDK4 during erythroid maturation can be extended to other hematopoietic lineages.

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