Increasing c-FMS (CSF-1 Receptor) Expression Decreases Retinoic Acid Concentration Needed to Cause Cell Differentiation and Retinoblastoma Protein Hypophosphorylation

Andrew Yen, Rhonda Sturgill, and Susi Varvayanis

Cancer Cell Biology Laboratory, Department of Pathology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

ABSTRACT

Increasing the expression of c-FMS (colony-stimulating factor 1 receptor) by introduction of a transgene reduced the concentration of retinoic acid or 1,25-dihydroxy vitamin D3 needed to cause myeloid or monocytic cell differentiation and hypophosphorylation of the retinoblastoma tumor suppressor protein (RB) typically associated with cell cycle G0 arrest and differentiation of HL-60 human myelomonoblastic precursor cells. The data are consistent with a model in which signals originating with retinoic acid and c-FMS integrate to cause differentiation, RB hypophosphorylation, and G0 arrest. Furthermore, these two signals can compensate for each other. Three HL-60 sublines described previously (A. Yen et al., Exp. Cell Res., 229: 111—125, 1996) expressing low (wild-type HL-60), intermediate, and high cell surface c-FMS were treated with various concentrations of retinoic acid. The lowest concentration tested, 10^{-8} M, induced significant differentiation of only the high c-FMS-expressing cells, with no accompanying hypophosphorylated RB or G0 arrest. The low and intermediate c-FMS-expressing cells showed no induced differentiation, hypophosphorylation of RB, or G0 arrest. A 10-fold higher retinoic acid concentration, 10^{-7} M, induced significant differentiation of both intermediate and high c-FMS-expressing cells. It induced RB hypophosphorylation only in high c-FMS-expressing cells but with no accompanying G0 arrest in any of the cells. The highest retinoic acid concentration, 10^{-6} M, elicited differentiation, hypophosphorylation of RB, and G0 arrest in low, intermediate, and high c-FMS-expressing cells. As the concentration of retinoic acid increased, cell differentiation, hypophosphorylation of RB, and G0 arrest were progressively elicited within this ensemble of cells with different c-FMS expression levels. Thus, for example, at the lowest concentration of retinoic acid, expression of high enough c-FMS still allowed differentiation. At higher concentrations, progressively less c-FMS was needed for differentiation. The apparent threshold for the sum of the retinoic acid plus c-FMS originated signals to elicit differentiation, hypophosphorylation of RB, and G0 arrest increased, in that order. Thus retinoic acid-induced cell differentiation, RB hypophosphorylation, and G0 arrest have different signal threshold requirements. 1,25-Dihydroxy vitamin D3, also a ligand for a member of the steroid thyroid hormone receptor superfamily, caused monocytic differentiation. Retinoic acid increased cell differentiation and hypophosphorylation of RB, and G0 arrest were progressively elicited within this ensemble of cells with different c-FMS expression levels. Thus, for example, at the lowest concentration of retinoic acid, expression of high enough c-FMS still allowed differentiation. At higher concentrations, progressively less c-FMS was needed for differentiation. The apparent threshold for the sum of the retinoic acid plus c-FMS originated signals to elicit differentiation, hypophosphorylation of RB, and G0 arrest increased, in that order. Thus retinoic acid-induced cell differentiation, RB hypophosphorylation, and G0 arrest have different signal threshold requirements. Thus, c-FMS-originated signal can enhance the cellular effect of the retinoic acid- or vitamin D3-originated signal. The two pathways appear to regulate each other. How these steroid and peptide hormone signaling pathways interact with each other is not known. One obvious question is whether upstream events might be involved that allow the c-FMS-originated signal to enhance cellular sensitivity to retinoic acid or vitamin D3, allowing cells to respond to lower doses of these ligands.

INTRODUCTION

Retinoic acid provides a signal that induces the growth arrest and myeloid differentiation of a human myeloblastic leukemia cell line, HL-60, in a process that depends on another signal originating with the c-FMS receptor (1). The RARs (2-4) and the related RXRs (5, 6) can homo- or heterodimerize and act as ligand-activated transcription factors (see Ref. 7 for review). Both RARs and RXRs must be ligand activated to induce differentiation (8). RARs and RXRs are members of the steroid-thyroid hormone receptor superfamily, as is the vitamin D receptor, which can also heterodimerize with RXRs. In contrast, c-FMS is a peptide hormone receptor of the platelet-derived growth factor family (see Refs. 9-11 for reviews). It is the receptor for colony-stimulating factor 1 and regulates the cell cycle and differentiation of myeloid-monocytic hematopoietic cells. c-FMS is a transmembrane tyrosine kinase receptor that signals through a prototypical mitogenic ras/raf-mediated kinase cascade (12), leading to phosphorylation and activation of MAPK and the phosphorylation of transcription factors such as Elk-1. Although such kinase cascade signaling pathways are prototypical mitogenic signaling pathways, in this instance they can also drive cells from proliferation to arrest. Retinoic acid can thus determine if this signal is mitogenic or growth arresting (1). Consistent with this, retinoic acid-induced myeloid differentiation and G0 arrest are accelerated by ectopic expression of activated Raf, a prototypical serine threonine kinase associated with proliferation and transformation (13). Vitamin D3-induced monocytic differentiation is likewise accelerated. Whereas retinoic acid can determine if c-FMS signaling promotes or inhibits mitogenesis, the putative c-FMS-originated signal can enhance the cellular effect of the retinoic acid-or vitamin D3-originated signal. Thus, the two pathways appear to regulate each other. How these steroid and peptide hormone signaling pathways interact with each other is not known. One obvious question is whether upstream events might be involved that allow the c-FMS-originated signal to enhance cellular sensitivity to retinoic acid or vitamin D3, allowing cells to respond to lower doses of these ligands.

The RB tumor suppressor gene is a putative immediate regulator of the cell cycle and, by extension, cell differentiation (see Refs. 14-16 for reviews). The regulatory activity is believed to be exercised through its phosphorylation by cyclin-driven kinases. However, recently reported nonconcordances between expression of cyclins and RB phosphorylation (17, 18), as well as nonconcordances between phosphorylation and the cell cycle phases (1, 13, 19, 20), and also expression levels and growth arrest (21-24), indicate that the function of RB is still unclear. This ambiguity is furthered by nonconcordances between apparent available free E2F, a putative principal binding partner of RB, and cell proliferation (see Refs. 20 and 25 for reviews). Given that the retinoic acid and FMS signaling pathways both regulate cell cycle arrest and differentiation, it becomes of potential interest to determine their effect on RB phosphorylation. Manipulation of either of these pathways provides a means of determining how RB phosphorylation and expression changes when cell proliferation and differentiation are thus altered.

HL-60 is a human myeloblastic leukemia cell line (26). It was originally designated as a cell line of acute promyelocytic origin, and later, it was reevaluated as myeloblastic (27). The cell line has amplified myc and activated ras, and it is p53 negative. It retains the capability to undergo myeloid or monocytic differentiation in response to a variety of agents (see Ref. 28 for review). The most commonly used inducers are retinoic acid (29), a myeloid inducer, and...
1,25-dihydroxy vitamin D3 (30), a monocytic inducer. Typical treatments with 10^{-6} M retinoic acid or 0.5 X 10^{-8} M vitamin D3 induce onset of phenotypic conversion and also G_{1}/G_{0} arrest by 48 h, a period corresponding to approximately two division cycles in the subline studied. Early events during the first ~24 h of this cascade are differentiation lineage independent, whereas late events during the second ~24 h specify myeloid or monocytic differentiation (31). Progressive differentiation and arrest result in almost entirely differentiated populations by 96 h. Analysis of RB expression produced unanticipated results. Unlike some other cells, RB is phosphorylated in all cell cycle phases; i.e., all of the RB protein in proliferating cells is phosphorylated (1, 13, 19). The degree of phosphorylation increases as does total RB per cell as HL-60 cells advance through progressive cell cycle phases (19). In contrast, cells that are induced to G_{1}/G_{0} arrest and differentiate express less RB per cell and have essentially only hypophosphorylated RB. However, it is possible in some cases to induce G_{0} arrest and differentiation prior to detectable hypophosphorylated RB (13). Hypophosphorylated RB thus does not necessarily precipitate cell cycle arrest or differentiation but rather appears as a feature of arrested, differentiated cells, much as might be anticipated with metabolic slow down, for example. In contrast, differentiation and arrest are consistently associated with an unanticipated reduced expression of this putative growth/cell cycle suppressor (21, 22). The novelty of some of these results makes HL-60 an intriguing cell line for further analysis of potential RB function and its coupling to either growth arrest or differentiation.

The retinoic acid- and c-FMS-originated signals may converge to regulate RB expression. In HL-60 cells, both proliferation and retinoic acid-induced myeloid differentiation or 1,25-dihydroxy vitamin D3-induced monocytic differentiation depend on a c-FMS-originated signal (1). In exponentially proliferating cells without retinoic acid, a c-FMS-originated signal promotes proliferation. Adding retinoic acid or 1,25-dihydroxy vitamin D3 causes the c-FMS-originated signal to now promote differentiation and G_{0} arrest. This differentiation and G_{0} arrest are associated with down-regulation of RB expression and conversion from the hyper- to the hypophosphorylated form (1, 8, 21, 22). Overexpression of the c-FMS transmembrane tyrosine kinase receptor or an activated RAF in stable HL-60 transfectants results in accelerated differentiation and arrest in response to retinoic acid and 1,25-dihydroxy vitamin D3 (1, 13, 32). Coupled to this, down-regulation of RB expression antecedent over cell cycle or cell differentiation changes is also accelerated, although conversion to the hypophosphorylated form is not. Thus it appears that the signals from retinoic acid or 1,25-dihydroxy vitamin D3 and c-FMS converge at some point to regulate RB expression and ultimately cell cycle arrest and differentiation. This motivates interest in the effects of the relative dose of each of these signals on RB.

The present studies determine the interactive effects of the retinoic acid- or 1,25-dihydroxy vitamin D3-originated signal and the c-FMS-originated signal on RB hypophosphorylation, cell cycle arrest, and cell differentiation. In these studies, the effect of overexpression of c-FMS, the receptor for colony-stimulating factor 1, on the ability of HL-60 cells to undergo myeloid or monocytic differentiation, convert their RB protein to the hypophosphorylated form, and undergo G_{0}/G_{1} specific growth arrest was analyzed using HL-60 sublines stably transfected with a c-FMS cDNA. Three HL-60 lines were studied, including wild-type HL-60, FMS/I, and Sort 10. The derivation and properties of the FMS/I and Sort 10 stable transfecteds have been previously described (1, 32). FMS/I was derived by stably transfecting HL-60 cells to overexpress ectopic c-FMS (1). Sort 10 was derived from FMS/I by successive iterations of fluorescence-activated cell sorting for the highest FMS-expressing cells in the population and amplification of the sorted cells in culture (32). The FMS/I cells have approximately 2-fold greater mean cell surface c-FMS expression than HL-60, whereas the Sort 10 cells have approximately 8-fold higher c-FMS expression than HL-60. As described previously (32), Sort 10 cells represent an asymptotic limit for the extent of allowable c-FMS overexpression in HL-60 cells.

**MATERIALS AND METHODS**

**Cells and Culture Conditions.** HL-60 human promyelocytic leukemia cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS in a humidified atmosphere of 5% carbon dioxide. Stock cultures were maintained in continuous exponential growth by reculturing every 2 or 3 days at an initial density of 0.2 X 10^{6} or 0.1 X 10^{6} cells/ml, respectively. The wild-type strain, designated F8, is not atypical of HL-60 cells that have undergone sustained passage, and has a doubling time of approximately 20 h, and proliferates exponentially in the range of approximately 0.1 X 10^{6} to 3.0 X 10^{5} cells/ml. The stable transfectant FMS/I overexpresses c-FMS detectable on the cell surface. Its creation and properties have been previously reported (1). FMS/I and its derivatives generated by fluorescence-activated cell sorting were maintained as stocks in the continuous presence of 400 μg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN) as described previously (1). Assays of cell differentiation, cell cycle phase distribution, and RB protein were performed on sequential cell samples from cultures initiated at a density of 0.2 X 10^{6} cells/ml with 20–30 ml per 75-cm² flask. Inducers were added to these cultures to make the stated final concentrations of retinoic acid or 1,25-dihydroxy vitamin D3 as described previously (31). Retinoic acid was added from a stock of 10^{-3} M in ethanol. VD3 (Solvay Duphar BV, Da Weesp, The Netherlands) was added from a stock of 10^{-3} M in ethanol. Carrier blanks had no effect on the measured parameters in control experiments. The experiments shown are each a typical case among three independent repeat experiments.

**Assays for Cell Proliferation and Differentiation.** Assays for cell proliferation and differentiation were performed as described previously (8, 31). Cell density in experimental cultures was measured by repeated counts with a hemacytometer. Viability was assessed by exclusion of 0.2% trypan blue dye and was routinely in excess of 90% in all cultures reported. The distribution of cells in the cell cycle was determined by flow cytometry using propidium iodide-stained nuclei. Cells (0.5 X 10^{6}) were harvested at each indicated time and resuspended in 0.5 ml of hypotonic propidium iodide (0.05 mg/ml propidium iodide, 1 mg/liter sodium citrate, and 0.1% Triton X-100) and stored refrigerated and protected from light until analyzed. Flow cytometric analysis was done with a multiparameter dual laser fluorescence-activated cell sorter (EPICS; Coulter Electronics, Hialeah, FL) using 200 mW of 488-nm excitation from a tunable argon ion laser. Functional differentiation to a mature monocyte lineage was assayed by phorbol-12-myristate-13-acetate (Sigma Chemical Co., St. Louis, MO) inducible intracellular reduction of nitroblue tetrazolium to formazan. Cells (0.2 X 10^{6}) were harvested at the indicated times and resuspended in 0.2 ml of 2 mg/ml nitroblue tetrazolium in PBS containing 200 ng/ml phorbol-12-myristate-13-acetate. The cells were incubated in a water bath for 20 min at 37°C and then scored using a hemacytometer for the percentage of cells containing intracellular superoxide-precipitated formazan.

**Western Analysis of RB.** Western analysis of RB protein expression and phosphorylation in whole cell lysates harvested at the indicated times were performed as described previously (1, 33). At indicated times, 1 X 10^{6} cells were harvested and fixed in methanol and stored at ~20°C until used. The cells were resuspended in 25–50 μl of loading buffer [6% SDS, 4 μg urea, 4 mM EDTA, 125 mm Tris (pH 6.9), 0.25% bromophenol blue, and 35 μl/ml β-mercaptoethanol], boiled, and gel-electrophoresed using a 4% stacking gel and 6% resolving gel (37.5:1, acrylamide:bis). 0.5 X 10^{6} cells were loaded per lane for PAGE. Proteins were electrophoretically from the gel to a nitrocellulose membrane, which was probed with a murine antihuman RB monoclonal antibody [RB Gene Product (mAb) 1 Monoclonal Antibody, Triton Diagnostics, Alameda, CA; or Zymed Laboratories, South San Francisco, CA]. Detection was done using enhanced chemiluminescence and a horseradish peroxidase-conjugated secondary antirabbit antibody (ECL kit; Amersham Ltd.). Film images of the membrane were densitometrically scanned to graphically represent the
relative amount of faster migrating unphosphorylated RB protein versus the slower migrating protein phosphorylated at multiple sites. The scan axis represents 20 mm on the gel.

c-FMS Analysis. Cells were immunofluorescently stained for cell surface c-FMS expression essentially as described previously (1, 32). To assay for cell surface FMS expression, 5 × 10^6 cells were harvested, washed once in 150 μl of PBS (pH 7.2) and gently resuspended, adding 15 μl of antibody against c-FMS (AB-2, 100 μg of IgG2b per ml of 0.05 M sodium phosphate buffer containing 0.1% sodium azide and 0.2% gelatin; Oncogene Science, Cambridge, MA). The suspension was gently mixed, incubated for 20 min on ice in the dark, and gently washed with 150 μl of PBS using a table-top centrifuge (800 rpm, 5 min). The cells were resuspended in 95 μl of PBS, to which 5 μl of fluorescein-conjugated goat anti-rat antibody, whole molecule (Cappel Division, Organon Teknika Corp., West Chester, PA, reconstituted per the manufacturer’s directions in sterile distilled water and then diluted 1:10 in normal goat serum/PBS) was added and then incubated on ice for 20 min. They were washed in 150 μl of PBS and resuspended in 500 μl of PBS. Flow cytometric analysis was performed with a multiparameter dual laser fluorescence-activated cell sorter (EPICS) using 200 mW of 488-nm excitation from a tunable argon ion laser. Emitted fluorescence was collected through a 547—502-nm band laser blocking filter and a 550-nm long-pass dichroic mirror. Green fluorescence was detected with a photomultiplier tube masked with a 525-nm band pass filter. Photomultiplier voltages ranged from approximately 1400 to 1700 V. Forward angle light scatter was used as a trigger signal and to eliminate debris for analysis. The ADC (analogue to digital converter) trigger was forward angle light scatter. c-FMS expression means were determined on the basis of 10,000 events analyzed by onboard statistical analysis packages.

Reticinoic Acid Uptake. Cellular uptake of trace labeled retinoic acid was measured using tritiated retinoic acid and liquid scintillation counting. HL-60 or Sort 10 cells (2 × 10^6) were resuspended in 10-ml cultures containing either 10^-6 M, 10^-7 M, 10^-8 M, or 0 M (control) labeled retinoic acid. A 10^-3 M labeled retinoic acid stock was made from 250 μl of 4.3 μM [3H]retinoic acid (46.0 Ci/mmol, 152 mCi/mg, 0.2 mCi/ml [3H]9-cis-retinoic acid; Amersham Ltd) plus 32.6 μl of 8.67 mM retinoic acid in ethanol. The resulting retinoic acid stock contained 0.38% [3H]retinoic acid. Stocks of 10^-4 M and 10^-5 M were made by dilution of the labeled 10^-3 M stock in ethanol. Ten μl of labeled retinoic acid stock were added to 10-ml cultures to generate final concentrations of 10^-6 M, 10^-7 M, or 10^-8 M. The concentration 10^-6 M corresponded to labeling with 0.18 μCi/ml, a relatively low dose unlikely to cause unanticipated effects due to radiation-induced damage during the 1 h of labeling. Cells were incubated in 15-ml conical centrifuge tubes for 1 h at 37°C in a humidified atmosphere of 5% CO2. The cells were centrifuged to a pellet and washed in 5 ml of cold PBS. The washed pellet was resuspended in 5 ml of scintillation fluid (Universol; ICN Radiochemicals, Irvine, CA), mixed, and transferred to scintillation vials for counting. Cells (2 × 10^6) treated with 10^-6 M retinoic acid took up approximately 1% of the total radioactivity (~8.2 × 10^5 cpm) available in culture, indicating that availability of retinoic acid is not rate limiting.

RESULTS

Reticinoic Acid Induced Myeloid Differentiation. Enhanced c-FMS expression enables cells to differentiate in response to lower concentrations of retinoic acid. HL-60, FMS/I, and Sort 10 cells were cultured with 0 μM (control), 10^-8 M, 10^-7 M, or 10^-6 M retinoic acid. The 10^-6 M dosage is the standard dosage most commonly used to elicit myeloid differentiation of wild-type HL-60 cells. The percentage of cells having differentiated was measured by the functional myelo-monocytic differentiation marker, inducible oxidative metabolism. Fig. 1 shows the percentage of cells that have differentiated during exposure to 0 μM (control), 10^-8 M, 10^-7 M, or 10^-6 M retinoic acid. Control cells underwent no significant differentiation during the 96-h course of observation. The generation time of HL-60 cells is approximately 20 h, and the period of observation thus corresponds to over four cell cycles. But as reported previously (32), the generation time of the transfectants is longer: the generation time for Sort 10 is up to approximately 29 h, for example. (The average after prolonged culture for multiple cryopreserved samples restored to culture can revert to approximately 23 h.) At 10^-8 M retinoic acid, the percentage of differentiated cells was indistinguishable from untreated controls for HL-60 and FMS/I, but a significant fraction of Sort 10 cells differentiated. The onset of the increase was detected at approximately 48 h. Thus, only the cells with the highest c-FMS expression exhibited any apparent differentiation in response to 10^-8 M retinoic acid. When the different cell lines were treated with a 10-fold higher concentration of retinoic acid, 10^-7 M, then a significant number of HL-60 cells differentiated with kinetics similar to that observed for Sort 10 cells at the lower (10^-8 M) retinoic acid concentration. Differentiation induced in FMS/I cells was enhanced relative to HL-60. The greatest amount of induced differentiation was by Sort 10 cells. Onset was again at approximately 48 h. Comparing these cell lines, the amount of differentiation increased as the amount of c-FMS expression increased. When the cells were treated with a further 10-fold higher concentration of retinoic acid, 10^-6 M, this relationship persisted. Sort 10 cells again exhibited the greatest fraction of cells differentiated at all times after onset of differentiation, except at the end of the observation period when the majority of cells in all cell lines had finally differentiated. Sort 10 cells consistently differentiated better than HL60, and FMS/I was consistently intermediate at different times, as well as at different inducer concentrations.

If the amount of differentiation at a specific time, 72 h, for each cell line is considered, then increasing the retinoic acid concentration consistently produced increasing differentiation. Likewise for each retinoic acid concentration, increasing FMS expression resulted in
of the RB is hypophosphorylated, as determined by integrating the densitometric scan. Treatment with a 10-fold higher concentration of retinoic acid, phosphorylated RB has greater relative mobility on electrophoresis than hyperphosphorylated RB, although it caused approximately 30% of the Sort cell lines showed any hypophosphorylated RB, indicating that over densitometric scans of Western blot images of the RB protein for hypophosphorylated RB can be consistently detected this way. Fig. 2 shows the hypophosphorylated RB is associated with cell differentiation (13). As shown previously, hypophosphorylated RB in HL-60 cells is distinguishable from hyperphosphorylated RB on a Western blot as a subpopulation of RB molecules with greater relative mobility (1, 13, 19, 24). As shown previously also (8), as little as 4% hypophosphorylated RB can be consistently detected this way. Fig. 2 shows the densitometric scans of Western blot images of the RB protein for HL-60, FMS/I, and Sort 10 cells treated with 0 M (control), 10^{-8} M, 10^{-7} M, and 10^{-6} M retinoic acid for 72 h. The X axis is 20 mm of the gel with the bottom of the gel oriented to the right. The optical density representing the relative amount of protein is the Y axis. Hypophosphorylated RB has greater relative mobility on electrophoresis than hyperphosphorylated RB and thus appears when present as a subpopulation to the right of the hyperphosphorylated RB peak. In the Sort 10 cells treated with 10^{-7} M retinoic acid, approximately 15% of the RB is hypophosphorylated, as determined by integrating the densitometric scan. More differentiation at 72 h, unless the retinoic acid dose was below the apparent threshold for cellular response. In this respect, the retinoic acid- and c-FMS-originated signals thus appeared to have similar additive effects on differentiation, affecting both the rate of differentiation and the maximum percentage differentiated.

Enhanced c-FMS expression allows cells to hypophosphorylate RB in response to lower concentrations of retinoic acid. It has been established previously that the RB protein in proliferating HL-60 cells is all in the hyperphosphorylated state (1, 13, 19, 24). Occurrence of hypophosphorylated RB is associated with cell differentiation (13). As shown previously, hypophosphorylated RB in HL-60 cells is distinguishable from hyperphosphorylated RB on a Western blot as a subpopulation of RB molecules with greater relative mobility (1, 13, 19, 24). As shown previously also (8), as little as 4% hypophosphorylated RB can be consistently detected this way. Fig. 2 shows the densitometric scans of Western blot images of the RB protein for HL-60, FMS/I, and Sort 10 cells treated with 0 M (control), 10^{-8} M, 10^{-7} M, or 10^{-6} M retinoic acid for 72 h. None of the untreated control cell lines showed any hypophosphorylated RB, but none of the cell lines showed hypophosphorylated RB with 10^{-7} M retinoic acid, indicating that differences observed at 72 h reflect at least in part differential kinetics. Thus, elevated c-FMS expression increases cellular sensitivity to retinoic acid-induced RB hypophosphorylation, but the signal threshold for eliciting hypophosphorylation is higher than that for inducing differentiation.

Interestingly, in some cases, down-regulation of RB appeared more responsive than hypophosphorylation to the retinoic acid- or c-FMS-originated signaling and more closely paralleled differentiation. For example, as described above, 10^{-8} M retinoic acid caused differentiation only in Sort 10 cells. At 72 h, there was still no RB hypophosphorylation, but RB expression was down-regulated in these cells. In another example, 10^{-7} M retinoic acid could cause detectable differentiation in HL-60 cells by 72 h, but no detectable RB hypophosphorylation. In contrast, RB expression was down-regulated. This is consistent with previous observations (13).

In contrast to the above, enhanced c-FMS expression did not allow cells to G_1/G_0 arrest in response to lower retinoic acid concentrations. Fig. 3 shows the percentage of cells with G_1/G_0 DNA measured by flow cytometry during exposure to 0 M (control), 10^{-8} M, 10^{-7} M, or 10^{-6} M retinoic acid. Untreated control cells showed no significant increase during the 96-h course of observation. This was paralleled by continued increases in cell density, except at 96 h, when growth was retarded due to depleted culture medium (data not shown). Treated with 10^{-5} M retinoic acid, none of the cell lines showed any apparent G_1/G_0 growth arrest evidenced by enhancement in relative numbers of G_1/G_0 cells. Likewise, this was paralleled by continued increases in cell density. Interestingly, there was no abatement at 96 h, possibly reflecting a growth-sustaining capability of low doses of retinoic acid reported previously for HL-60 cells. Increasing the retinoic acid concentration 10-fold to 10^{-7} M caused no apparent arrest. Sort 10 cells showed no enrichment in G_1/G_0 cells distinguishable from FMS/I or HL-60. There was a slight but reproducible increase in percentage of G_1/G_0 cells at late times, which was similar for all the cell lines. This was not considered an indication of arrest because there was no significant inhibition of increasing cell density throughout the 96-h period of observation (data not shown). The slight changes in percentages may reflect shifts in the relative durations of cell cycle phases as the cells continue to proliferate. Increasing the concentration of retinoic acid 10-fold further to the standard concentration, 10^{-6} M, used to induce differentiation and G_1/G_0 arrest of wild type HL-60 cells, caused an enrichment in G_1/G_0 cells for all of the cell lines, consistent with a previous report (32). Thus, enhanced c-FMS expression does not apparently enable cells to G_1/G_0 growth arrest in response to a lower concentration of retinoic acid.

The trend of the above data indicates that there is a c-FMS-dependent threshold for retinoic acid to elicit differentiation, RB hypophosphorylation, or G_1/G_0 growth arrest. Differentiation can be elicited at the lowest (10^{-8} M) concentration, RB hypophosphorylation at a higher (10^{-7} M) concentration, and G_1/G_0 growth arrest at the highest (10^{-6} M) concentration tested. Increasing the retinoic acid

Fig. 3 shows the percentage of cells with G_1/G_0 DNA measured by flow cytometry during exposure to 0 M (control), 10^{-8} M, 10^{-7} M, or 10^{-6} M retinoic acid. Untreated control cells showed no significant increase during the 96-h course of observation. This was paralleled by continued increases in cell density, except at 96 h, when growth was retarded due to depleted culture medium (data not shown). Treated with 10^{-5} M retinoic acid, none of the cell lines showed any apparent G_1/G_0 growth arrest evidenced by enhancement in relative numbers of G_1/G_0 cells. Likewise, this was paralleled by continued increases in cell density. Interestingly, there was no abatement at 96 h, possibly reflecting a growth-sustaining capability of low doses of retinoic acid reported previously for HL-60 cells. Increasing the retinoic acid concentration 10-fold to 10^{-7} M caused no apparent arrest. Sort 10 cells showed no enrichment in G_1/G_0 cells distinguishable from FMS/I or HL-60. There was a slight but reproducible increase in percentage of G_1/G_0 cells at late times, which was similar for all the cell lines. This was not considered an indication of arrest because there was no significant inhibition of increasing cell density throughout the 96-h period of observation (data not shown). The slight changes in percentages may reflect shifts in the relative durations of cell cycle phases as the cells continue to proliferate. Increasing the concentration of retinoic acid 10-fold further to the standard concentration, 10^{-6} M, used to induce differentiation and G_1/G_0 arrest of wild type HL-60 cells, caused an enrichment in G_1/G_0 cells for all of the cell lines, consistent with a previous report (32). Thus, enhanced c-FMS expression does not apparently enable cells to G_1/G_0 growth arrest in response to a lower concentration of retinoic acid.
c-FMS REGULATES RB, CELL CYCLE, AND DIFFERENTIATION

myelo-monocytic differentiation marker, oxidative metabolism. $0.5 \times 10^{-8} \text{ M VD3}$ caused a low but reproducible percentage of all cell lines to differentiate by late times. The extent was greater for the two transfected cells, Sort 10 and FMS/I, compared to the HL-60 parent. Using $0.5 \times 10^{-7} \text{ M VD3}$ increased the percentage of differentiated cells for all cell lines, but Sort 10 showed the greatest percentage of differentiated cells. Using $0.5 \times 10^{-6} \text{ M VD3}$, a concentration that is typically used to elicit monocytic differentiation, all cell lines differentiated, consistent with a previous report (32). The FMS/I cells differentiated to a greater extent than HL-60 parents at any time, and the Sort 10 cells differentiated likewise to a greater extent than the FMS/I cells. As for retinoic acid, at lower VD3 concentrations, enhanced c-FMS expression increased differentiation. As with retinoic acid, increasing c-FMS expression could compensate to some extent for a low VD3 concentration and vice versa.

Enhanced c-FMS expression also caused RB hypophosphorylation in response to lower concentrations of VD3. Fig. 5 shows the densitometric scans of RB protein Western blots for HL-60, FMS/I, and Sort 10 cells treated with $0 \text{ M (control)}$, $0.5 \times 10^{-8} \text{ M}$, $0.5 \times 10^{-7} \text{ M}$, or $0.5 \times 10^{-6} \text{ M VD3}$ for 48 h. Cells treated with $0 \text{ M (control)}$ or $0.5 \times 10^{-8} \text{ M VD3}$ showed no hypophosphorylated RB. A higher concentration of $0.5 \times 10^{-7} \text{ M}$ induced hypophosphorylated RB in Sort 10 cells, but not HL-60 or FMS/I. This is analogous to the case of retinoic acid-induced RB hypophosphorylation at 72 h. VD3, at $0.5 \times 10^{-6} \text{ M}$, a concentration typically used to elicit HL-60 monocytic differentiation and arrest, caused all the cell lines to express hypophosphorylated RB. At 24 h, none of the cell lines treated with any of these VD3 concentrations exhibited hypophosphorylated RB (data not shown). At 72 h, none of the cells treated with $0.5 \times 10^{-8} \text{ M}$ showed detectable hypophosphorylated RB (data not shown). But treatment with $0.5 \times 10^{-7} \text{ M}$ induced hypophosphorylated RB in all of the cell lines. Comparing HL-60, FMS/I, and Sort 10, the relative amount of hypophosphorylated RB increased with increasing c-FMS expression when cells were treated with $0.5 \times 10^{-6} \text{ M VD3}$ (Fig. 6). At 96 h, all of the tested VD3 concentrations induced hypophosphorylated RB in each of the cell lines (data not shown). For each cell line, the percentage of hypophosphorylated RB increased with increasing VD3 concentration, and at each concentration, the percentage of hypophosphorylated RB increased with increasing c-FMS expres-

### Table 1

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concentration allows cells expressing lower c-FMS to respond. Table 1 summarizes the results. Thus, to some extent it appears that the retinoic acid- and c-FMS-originated signals can compensate for each other as if it is their sum that determines response.

**VD3-induced Monocytic Differentiation.** The above findings for retinoic acid motivate the question of whether these effects extend to VD3, which induces monocytic differentiation through a related receptor. The effects of c-FMS overexpression are qualitatively similar for VD3 and retinoic acid, but in VD3 they are not as pronounced. HL-60, FMS/I, and Sort 10 cells were cultured with $0 \text{ M (control)}$, $0.5 \times 10^{-8} \text{ M}$, $0.5 \times 10^{-7} \text{ M}$, or $0.5 \times 10^{-6} \text{ M VD3}$ in lieu of retinoic acid, and cell differentiation was assayed at as above at sequential times. Fig. 4 shows the percentage of cells that expressed the functional

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**Fig. 3.** Percentage of cells in G1/G0 for HL-60 sublines expressing progressively more c-FMS. The percentage of cells with G1/G0 DNA was determined by flow cytometry. Percentage of cells in G1/G0 (Y axis) during treatment (X axis, h) of HL-60 (parental cells; ○), FMS/I (▲), and Sort 10 (■) transfectants with $0 \text{ M (control)}$, $10^{-7} \text{ M (top left)}$, $10^{-6} \text{ M (top right)}$, $10^{-7} \text{ M (bottom left)}$, and $10^{-6} \text{ M (bottom right)}$ retinoic acid.

**Fig. 4.** Vitamin D3-induced differentiation of HL-60 sublines expressing progressively more c-FMS. Differentiation was measured by the percentage of cells expressing inducible oxidative metabolism detected by intracellular NBT reduction. Percentage of cells that have differentiated (Y axis) during treatment (X axis, h) of HL-60 (parental cells; ○), FMS/I (▲), and Sort 10 (■) transfectants with $0.5 \times 10^{-8} \text{ M (left)}$, $0.5 \times 10^{-7} \text{ M (middle)}$, and $0.5 \times 10^{-6} \text{ M (right)}$ retinoic acid.
c-FMS REGULATES RB, CELL CYCLE, AND DIFFERENTIATION

inhibited cell density increases. Like retinoic acid, the intermediate VD3 concentration induced a modest $G_1/G_0$ enrichment (by up to ~20% in some cases) but no associated abatement in increasing cell density. This was consequently not taken to indicate $G_1/G_0$-specific growth arrest, as occurred at the highest VD3 concentration, but rather a redistribution of cell cycle phases as reported previously for these cells (32).

As for retinoic acid, the lowest concentration of VD3 could elicit cell differentiation if sufficient c-FMS receptors were overexpressed. An intermediate concentration was needed to elicit RB hypophosphorylation, and this occurred first in only the highest c-FMS-expressing cells. The highest concentration was needed to elicit $G_1/G_0$-specific growth arrest. Thus, as for retinoic acid, the signal threshold for response was lowest for cell differentiation, higher for RB hypophosphorylation, and highest for $G_1/G_0$-specific growth arrest. The effects of c-FMS overexpression on VD3-induced monocytic differentiation are thus similar to those for retinoic acid-induced myeloid differentiation. In conclusion, c-FMS enhanced the sensitivity to both these inducers, indicating that the effects with retinoic acid induced myeloid differentiation extend to the case of VD3-induced monocytic differentiation.

Cellular Retinoic Acid Uptake. Overexpression of c-FMS had no significant effect on uptake of exogenous retinoic acid (Table 2). Exponentially proliferating HL-60 or Sort 10 cells were cultured for 1 h with 0 M (control), $10^{-8}$ M, $10^{-7}$ M, or $10^{-6}$ M trace tritium-labeled retinoic acid. An equal number of labeled cells were harvested in each case, washed, and solubilized for radioactive liquid scintillation counting. Fig. 8 shows the results. Cellular uptake of retinoic acid was approximately proportional to the exogenous retinoic acid concentration. Successive reductions in exogenous concentrations by 10-fold caused comparable 10-fold reductions in uptake. Uptake by HL-60 cells, which expressed low c-FMS, and by Sort 10 cells, which expressed high c-FMS, was comparable for each concentration of exogenous retinoic acid tested.
DISCUSSION

The results of the present study indicate that increasing c-FMS expression increases the sensitivity of HL-60 promyelocytic leukemia cells to the myeloid differentiation inducer, retinoic acid. The cellular responses analyzed were cell differentiation, cell cycle arrest, and RB hypophosphorylation, a putative regulator of such processes. Cells with progressively higher c-FMS expression could respond to progressively lower concentrations of retinoic acid. Cell differentiation could be elicited by the lowest concentration tested, without accompanying RB hypophosphorylation or G1/G0-specific growth arrest, if c-FMS expression was high enough. A higher concentration of retinoic acid elicited cell differentiation, even for cells expressing less c-FMS, and also RB hypophosphorylation, without G1/G0-specific growth arrest, if c-FMS expression was sufficiently high. The highest concentration tested corresponded to that routinely used to elicit HL-60 myeloid differentiation and caused cell differentiation, RB hypophosphorylation, and G1/G0-specific growth arrest, even for cells expressing low c-FMS levels. Similar although not as pronounced results were observed with the monocytic differentiation inducer, 1,25-dihydroxy vitamin D3, indicating that c-FMS had similar regulatory effects on both of the differentiation lineages available to HL-60 promyelocytic leukemia cells.

There was an apparent threshold in cumulative retinoic acid plus c-FMS-originated signal needed to elicit cellular response. The threshold to elicit cell differentiation, RB hypophosphorylation, and cell cycle arrest progressively increased. Within the range of ligand (retinoic acid or 1,25-dihydroxy vitamin D3) concentrations or c-FMS expression levels tested, cell differentiation could be elicited without occurrence of detectable RB hypophosphorylation or cell cycle arrest. RB hypophosphorylation could be elicited without cell cycle arrest, but not without differentiation. Cell cycle arrest could only be elicited with cell differentiation and RB hypophosphorylation. This progression of effects from cell differentiation to RB hypophosphorylation to cell cycle specific arrest paralleled increasing concentrations of retinoic acid or VD3. In the case of cell differentiation and RB hypophosphorylation, it was apparent that within the range of ligand concentration or c-FMS expression tested, for any given concentration of retinoic acid or VD3, increasing c-FMS expression paralleled increasing extent of response. Thus, it appears as if the retinoic acid-originated and c-FMS-originated signals integrate, summing to elicit cell differentiation or RB hypophosphorylation. Thus, the apparent signal thresholds for causing cell differentiation, RB hypophosphorylation, and cell cycle-specific growth arrest increase, in that order. This rules out simple models of regulation by retinoic acid, whereby the ligand-activated transcription factor induces expression of a central regulatory molecule causing pleiotropic effects, specifically differentiation, RB hypophosphorylation, and G1/G0-specific arrest.

The present results indicate that there is an intersection between the retinoic acid- and vitamin D3-originated signals and the c-FMS-originated signals. The identity of this mechanism is not known. Because both retinoic acid and vitamin D3 are similarly involved and both putatively induce common essential early events (31, 34), the mechanism likely involves early events common to both inducers.

Table 2: Uptake of retinoic acid by HL-60 and c-FMS-transfected HL-60 cells

<table>
<thead>
<tr>
<th>Subline</th>
<th>Control</th>
<th>10^-8 M</th>
<th>10^-7 M</th>
<th>10^-6 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>4,598 ± 5 M</td>
<td>1,320 ± 62</td>
<td>10,992 ± 1,102</td>
<td></td>
</tr>
<tr>
<td>Sort 10</td>
<td>1,196 ± 23</td>
<td>1,196 ± 213</td>
<td>8,914 ± 1,640</td>
<td></td>
</tr>
</tbody>
</table>

Recent literature suggests a variety of potential mechanisms by which the retinoic acid/vitamin D3 and c-FMS signal transduction pathways may intersect. Such an intersection may occur at the retinoic acid/vitamin D steroid receptor, at intermediate signal transduction molecules downstream of c-FMS, or at promoters targeted by the two signal transduction pathways.

Phosphorylation of the steroid receptor may be one intersection of steroid hormone signaling with a kinase cascade. The RARα is phosphorylated, although in embryonal carcinoma cells, the phosphorylation is not affected by retinoic acid (35). Likewise, in HL-60 cells, RARα is also phosphorylated, but the relative amount of phosphorylated RARα appears unchanged by retinoic acid, based on detecting a phosphorylation induced gel electrophoretic mobility shift of RARα by Western analysis. RARβ- and RARγ are also phosphorylated in embryonal carcinoma cells (36, 37). Using ectopic expression, RARα can be phosphorylated by protein kinase A, causing enhanced transcriptional activation of a reporter by ligand-activated RARα (38).

Likewise, the vitamin D receptor can also be phosphorylated by PKC, a kinase implicated in signal transduction by typical transmembrane tyrosine kinase receptors for mitogenic growth factors (39). The vitamin D receptor can also be phosphorylated by casein kinase (40), a kinase known to target nuclear proto-oncogenes myc, myb, and max and RNA polymerases I and II, thereby also enhancing transcriptional activation. In general, the phosphorylation of steroid hormone receptors has been implicated in their ligand activation, nuclear transport, and DNA binding (41). Thus, signaling molecules downstream of c-FMS may affect such processes for the retinoic acid or vitamin D receptors.

The intersection of retinoic acid/vitamin D3 and c-FMS signaling...
may also involve retinoic acid or vitamin D3 control of signaling molecules typically downstream of transmembrane tyrosine kinase receptors, such as c-FMS. Ligand-activated c-FMS binds the adapter molecules Grb2 and SOS1, associated with RAS activation (42), implicating the signaling molecules typically associated with RAS/RAF mediated signaling. And RAF has demonstrated regulatory effects on retinoic acid- or vitamin D3-induced HL-60 differentiation (13). Retinoic acid can specifically increase PKCo levels in murine melanoma cells (43). In U937 monoblastoid leukemia cells, low doses of <1 nM retinoic acid enhanced protein phosphorylation and cell differentiation by 12-O-tetradecanoylphorbol-13-acetate, an activator of PKC (44). Furthermore, in HL-60 cells, retinoic acid increased expression of the src-like kinase fgr, whereas vitamin D3 increased the expression of the src-like kinases fyn, fgr, and lyn (45). Thus, retinoic acid can have a direct effect on signaling molecules. Likewise, vitamin D3 can also affect downstream effectors of transmembrane tyrosine kinases. Vitamin D3 can activate RAF within 1 min of stimulation in a 12-O-tetradecanoylphorbol-13-acetate or staurosporine-sensitive process suggesting a PKC dependence (46). Further downstream, vitamin D3 can, for example, enhance DNA binding activity of AP-1 (47).

Finally, the intersection of the retinoic acid/vitamin D3- and c-FMS-originated signals may occur at the promoter(s)-targeted by these signals. The M, 265,000 CREB-binding protein required for AP-1 activation is part of an integrator complex that also mediates transcriptional activation by RAR (48). This provides a potential mechanism for the observed ability of retinoic acid to act as a negative regulator of AP-1 (49). In this case, the RAR may deprive AP-1 sites of the needed CBP. One can speculate, however, that if the RAR binds in proximity of an AP-1, then a factor such as CBP, which is attracted to AP-1, for example, might also positively regulate transcriptional activation by RAR. Thus, the AP-1 activation downstream of some prototypical transmembrane tyrosine kinase receptor might affect retinoic acid signaling, and potentially vice versa. Such a process might, for example, be the basis of the integration of the retinoic acid/vitamin D- and c-FMS-originated signals observed here.

The present results motivate a re-evaluation of current ideas on the role of RB in regulating the cell cycle and, putatively, differentiation. RB is thought to regulate the cell cycle and, putatively, cell differentiation through its phosphorylation. In this present paradigm, the phosphorylation of RB, possibly at the G1 restriction point (50, 51) by cyclin D1, E1, or A-dependent kinases (52, 53), is believed to cause progression to S phase, due in large part to the release of transcription factors binding only the unphosphorylated RB (see Refs. 54 and 55 for review). Along with other recent data not entirely consistent with the anticipations of this paradigm, (13, 17-25), two pieces of the present data specifically mitigate against this paradigm. One is that, although HL-60 cells have a substantial G1 phase, with approximately 45% of exponentially proliferating cells in G1, there is no evident hypophosphorylated RB; i.e., all of the RB is hyper phosphorylated. The other is that it is possible to cause appearance of significant, ~15% hypophosphorylated RB without precipitating growth arrest. Thus, RB phosphorylation or dephosphorylation per se does not immediately regulate G1 to S cell cycle progression or G0 arrest. Furthermore, because differentiation can occur without observable hypophosphorylated RB, phosphorylation shift also does not appear to be causal for cell differentiation. Interestingly, in proliferating HL-60 cells, the amount and degree of phosphorylation of RB progressively increase as cells advance through the cell cycle (19, 21). Furthermore, when HL-60 cells differentiate, the amount of RB protein per cell decreases as it shifts to the unphosphorylated state, as cells G0 arrest and differentiate (1, 13, 19). In these instances, the amount of RB and its phosphorylation appear to generally reflect cellular anabolic activity, and may be a regulator thereof. Several recent findings suggest this possibility. RB can be found concentrated in nucleoli (56) and can interact with the UBF transcription factor (57) and inhibit RNA polymerases (56, 57, 58). Thus, RB may function as a general regulator of transcriptional activity. In the particular cases of E2F, Sp1, AP-1, or p53 dependent transcriptional activation, a RB-GAL4 fusion protein can repress transcriptional activation by this range of transcription factors (58). Thus, RB may have a more global regulatory function than just G1/G0 control by regulating cellular anabolic levels.

REFERENCES


Increasing c-FMS (CSF-1 Receptor) Expression Decreases Retinoic Acid Concentration Needed to Cause Cell Differentiation and Retinoblastoma Protein Hypophosphorylation

Andrew Yen, Rhonda Sturgill and Susi Varvayanis


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