BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **223,** 73–79 (1996)
ARTICLE NO. 0848

Enzymatic Pattern of Aldehyde Metabolism during HL-60 Cell Differentiation

G. Barrera, S. Pizzimenti, G. Muzio, M. Maggiora,* A. Garramone,* F. Biasi, M. U. Dianzani, and R. A. Canuto*

*Dipartimento di Medicina e Oncologia Sperimentale, Università di Torino, 10125 Turin, Italy; and *Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Ospedale S. Luigi Gonzaga, Orbassano (Turin) Italy*

Received April 16, 1996

A number of metabolic changes, including modification of different enzyme activities, are linked to the acquisition of differentiated phenotype in HL-60 cells. Enzymes metabolizing aldehydes contribute to maintaining the intracellular steady-state concentration of aldehydes derived from lipid peroxidation. 4-Hydroxynonenal is one of the most important aldehydes produced by this process, and it is able to inhibit proliferation and induce differentiation of HL-60 human leukemic cells. We have now demonstrated that, after induction of HL-60 cell differentiation by 4-hydroxynonenal or DMSO, glutathione transferase activity increases in parallel to the degree of differentiation, whereas aldehyde dehydrogenase, aldehyde reductase and alcohol dehydrogenase are not affected by differentiation induction. Moreover, in 4-hydroxynonenal- or DMSO-treated cells, the concentration of reduced glutathione decreases five days after treatment. The rise of glutathione transferase activity, as well as the decrease of reduced glutathione, are possibly linked to the increase of detoxification capability of differentiated cells.

Cell differentiation elicits a number of metabolic changes including modification of different enzyme activities linked to the acquisition of a differentiated phenotype (1). HL-60 cells have become an archetype model for studying terminal differentiation of myeloid cells “in vitro” (2). They can be induced to differentiate either in monocytic- or in granulocytic-like cells by a variety of compounds such as TPA, retinoic acid and DMSO (1–4). Moreover, we have recently reported that 4-hydroxynonenal (HNE), an aldehyde produced from lipid peroxidation, blocks cell growth and induces a granulocytic differentiation of HL-60 cells at concentrations similar to those detected in a number of normal non proliferating cells (5,6). Enzymes metabolizing aldehydes produced during lipid peroxidation (aldehyde dehydrogenase, aldehyde reductase, alcohol dehydrogenase and glutathione-S-transferase) contribute to maintain the steady-state aldehyde concentration inside the cells (7). Studies on the activities of aldehyde metabolizing enzymes have been performed in normal tissues like liver (7,8), kidney (9) and retina (10) as well as in hepatoma cell lines (7,11,12) and in liver tissue during chemically-induced carcinogenesis (13); little information is however available for myeloyd cells. Generally in tumour tissues a marked increase of aldehyde dehydrogenase and aldehyde reductase activities has been reported (11,12) in direct correlation with the degree of deviation, whereas glutathione-S-transferase activity was found to be reduced in hepatoma cell lines (11) but increased during liver carcinogenesis (13). Some studies on HL-60 cell line demonstrated that differentiation leads to the acquisition of most of polymorphonuclear leukocytes functions: chemotaxis, phagocytosis, respiratory burst activity, and bacterial killing (1–4). During induced and spontaneous HL-60 cell differentiation NADPH-depended superoxide production increases as well as the maturation of O_2 generating system (14). On the other hand, glutathione peroxidase activity also increases indicating a rapid modulation oof cellular genes involved in the protection against hydrogen peroxide and related toxic oxidants (15).

The level of lipid peroxidation and the role of aldehyde metabolizing enzymes during differentiation are not yet well characterized: it is well established that tumour cells display a very low level of lipid peroxidation, generally inversely related to the degree of deviation, and a high resistance to induction of oxidative stress, resulting in low steady-state concentrations of endogenous lipid

0006-291X/96 $18.00

peroxidation products (16). During myeloid differentiation the potential changes of redox state might suggest a possible alteration of the lipid peroxidation level and/or of the catabolism rate of its products.

In this research, the pattern of aldehyde metabolizing enzymes, the HNE metabolism and the level of reduced glutathione (GSH) have been evaluated in HL-60 cells before and after induction of differentiation by HNE and DMSO.

MATERIALS AND METHODS

Cells and culture conditions. HL-60 cells (DSM, German collection of microorganisms and cell cultures, Braunschweig, Germany) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air in RPMI 1640 medium (Biochrom KG, Berlin) supplemented with 2 mM glutamine, antibiotics and 10% FCS (Biochrom KG, Berlin). Growth rate and cell viability were monitored daily by the trypan blue exclusion test.

Cell treatments. HL-60 cells were seeded at 300,000/ml and cultured for 5 days. HNE, kindly provided by Prof. H. Esterbauer, (University of Graz, Austria) was prepared as previously described (5). At the beginning of each experiment, 1 μM HNE was added 10 times to the cultures at 45 min intervals. HNE concentrations were monitored by HPLC after each addition, and never exceeded 1 μM (data not shown). HNE was no longer detectable in the culture medium 45 min after the last addition (7.5 hours from the beginning of experiment).

Cells treated with 1.25% DMSO (Sigma Chemical Co.) were exposed to the chemical for 5 days.

Phagocytosis assay. Phagocytosis was evaluated by counting the number of HL-60 cells that engulfed opsonized zymosan (Sigma Chemical Co.) as previously described (5).

Lipid peroxidation. Lipid peroxidation was measured as MDA production by the spectrophotometric thiobarbituric acid assay (17). Cells (20×10^6 for each sample) were collected before the treatments with HNE or DMSO and after 5 days, washed twice with PBS and resuspended in 4 ml of PBS at a concentration of 5×10^6 cells/ml. Each sample was incubated for 1 hour at 37°C with or without prooxidant agents: 2.5 mM ADP plus 0.1 mM $FeCl_3$ or 0.5 mM ascorbic acid plus 20 μM $FeSO_4$ were used. 0.7 ml of cells were collected before incubation and after 30 min or 1 hour, added to 0.7 ml of 10% TCA in ice and centrifuged at 2000 rpm at 4°C for 10 min in a Beckman J-6B centrifuge. Supernatants were used for MDA determinations (17).

HNE consumption. Control and treated cells were harvested 5 days after induction. 10 μM HNE was added to HL-60 cell suspension (10^6 cells/ml). Aliquots (0.5 ml) were harvested immediately after addition (TO) and after 5, 10, 15, 30, 45 and 60 min, added to an equal volume of acetonitrile/acetic acid 96:4 (v/v) and centrifuged at 2,000 rpm for 10 min in a Beckman J-6B centrifuge. The clear supernatants were used for HPLC analysis. The separation conditions were as previously described (18).

Preparation of cytosolic fractions. Cells (about 250×10^6) treated with HNE or DMSO were harvested 7.5 hours after the beginning of experiments and after 5 days. Cell homogenates were obtained by one freeze-thaw cycle followed by a 20 min incubation in a hypotonic solution (17.5 mM sucrose, 55 mM mannitol, 5 mM Tris-HCl pH 7.4, 0.5 mM EGTA and 0.025 % (w/v) bovine albumine (fraction V, fatty acid free); then they were diluted to 20% w/v with sucrose/mannitol to obtain an isotonic solution (70 mM sucrose, 220 mM mannitol, 20 mM Tris-HCl pH 7.4, 2 mM EGTA and 0.1% bovine albumine) and mildly sonicated. Homogenates were centrifuged in a Beckman L8-55 centrifuge at 105.000 g for 60 min to obtain cytosolic fraction.

Enzyme assays. Aldehyde dehydrogenase (ALDH; EC 1.2.1.3), aldehyde reductase (ALRD; EC 1.1.1.2), alcohol dehydrogenase (ADH; EC 1.1.1.1.) and glutathione-S-transferase (GST; EC 2.5.1.18) were assayed in cytosolic fractions as previously described (4) by using HNE as substrate, at the concentration of 0.05 mM for GST assay and 0.1 mM for the other enzymes.

Glutathione determination. Intracellular glutathione (GSH) was assayed with Elmann's reagent as reported by other authors (19).

Protein determination. Proteins were determined by biuret method (20).

Statistical analysis. All data are expressed as mean ± SD. Variance analysis followed by the Bonferroni test was carried out to evaluate the differences between group means.

RESULTS

The degree of differentiation obtained after either HNE or DMSO treatments was evaluated by measuring the inhibition of cell growth and the induction of phagocytosis (fig. 1). According with previous studies (5), HNE and DMSO provoked a reduction of cell growth without affecting cell viability. After differentiation induction the proportion of phagocytosing cells gradually increased; 5 days after treatments it was 50% and 25% with DMSO and HNE respectively.

The metabolic pattern of HNE in uninduced and in induced HL-60 cells is shown in table 1 and

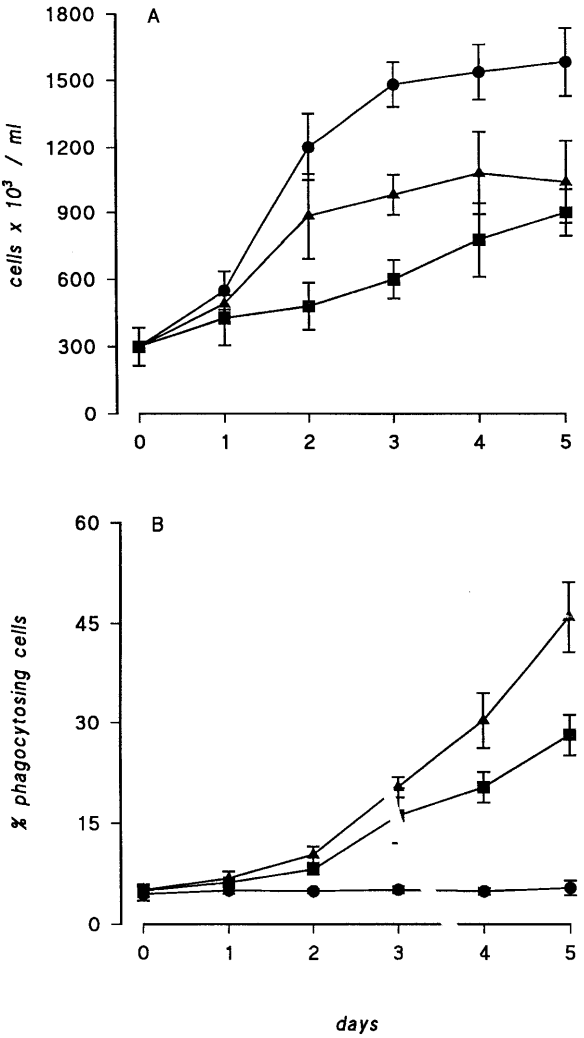

FIG. 1. Panel A: growth of HL-60 cells. > Control cells, ■ cells treated for 10 times, at intervals of 45 minutes, with 1 μM HNE, ▲ cells treated with 1.25% DMSO. Results are the mean ±S.D. of four separate experiments. Panel B: Phagocytosis: ● Control cells, ■ cells treated with 1 μM HNE for 10 times, at intervals of 45 minutes, ▲ cells treated with 1.25% DMSO. Results are the mean ±S.D. of 3 separate experiments.

2. Enzyme activities were detected at the end of HNE treatments (7.5 hours from the beginning of experiment) and after 5 days. On the whole, the enzyme activities were lower than those reported for other cell types (11,13). ALDH (NAD- or NADP-dependent) and ADH activities remained unchanged after HNE and DMSO treatments, whereas GST activity increased. This increase was evident since 7.5 hour, (from 5.22 nmol of HNE consumed/min/mg of protein in control cells to 7.085 and 10.772 in HNE- and DMSO-treated cells, respectively), and it was unchanged at day 5. The time course of GST activity and the level of GSH were investigated at different intervals from the beginning of treatments (fig. 2). Enzyme activity increased since 45 minutes, peaked at 7.5 hours and remained almost constant in the following days (fig. 2 A). The level of GSH was similar to the control until day 3, whereas decreased at day 5 (fig. 2B).

HNE consumption, detected by HPLC 5 days after the beginning of experiments, was poorly affected by HNE or DMSO treatments with respect to the control cells (fig. 3). However, a little,

TABLE 1
HNE Metabolizing Enzymes in HL-60 Cells

ENZYMES	CONTROL	HNE 7.5h	HNE 5d
ALDH NAD	3.205 ± 1.187[a]	2.582 ± 0.600[a]	3.362 ± 1.300[a]
ALDH NADP	0.903 ± 0.401[a]	1.118 ± 0.106[a]	0.642 ± 0.240[a]
ADH	0.548 ± 0.388[a]	0.314 ± 0.081[a]	0.350 ± 0.119[a]
ALRD	0.444 ± 0.230[a]	0.401 ± 0.249[a]	0.424 ± 0.253[a]
GST	5.277 ± 0.211[a]	7.085 ± 1.085[b]	7.408 ± 0.842[b]

Means ± S.D. of four experiments expressed as nmol of NAD(P) reduced/min/mg of protein for ALDH, as nmol of NAD(P)H consumed/min/mg of protein for ADH and ALRD, respectively, and as nmol and HNE consumed/min/mg of protein for GST. Means of three groups (control, HNE 7.5h, HNE 5d) with different letters are statistically different ($p < 0.05$) from one another as determined by variance analysis followed by the Bonferroni test.

even if not statistically significant, increase in the time-course of HNE disappearance has been noted in both HNE and DMSO induced cells.

In HL-60 cells the level of lipid peroxidation remains undetectable, even after exposition to prooxidant stimuli. This pattern remained unchanged after both DMSO and HNE treatments (data not shown).

DISCUSSION

Differentiation elicits a number of biochemical and morphological changes in HL-60 cells which loose their replicative ability and acquire phagocytic properties (1). In our experiments, treatment with DMSO or HNE caused both a decrease in proliferation and an increase in the proportion of Hl-60 cells that acquire phagocytic properties, according to our previous observations (5).

ALDH (NAD- or NADP-dependent), ALRD and ADH do not show any significant changes after induction of differentiation. All these activities in HL-60 cells are lower than those detected in other normal or tumour tissues (11–13). In fact, in HL-60 cells ALDH activity is about one third of that present in hepatocytes, ALRD activity is about 15 times lower and ADH is almost 100 times lower than in liver (7). This may be consistent with the extremely low values of endogenous HNE production through lipid peroxidation. Among the aldheyde metabolizing enzymes, only GST activity was found to increase after HNE or DMSO treatments. Cytosolic GSTs are a family of important detoxification isoenzymes (21) one of them (8:8) is known to be able to use HNE as a substrate (22). GST-catalyzed reactions involve the direct coupling of glutathione to electrophilic drugs, carcinogens and endogenous compounds (21). The increase in GST activity with HNE as a substrate, in HNE-treated cells, might be interpretated as a consequence of a substrate-linked induction. However, the cells remain in the presence of added HNE only for 7.5 hours, after this

TABLE 2
HNE Metabolizing Enzymes in HL-60 Cells

ENZYMES	CONTROL	DMSO 7.5h	DMSO 5d
ALDH NAD	3.205 ± 1.187[a]	3.378 ± 0.859[a]	4.700 ± 1.045[a]
ALDH NADP	0.903 ± 0.401[a]	0.790 ± 0.068[a]	0.658 ± 0.422[a]
ADH	0.548 ± 0.388[a]	0.310 ± 0.047[a]	0.459 ± 0.043[a]
ALRD	0.444 ± 0.230[a]	0.250 ± 0.091[a]	0.292 ± 0.100[a]
GST	5.277 ± 0.211[a]	10.772 ± 1.569[b]	10.789 ± 1.090[b]

Means ± S.D. of four experiments expressed as nmol of NAD(P) reduced/min/mg of protein for ALDH, as nmol of NAD(P)H consumed/min/mg of protein for ADH and ALRD, respectively, and as nmol of HNE consumed/min/mg of protein for GST. Means of three groups (control, DMSO 7.5h, DMSO 5d) with different letters are statistically different ($p < 0.05$) from one another as determined by variance analysis followed by the Bonferroni test.

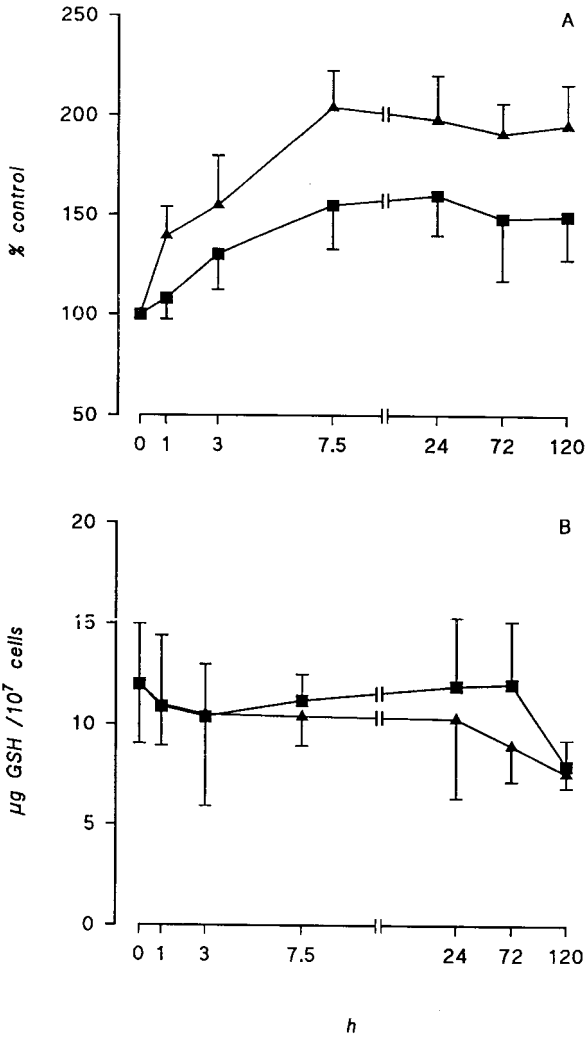

FIG. 2. Panel A: time course of GST activity after HNE ■ and DMSO ▲ treatments. The values, expressed as percentage of control are the mean ± S.D. of 3 separate experiments. Panel B: levels of GSH ($\mu g/10^7$ cells) in HL-60 cells after HNE ■ and DMSO ▲. The values are the mean ± S.D. of 3 separate experiments.

period the aldehyde is not longer detectable in the culture medium (5). GST activity remains however at high levels even 5 days after the treatment, in absence of aldehyde in the culture medium. This suggests that GST increase is more probably linked to the acquisition of the differentiated phenotype rather than to substrate depending induction. On the other hand, the fact that an even higher increase is observed in DMSO-treated cells is in favour of this interpretation.

HNE or DMSO treatments induce the increase of GST activity already evident after 3 and 7.5 hours from the beginning of the experiments. It has recently been reported that the induction of differentiation along the monocytic or granulocytic pathway in HL-60 cells resulted in a rapid modulation of enzymes linked to the increase of the detoxification capability in differentiated cells (19).

Intracellular GSH content also decreases 5 days after HNE and DMSO treatments. Probably, the decrease of GSH is a consequence of the persistence of the high GST activity during the days following the treatments.

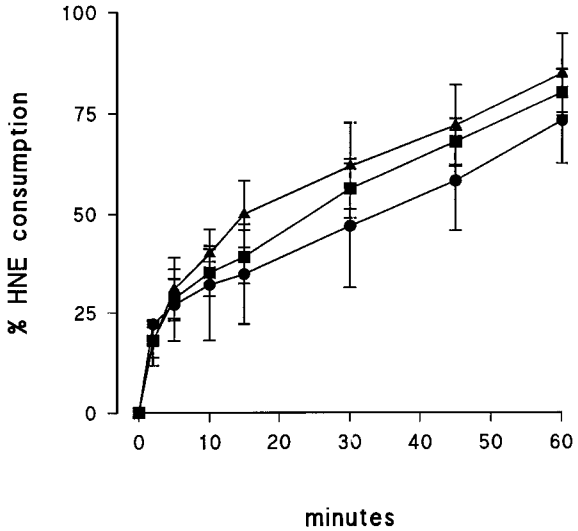

FIG. 3. Consumption of 10 μM HNE by HL-60 cells: ● control cells, ■ cells treated with 1 μM HNE (10 treatments), ▲ cells treated with 1.25 DMSO. Cells were collected 5 days after treatments. Results are the mean ± S.D. of three separate experiments. For experimental details see Materials and Methods.

The values of GST activity in HL-60 cells, even though doubled after DMSO induction, are lower than in liver in which GST has by far the highest specificity for HNE, its bioconversion by GST represents 50–60% of the total HNE removal by hepatocytes (12). This may account for the slow disappearance of HNE from the HL-60 cell suspension with respect to the liver cells and for the poor effect on HNE metabolism observed in HNE- and DMSO-treated cells. The levels of both basal and induced lipid peroxidation in HL-60 cells are undetectable and the induction of differentiation do not modify the susceptibility of HL-60 cells to peroxidize.

ACKNOWLEDGMENTS

This work was supported by the Italian Association for Cancer Research, C.N.R. (A.C.R.O. project), and M.U.R.S.T.

REFERENCES

1. Collins, S. J., Ruscetti, F. W., Gallaghen, R. E., and Gallo, R. C. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2458–2462.
2. Collins, S. J., Gallo, R. C., and Gallagher, R. E. (1977) *Nature* **270,** 347–349.
3. Rovera, G., Santoli, D., and Damsky, C. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76,** 2779–2783.
4. Breitman, T. R., Selonick, E. S., and Collins, S. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2936–2940.
5. Barrera, G., Di Mauro, C., Muraca, R., Ferrero, D., Cavalli, G., Fazio, V. M., Paradisi, L., and Dianzani, M. U. (1991) *Exp. Cell Res.* **197,** 148–152.
6. Barrera, G., Muraca, R., Pizzimenti, S., Serra, A., Rosso, C., Saglio, G., Farace, M. G., Fazio, V., and Dianzani, M. U. (1994) *Biochem. Biophys. Res. Commun.* **203,** 553–561.
7. Canuto, R. A., Ferro, M., Muzio, G., Bassi, A. M., Leonarduzzi, G., Maggiora, M., Adamo, D., Poli, G., and Lindahl, R. (1994) *Carcinogenesis* **15,** 1359–1364.
8. Hartley, D. P., Ruth, J. A., and Petersen, D. R. (1995) *Arch. Biochem. Biophys.* **316,** 197–205.
9. Ullrich, D., Grune, T., Henke, W., Esterbauer, H., and Siems, W. G. (1994) *FEBS-Lett.* **352,** 84–86.
10. Singhal, S. S., Awasthi, S., Srivastava, S. K., Zimniak, P., Ansari, N. H., and Awasthi, Y. C. (1995) *Invest. Ophthalmol. Vis. Sci.* **36,** 142–150.
11. Canuto, R. A., Muzio, G., Bassi, A. M., Biocca, M. E., Poli, G., Esterbauer, H., and Ferro, M. (1991) *in* Enzymology and Molecular Biology of Carbonyl Metabolism (Weiner, H., Wermuth, B., and Crabb, D. W., Eds.), Vol. 3, pp. 75–84, Plenum, New York.
12. Grune, T., Siems, W. G., Zollner, H., and Esterbauer, H. (1994) *Cancer Res.* **54,** 5231–5235.
13. Canuto, R. A., Muzio, G., Biocca, M. E., and Dianzani, M. U. (1989) *Cancer Letts.* **46,** 7–13.
14. Newburger, R. E., Speier, C., Borregaard, N., Walsh, C. E., Whitin, J. C., and Simons, E. R. (1984) *J. Biol. Chem.* **259,** 3771–3776.

15. Shen, Q., Chada, S., Withitney, C., and Newburger, P. E. (1994) *Blood* **84,** 3902–3908.
16. Dianzani, M. U. (1993) *Critical reviews in Oncology/Hematology* **15,** 125–147.
17. Slater, T. F., and Sawyer, B. C. (1971) *Biochem. J.* **123,** 805–814.
18. Barrera, G., Biasi, F., Fazio, V. M., Paradisi, L., and Dianzani, M. U. (1991) *in* Chemical Carcinogenesis 2 (Columbano, A., *et al.*, Eds.), pp. 337–342, Plenum, New York.
19. Chiarpotto, E., Biasi, F., Aragno, M., Scavazza, A., Danni, O., Dianzani, M. U., and Poli, G. (1995) *Alcohol and Alcoholism* **30,** 37–45.
20. Gornall, A. G., Bardawill, C. J., and David, M. (1949) *J. Biol. Chem.* **177,** 751–766.
21. Waxman, D. J. (1990) *Review Cancer Res.* **50,** 6449–6454.
22. Poot, M., Verkerk, A., Koster, J. F., Esterbauer, H., and Jorgkind, J. F. (1987) *Eur. J. Biochem.* **162,** 287–291.