Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 400-407

A NON-CHARACTERISTIC RESPONSE OF L1210 CELLS TO LOVASTATIN

Kory J. Engelke and Miles P. Hacker

Department of Pharmacology and Vermont Cancer Center
University of Vermont, Burlington, Vermont 05405

Received July 1, 1994

SUMMARY Isoprenylated proteins are believed to play an important role in DNA synthesis and subsequent cell cycle progression. Inhibition of the biosynthesis of these isoprenylated proteins results in a decrease in DNA synthesis and a characteristic G_1 blockade of the cell cycle. This inhibition can be achieved by incubation of cells in the presence of a hydroxy-methylglutaryl-coenzyme A reductase inhibitor (lovastatin) and can be reversed by addition of exogenous mevalonate. When incubated in the presence of lovastatin, L1210 wild-type cells are not inhibited at G_1 but rather progress to a G_2/M blockade, which is reversible with exogenous mevalonate. Thymidine incorporation has also shown that DNA synthesis is occurring until this blockade is achieved. However, when L1210 cells resistant to cisplatin are incubated in the presence of lovastatin, the characteristic G_1 cell cycle blockade and DNA synthesis inhibition occur. The understanding of this mechanism and the role that isoprenylated proteins play in the regulation of DNA synthesis and cell cycle progression may gain great insight into the abnormal control of the cancer cell.

What controls the cell cycle and enables the to cell enter into the cell cycle is very obscure. Cholesterol was first shown to be essential for cell growth and DNA synthesis (1-2). Later, a precursor of cholesterol (mevalonate) was shown to be responsible for this DNA replication (3-4). Thus, when cells are starved for mevalonate by various methods, there is decreased DNA synthesis, cell growth stops and the cell is prevented from entering into S phase of the cell cycle (there is a G_1 blockade) [3-10]. Upon the addition of mevalonate, but not cholesterol, cell cycle progression, subsequent DNA replication and cell growth reoccurs (3-4,6-8,10).

Intracellular mevalonate is the product of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (E.C. 1.1.1.34). This enzyme is the rate limiting step in the production of cholesterol (3,11) and can be competitively inhibited by the fungal metabolite lovastatin (11). When many normal and tumorigenic cells are incubated in the presence of lovastatin, there is an inhibition of DNA synthesis and cell growth and a characteristic G_1

0006-291X/94 $5.00

blockade in the cell cycle is achieved (5-9) which is reversible only after the addition of additional mevalonate or the removal of lovastatin (5,7-8,10). Exceptions to this rule do exist. It has been reported in the literature that human myeloid leukemia cells have a "paradoxical increase in DNA synthesis" when incubated in the presence of compactin, another HMG-CoA reductase inhibitor, for unknown reasons and mechanisms (12).

Mevalonate plays a pivotal role in the cell (see 13 for review). It is a precursor for many cellular products including cholesterol (13) as well as the prenyl groups of isoprenylated proteins (14). These isoprenylated proteins make up a very important group of proteins which include some of the following: the ras proteins, the small GTP-binding proteins and the γ-subunit of the heterotrimeric G-proteins (isoprenylated proteins are reviewed in 15, 16-19). Of considerable interest is that the guanine nucleotide binding proteins have been implicated in the control of cell growth (20-21). Mevalonate has also been implicated in the maintenance of cellular shape and adherence in several adherent cell lines (6-7,22). If the cells are incubated in the absence of mevalonate, there is a rounding of the cells which is reversible upon the addition of mevalonate (6-7,22).

We present here the effect of lovastatin on a murine leukemia cell line (L1210) and two variants incubated in the presence of lovastatin. L1210/0 cells are a suspension growing cell line with a doubling time of 8-12 hours. Many L1210 variants have been developed in our laboratory and two (L1210/DDP and L1210/DACH) have been included in the present study. The L1210/DDP cells are resistant to the anti-cancer drug cisplatin, are slightly more adherent and have a longer doubling time (18-24 hours) than the parent cell line. The L1210/DACH cells are resistant to the cisplatin derivative diamino-cyclohexane platinum (II) sulfate and retain the characteristics of the parent cell line (growing in suspension and a doubling time of 8-12 hours). The results show that the L1210/0 and L1210/DACH cells are blocked in G_2/M whereas the L1210/DDP cells are blocked in the more characteristic G_1 phase of the cell cycle when they are incubated with lovastatin.

MATERIALS AND METHODS

Cells and Culture Conditions L1210/0, L1210/DDP and L1210/DACH cells were seeded at a concentration of $1x10^5$ cells/ml and grown in McCoy's 5A media supplemented with 5% defined horse serum (L1210/0) or 5% fetal bovine serum (L1210/DDP and DACH). CHO cells were seeded at a concentration of $5x10^4$ cells/ml and grown in Ham's F12 media supplemented with 5% fetal calf serum for 24 hours before the addition of lovastatin.

Lovastatin (a gift from A. Alberts, Merck, Inc.) was activated as previously described (8) and was added to the cell cultures at the time of seeding for the L1210 cells or after a 24 hour incubation for the CHO cells.

Mevalonate (Sigma, St. Louis, MO) was added after incubating the cells with and without lovastatin for 24 hours.

Cholesterol and squalene (Sigma and Aldrich, Milwaukee, WI, respectively) were added to the

cell cultures at the time of seeding.

Cell Cycle Analysis: Cells were harvested, washed once with ice cold PBS, counted, fixed with ice cold 95% EtOH and stored at 4°C for at least 2 hours. The fixed cells were then pelleted and resuspended in PBS, treated with RNAse, and stained with propidium iodide. The cells were then analyzed on a Coulter Elite Flow Cytometer within 24 hours.

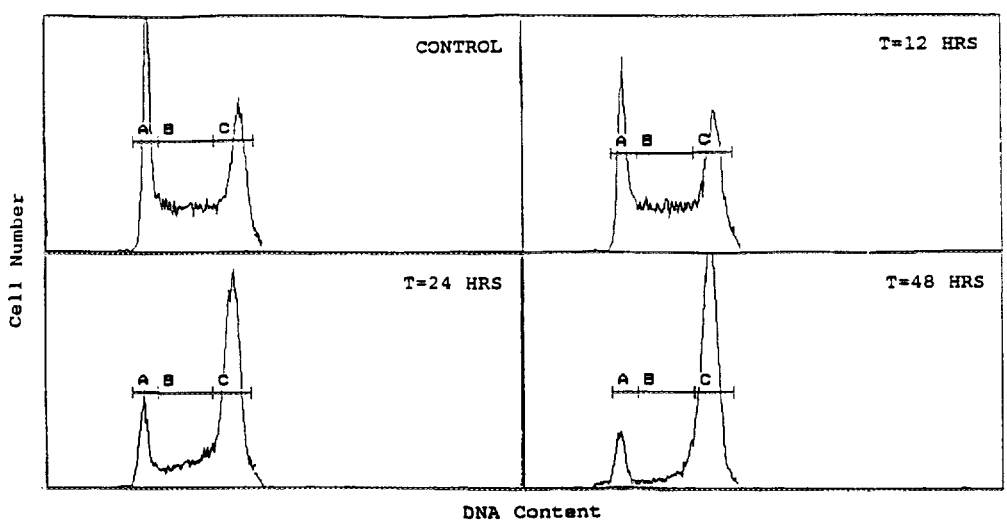
Thymidine Incorporation: Cells were incubated in the absence/presence of lovastatin for the indicated times in 96 well flat bottom plate. [^{3}H]-Thymidine was added and cells were incubated at 37°C for an additional hour. Cells were then harvested on a Matrix harvester and counted on a Matrix direct beta counter.

All cells were counted in a hemocytometer using trypan blue as an indicator of viability.

RESULTS AND DISCUSSION

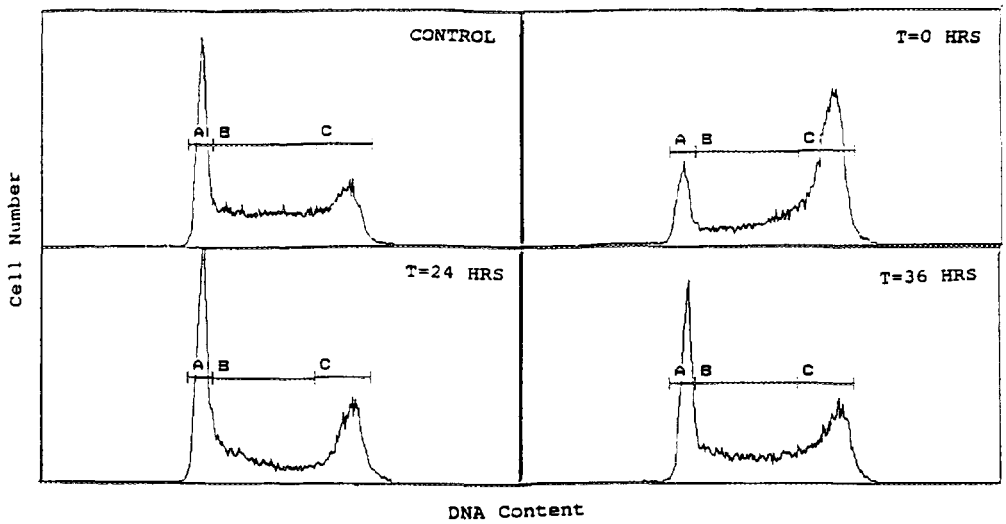
Figure 1 shows the results of the L1210/0 cells incubated in the presence of 40 uM lovastatin for up to 48 hours. With increased time of lovastatin treatment, an increase in the number of cells in G_2/M phase of the cell cycle and a decrease in the number of cells in the G_1 phase is observed. (There are approximately seven times as many cells in G_2/M phase than are in the G_1 phase by 48 hours.)

This blockade is reversed by the addition of 4 mM mevalonate, as shown in figure 2. With increased time of mevalonate exposure, the cells are released from G_2/M and progress into G_1 and S.



CONDITION	% G_1 PHASE (A)	% S PHASE (B)	% G_2/M PHASE (C)
CONTROL	32.0	29.9	38.1
T=12 HRS	28.7	31.7	39.6
T=24 HRS	16.2	20.5	63.4
T=48 HRS	11.0	12.5	76.5

FIGURE 1.
Cell cycle analysis of L1210/0 cells incubated in presence of 40 μM lovastatin. Hours indicated are after addition of lovastatin. (Control cells are not treated with lovastatin.) A=G_1 Phase; B=S Phase; C=G_2/M Phase.



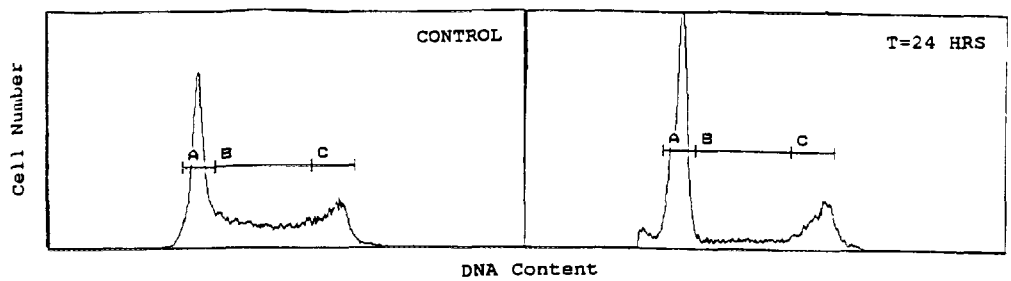
CONDITION	% G_1 PHASE (A)	% S PHASE (B)	% G_2/M PHASE (C)
CONTROL	31.6	41.3	26.4
T=0 HRS	14.3	25.9	57.9
T=24 HRS	39.2	28.9	29.6
T=36 HRS	29.3	34.7	33.3

FIGURE 2.
Cell cycle analysis of L1210/0 cells incubated in presence of 40 μM lovastatin for 24 hours and then rescued with 4 μM mevalonate (T=0 HRS). Hours indicated are after addition of mevalonate. (Control cells are not treated with lovastatin or mevalonate.) A=G_1 Phase; B=S Phase; C=G_2/M Phase.

As a control, CHO cells, which have been reported in the literature to block in G_1 when incubated in the presence of lovastatin (5), were treated with lovastatin. As expected, the CHO cells demonstrated the characteristic G_1 blockade in the cell cycle (figure 3). Also, microscopic examination of the cells after 12 hours of lovastatin treatment revealed that the cells displayed the characteristic rounded state associated with mevalonate depletion (data not shown).

When the L1210 variant L1210/DDP is incubated in the presence of 40 uM lovastatin, the characteristic G_1 blockade in the cell cycle is achieved (figure 4). By microscopic examination, these cells were also rounded in appearance by 12 hours (data not shown). When the other L1210 variant, L1210/DACH, is incubated in the presence of 40 uM lovastatin, they behave like the parent L1210/0. As can be seen in figure 5, these cells are blocked in the non-characteristic G_2/M phase of the cell cycle.

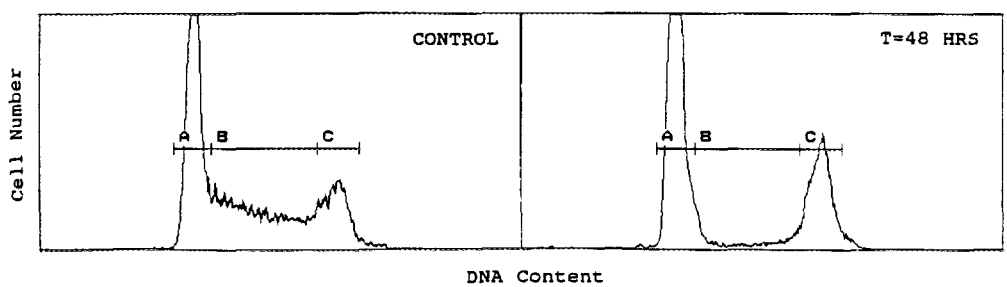
The only cells which can replicate their DNA (thus incorporate [^{3}H]thymidine) are those cells in S phase. As can be seen in figures 1, 4, and 5, many of the lovastatin untreated cells are in G_1 phase. Therefore, if the cells are blocked at G_1, there will be no progression into S



CONDITION	% G_1 PHASE (A)	% S PHASE (B)	% G_2/M PHASE (C)
CONTROL	42.2	34.0	20.6
T=24 HRS	58.2	13.0	19.9

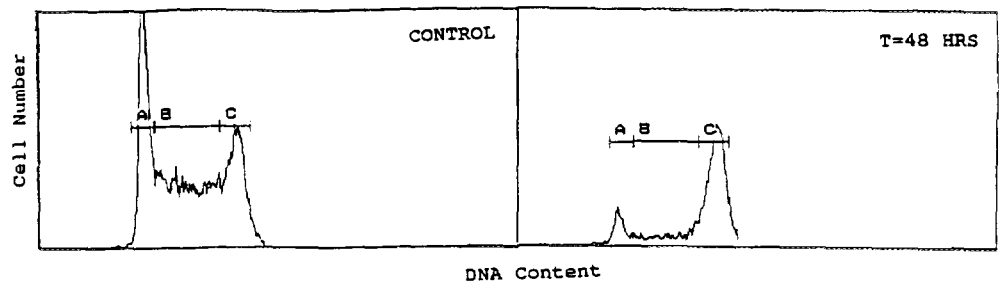
FIGURE 3.
Cell cycle analysis of CHO cells incubated in presence of 10 μM lovastatin. Hours indicated are after addition of lovastatin. (Control cells are not treated with lovastatin.) A=G_1 Phase; B=S Phase; C=G_2/M Phase.

phase and no [^{3}H]thymidine will be incorporated except in those cells which are already in S phase. This is observed with the L1210/DDP cells which cease to incorporate [^{3}H]thymidine after 6 hours (figure 6A and 6B). However, if the cells are blocked in G_2/M , any cell that is not already in G_2/M can proceed through G_1 into S and incorporate [^{3}H]thymidine, as is observed with the L1210/0 and L1210/DACH cells. Both of these cells continue to incorporate [^{3}H]thymidine until 24 hours after the addition of lovastatin (figure 6A and 6B), concurrent with the above hypothesis.



CONDITION	% G_1 PHASE (A)	% S PHASE (B)	% G_2/M PHASE (C)
CONTROL	46.8	35.0	17.2
T=48 HRS	66.2	7.5	24.6

FIGURE 4.
Cell cycle analysis of L1210/DDP cells incubated in presence of 40 μM lovastatin. Hours indicated are after addition of lovastatin. (Control cells are not treated with lovastatin.) A=G_1 Phase; B=S Phase; C=G_2/M Phase.



CONDITION	% G_1 PHASE (A)	% S PHASE (B)	% G_2/M PHASE (C)
CONTROL	33.1	41.5	25.4
T=48 HRS	12.4	18.4	69.2

FIGURE 5.
Cell cycle analysis of L1210/DACH cells incubated in presence of 40 μM lovastatin. Hours indicated are after addition of lovastatin. (Control cells are not treated with lovastatin.) A=G_1 Phase; B=S Phase; C=G_2/M Phase.

The addition of 100 μg/ml cholesterol and/or 100 μM squalene following lovastatin treatment had no effect on any of the blockades achieved by lovastatin in any of the cell lines tested (data not shown).

Our results demonstrate that the L1210/0 and L1210/DACH cells are not like the "normal" cells which have been previously described in the literature in that a G_1 blockade does not occur when these two L1210 cell lines are incubated with lovastatin. One of the differences between our results and those which were reported is that the L1210/0 and L1210/DACH cells are both suspension cell lines while all previously studied cell lines were adherent. Since adherent cells appear rounded in the presence of lovastatin, it has been hypothesized that mevalonate may play a role in the maintenance of cell shape in adherent cells (22). Lovastatin induced depletion of cellular mevalonate, and thus isoprenylated proteins, is evidenced by the change in cell shape as well as by the fact that the cells lack sufficient isoprenylated signal to allow the cell to enter the cell cycle resulting in a characteristic G_1 blockade.

In contrast, the L1210/0 and L1210/DACH cells are a true suspension cell line and therefore do not need the isoprenylated proteins for maintenance of cell shape and adherence. It is possible that this difference could result in the L1210/0 and L1210/DACH cells having enough of the isoprenylated signal to allow the cell to enter into the S phase of the cell cycle only to get blocked at the end of the cycle (G_2/M). Since the L1210/DDP cells are slightly more adherent than the L1210/0 or L1210/DACH cells, they consume isoprenylated proteins for adherence and have insufficient isoprenylated protein pools to allow entry into the S phase of the cell cycle. Understanding the mechanism of why these two L1210 cells enter into the cell

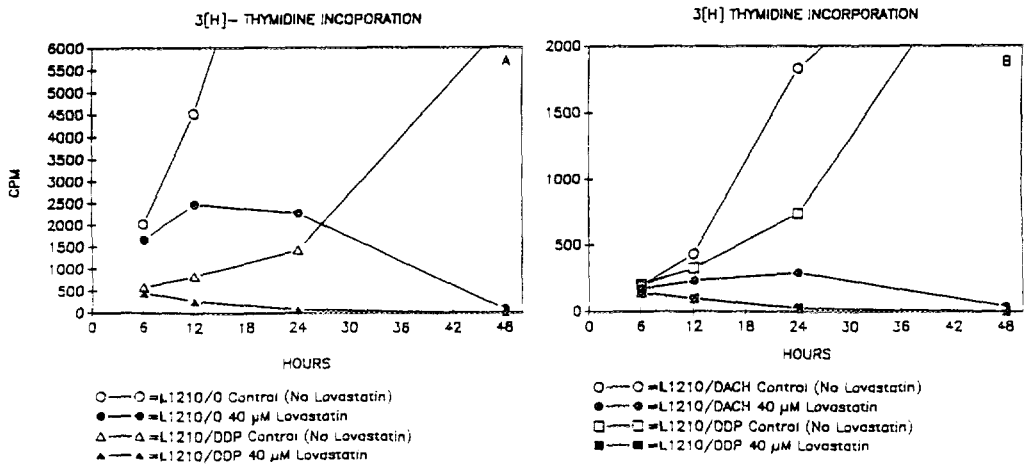


FIGURE 6.
^{3}H-Thymidine incorporation of L1210/0, L1210/DDP, and L1210/DACH cells incubated with 40 μM lovastatin. Hours indicated are incubation with lovastatin. (Control cells are not incubated with lovastatin.)

cycle (attain a G_2/M blockade) when incubated in the presence of lovastatin may help to ascertain what role mevalonate and isoprenylated proteins play in DNA synthesis and cell cycle progression and may ultimately help in understanding what is occurring in the cancer cell which leads the cell to uncontrolled growth.

REFERENCES

1. Chen, HW, Kandutch, AA, Waymouth, C (1974) Nature (London) 251:419-421.

2. Brown, MS, Goldstein, JL (1973) J. Biol. Chem. 249:7306-7314.

3. Quesney-Huneeus, V, Wiley, MH, Siperstein, MD (1979) Proc. Natl. Acad. Sci. USA 76:5056-5060.

4. Quesney-Huneeus, V, Galick, HA, Siperstein, MD, Erickson, SK, Spencer, TA, Nelson, JA (1983) J. Biol. Chem. 258:378-385.

5. Doyle, JW, Kandutch, AA (1988) J. Cell Physiol. 137:133-140.

6. Maltese, WA, Sheridan, KM (1987) J. Cell Physiol. 133:471-481.

7. Fairbanks, KP, Barbu, VD, Witte, LD, Weinstein, IB, Goodman, DS (1986) J. Cell Physiol. 127:216-222.

8. Keyomarsi, K, Sandoval, L, Band, V, Pardee, AB (1991) Cancer Res. 51:3602-3609.

9. Jakobisiak, M, Bruno, S, Skierski, JS, Darzynkiewicz, Z (1991) Proc. Natl. Acad. Sci. USA 88:3628-3632.

10. Sinensky, M, Logel, J (1985) Proc. Natl. Acad. Sci. USA 82:3257-3261.

11. Alberts, AW, Chen, J, Kuron, G, Hunt, V, Huff, J, Hoffman, C, Rothrock, J, Lopez, M, Joshua, H, Harris, E, Patchett, A, Monaghan, R, Currie, S, Stapley, E, Albers-Schonberg, G, Hensens, O, Hirshfield, J, Hoogsteen, K, Liesch, J, Springer, J (1980) Proc. Natl. Acad. Sci. USA 77:3957-3961.

12. Hohl, RJ, Larson, RA, Mannickarottu, V, Yachnin, S (1991) Blood 77:1064-1070.

13. Goldstein, JL, Brown, MS (1990) Nature (London) 343:425-430.

14. Schmidt, RA, Schneider, CJ, Glomset, JA (1984) J. Biol. Chem. 259:10175-10180.

15. Maltese, WA (1990) FASEB J. 4:3319-3328.

16. Maltese, WA, Sheridan, KM, Repko, EM, Erdman, RA (1990) J. Biol. Chem. 265:2148-2155.

17. Kawanta, M, Farnsworth, CC, Yoshida, Y, Gelb, MH, Glomset, JA, Takon, Y (1990) Proc. Natl. Acad. Sci. USA 87:8960-8964.

18. Farnsworth, CC, Kawata, M, Yoshida, Y, Takai, Y, Gelb, MH, Glomset, JA (1991) Proc. Natl. Acad. Sci. USA 88:6196-6200.

19. Khrosraui-Far, R, Lutz, RJ, Cox, AD, Conroy, L, Browne, JR, Sinensky, M, Balch, WE, Bass, JE, Der, CJ (1991) Proc. Natl. Acad. Sci. USA 88:6264-6268.

20. Gilman, AG (1987) Annu. Rev. Biochem. 56:615-649.

21. Bourne, HR, Sanders, DA, McCormick, F (1990) Nature (London) 348:125-132.

22. Schmidt, RA, Glomset, JA, Wight, TN, Habenicht, AJR, Ross, R J. Cell Biol. (1982) 95:144-153.