These studies establish the feasibility of designing inhibitors of PTH in vivo, and may facilitate further studies of the mechanism of PTH action. Such analogs may ultimately prove useful as diagnostic and therapeutic agents in clinical disorders of the parathyroid glands.

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References and Notes

1. H. Heath, III, S. F. Hodgson, M. A. Kennedy, *N. Engl. J. Med.* **302**, 189 (1980).
2. G. W. Tregear, J. van Reitschoten, E. Greene, H. T. Keutmann, H. D. Niall, B. Reit, J. A. Parsons, J. T. Potts, Jr., *Endocrinology* **93**, 1349 (1973).
3. J. T. Potts, Jr., H. M. Kronenberg, M. Rosenblatt, *Adv. Protein Chem.* **35**, 323 (1982).
4. D. Goltzman, A. Peytremann, E. Callahan, G. W. Tregear, J. T. Potts, Jr., *J. Biol. Chem.* **250**, 3199 (1975).
5. M. Rosenblatt, E. N. Callahan, J. E. Mahaffey, A. Pont, J. T. Potts, Jr., *ibid.* **252**, 5847 (1977).
6. S. R. Nussbaum, M. Rosenblatt, J. T. Potts, Jr., *ibid.* **255**, 10183 (1980).
7. S. R. Goldring, J. E. Mahaffey, M. Rosenblatt, J.-M. Dayer, J. T. Potts, Jr., S. M. Krane, *J. Clin. Endocrinol. Metab.* **48**, 655 (1979).
8. D. A. Gray, J. A. Parsons, J. T. Potts, Jr., M. Rosenblatt, R. W. Stevenson, *Br. J. Pharmacol.* **76**, 259 (1982).
9. J. A. McGowan, T. C. Chen, J. Fragola, J. B. Puschett, M. Rosenblatt, *Science* **219**, 67 (1983).
10. G. V. Segre, G. L. Tully, III, M. Rosenblatt, J. A. Laugharn, B. Reit, J. T. Potts, Jr., *Calcif. Tissue Int.* **28**, 171 (Abstr.) (1979).
11. N. Horiuchi, M. Rosenblatt, H. T. Keutmann, J. T. Potts, Jr., M. F. Holick, *Am. J. Physiol. Endocrinol. Metab.*, in press.
12. K. J. Martin, E. Bellorin-Font, J. Freitag, M. Rosenblatt, E. Slatopolsky, *Endocrinology* **109**, 956 (1981).
13. M. Rosenblatt and J. T. Potts, Jr., *Calcif. Tissue Int.* **33**, 153 (1981).
14. F. R. Bringhurst and M. Rosenblatt, unpublished data.
15. M. Rosenblatt, G. V. Segre, G. A. Tyler, G. L. Shepard, S. R. Nussbaum, J. T. Potts, Jr., *Endocrinology* **106**, 545 (1980).
16. M. Rosenblatt, M. D. Coltrera, G. L. Shepard, D. A. Gray, J. A. Parsons, J. T. Potts, Jr., *Biochemistry* **20**, 7246 (1981).
17. R. B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149 (1963).
18. G. Barany and R. B. Merrifield, in *The Peptides*, E. Gross and J. Meienhofer, Eds. (Academic Press, New York, 1979–1980), vol. 2, pp. 1–284.
19. M. Rosenblatt, G. W. Tregear, G. L. Shepard, G. A. Veroni, J. T. Potts, Jr., *Arch. Biochem. Biophys.* **199**, 286 (1980).
20. G. A. Tyler and M. Rosenblatt, *J. Chromatogr.*, in press.
21. N. Horiuchi, T. Suda, H. Takahashi, E. Shimazawa, E. Ogata, *Endocrinology* **101**, 969 (1977).
22. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* **66**, 374 (1925).
23. B. L. Brown, J. D. M. Albano, R. P. Ekins, A. M. Spherzi, *Biochem. J.* **121**, 561 (1971).
24. W. H. Sawyer, P. K. T. Pang, J. Seto, M. McEnroe, B. Lammek, M. Manning, *Science* **212**, 49 (1981).
25. G. Engberg, T. H. Svensson, S. Rosell, K. Folkers, *Nature (London)* **293**, 222 (1981).
26. D. G. Johnson, C. U. Goebel, V. J. Hruby, M. D. Bergman, D. Trivedi, *Science* **215**, 1115 (1982).
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DNA Methylation Decreases in Aging but Not in Immortal Cells

Abstract. *When normal diploid fibroblasts from mice, hamsters, and humans were grown in culture, the 5-methylcytosine content of their DNA's markedly decreased. The greatest rate of loss of 5-methylcytosine residues was observed in mouse cells, which survived the least number of divisions. Immortal mouse cell lines had more stable rates of methylation.*

Normal diploid cells do not replicate indefinitely in vitro unless they are altered by some heritable transformation (*1*) to produce immortal cell lines that may or may not be tumorigenic in nude mice (*2*). The mechanism underlying the spontaneous conversion of cells from senescence to immortality is unknown, but alterations in gene expression are probably involved in the process.

The methylation of DNA influences vertebrate gene expression and methylation patterns are tissue-specific (*3*). The patterns are inherited with a high but not absolute fidelity (*4, 5*), and alterations in them could cause changes in gene expression that might be involved in aging in culture. We have therefore determined the levels of cytosine methylation in senescing cells from mice, hamsters, and humans and compared them to cell lines derived from the same species.

The growth kinetics of normal populations of diploid cells from these three species were followed (Fig. 1). The number of population doublings (computed from the increases in cell number without correction for plating efficiency) that the cells could undergo before senescence was lowest for mouse embryo (C3HME) cells that showed a maximum of four to eight population doublings. Spontaneous regrowth of the culture occurred after 12 passages, which is characteristic of the well-known abilities of mouse cells to form immortal lines (*2*). Syrian hamster embryo (SHE) cells were maintained for 25 doublings before decreased plating efficiency and cell death caused a decrease in the computed growth curve. Human fibroblasts can replicate for 100 population doublings (*1*). Human fetal lung fibroblasts (IMR 90) were followed for more than 40 doublings without change in growth rate (Fig. 1).

The levels of DNA methylation in these cultures were measured as a function of population doubling (Fig. 2A). The percentage of cytosine residues modified to 5-methylcytosine in freshly explanted mouse fibroblasts was 3.8 percent; the ratio decreased dramatically with increased time in culture. This decrease was in marked contrast to the situation with two immortal mouse cell lines in which the level of methylation was stable, or increased slightly, over many passages representing a large number of cell divisions (Fig. 2B).

Hamster fibroblasts, which underwent approximately 25 population doublings (Fig. 1), showed a lower rate of loss of 5-methylcytosine as a function of time in culture and human fibroblasts showed the lowest rate of decrease (Fig. 2A). Thus, the cell populations that rapidly senesced in culture showed the greatest rate of loss of methyl groups from their DNA's.

The 5-methylcytosine content of other cell types available in the laboratory was also determined to assess the generality of these findings (Table 1). The rapidly

Table 1. Methylcytosine content of cells.

Cell line	Description	Passage number	5-Methylcytosine* (%)	Number of samples
		Mouse cells		
C3H C1V	Immortal	5	2.95 ± 0.19	3
		7	3.82 ± 0.31	3
		11	3.66	2
		23	3.64	2
		Hamster cells		
A(T_1) C13	Oncogenic and immortal	Unknown	2.25 ± 0.24	5
		Human cells		
T-1	Normal diploid	8	3.00	2
		22	2.88	2
		36	2.37	2
HT1080	Oncogenic and immortal	44	2.32	2

*The percentage of 5-methylcytosine (5mC) was calculated as follows: [(5mC)/(5mC + cytosine] × 100. Values represent the average of at least two samples, and standard deviations are provided where appropriate.

dividing population of C3HC1V cells isolated in the experiment shown in Fig. 1 had a cytosine modification level of 2.95 percent at passage 5 which increased to 3.82 percent at passage 7 and stabilized at 3.65 percent between passages 11 and 23. This value was similar to those of the established mouse lines (Fig. 2B), but we do not know whether the increase was the result of de novo methylation or the selection of cells with high methylation levels from the aging population. A hamster cell line A(T_1)C13, which was derived by oncogenic transformation of hamster fibroblasts in vitro (*6*), showed a decreased 5-methylcytosine content relative to freshly explanted fibroblasts (Fig. 2A).

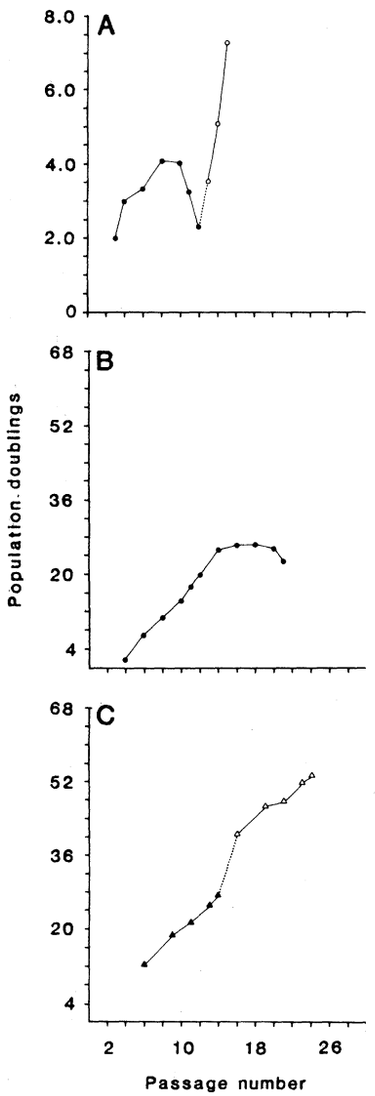

Fig. 1. Representative population growth curves. (A) Mouse. Freshly prepared C3HME cells (●) from C3H/HEN(MTV−) mice were passaged by trypsinization every 3 to 4 days; 3×10^5 cells were seeded per T-75 flask at each passage. The surviving 2.5×10^4 cells at passage 12 were seeded into a 60-mm dish and fed twice a week for 2 weeks, at which time a colony (C3HC1V) developed. C3HC1V cells (○) were then passed as above. (B) Hamster. Golden Syrian hamster embryo (SHE) cells were prepared from 10-day embryos; 2×10^5 cells were seeded per T-25 flask at each passage. (C) Human. Human fetal lung fibroblasts (IMR 90) were obtained from the Institute for Medical Research (Camden, New Jersey) at passages 6 (▲) and 16 (△) with known division histories. The cells were passaged by trypsinization every 7 days and 3×10^5 cells were seeded per T-75 flask.

The decrease in DNA methylation with passage of the IMR 90 cells (Fig. 2A) was also found with a strain of human foreskin fibroblasts (T-1). Since normal human immortal lines are not available, we measured the 5-methylcytosine content of a human tumor cell line HT1080 (*7*), which showed a much decreased 5-methylcytosine content relative to diploid fibroblasts.

Our results clearly demonstrate that decreases in the levels of DNA methylation occur with time in culture in diploid mouse cells, in contrast to the situation with immortal cell lines. The increased levels of cytosine methylation of the newly derived mouse line relative to the senescing cultures was interesting and suggested that cells may require a certain level of methylation to survive in culture. However, tumorigenic hamster and human lines had lowered 5-methylcytosine levels compared to their diploid progenitors so that alterations in methylation patterns may be more important than the final 5-methylcytosine content reached.

The present data do not establish whether the loss of methyl groups was related to the number of divisions or simply to time in culture. However, methylation patterns most probably change after the synthesis of DNA (*3*). Division may therefore lead to infidelity of maintenance methylation, as has been demonstrated at selective sites for other dividing cells in culture. Significant variability in methylation at specific sites in γ-globin gene sequences (*8*) and in X-chromosome sequences (*9*) of cloned and serially passaged human fibroblasts have been reported. The work of Wigler *et al.* (*4*) suggested that mammalian cells were capable of maintaining methylation of foreign DNA with 95 percent fidelity per site per generation.

The observed loss of 5-methylcytosine in aging cells might be partially accounted for by selective decreases of highly repetitive DNA sequences (*10*) known to contain the highest percentage of the compound (*3*). However, the loss of selective DNA sequences cannot completely account for the rapid decrease or for the total reduction in 5-methylcytosine content observed in aging C3HME cells or in SHE cells (Fig. 2A). Highly repetitive DNA constitutes only a small portion of mammalian DNA and contains less than 20 percent of the total 5-methylcytosine (*11*). The decreases observed here, which are more extensive than this, may therefore reflect the combined effects of removal of repetitive DNA sequences and infidelity in the maintenance of 5-methylcytosine patterns.

Some data are available on possible changes in DNA methylation during aging in vivo. Romanov and Vanyushin (*12*) reported decreases in 5-methylcytosine in the DNA of aging cattle and salmon, but Ehrlich *et al.* (*13*) failed to

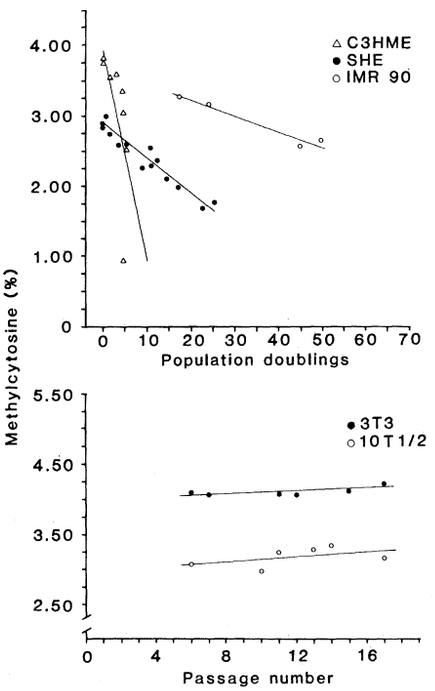

Fig. 2. The rate of DNA methylation in aging cells. Nonconfluent cells were grown for 24 hours in the presence of 30 to 100 μCi of [6-^{3}H]uridine in 2 ml of medium. The cells were lysed, and the percentage of 5-methylcytosine was determined by two-dimensional thin-layer chromatography after hydrolysis of the DNA to bases (*15*). (A) Amounts of 5-methylcytosine were determined for normal diploid cells after various population doublings. Curves were drawn by linear regression analysis of the data for mouse (△) (slope = −0.136 ± 0.153), hamster (●) (slope = −0.043 ± 0.005), and human (○) (slope = −0.021 ± 0.004) cells (the ± is standard deviation of slope). The mouse and hamster data represent the compilation of data from two separate experiments and each point represents the average of two samples. (B) The amount of 5-methylcytosine in the DNA of BALB/3T3A31 C1 1-13 cells (●) and C3H/10T½ C1 8 cells (○) was measured at different passages. Each passage represents a minimum of three to ten doublings. Linear regression analysis provided positive slopes of 0.010 ± 0.003 for BALB/3T3 cells and 0.016 ± 0.014 for C3H/10T½ C1 8 cells. Each point represents the average of at least two samples.

detect such decreases in tissues obtained from young and old humans. Ehrlich *et al.* (*13*) also found no significant changes in the 5-methylcytosine content of human fibroblasts allowed to undergo approximately 10 to 15 doublings. However, from our data, this may not have represented a sufficiently long time span for measurable differences to be apparent in human cells.

The decreases in 5-methylcytosine that we have observed in cultured cells from three different species may account for aberrant gene expression in aging cultures (*14*). However, it remains to be seen whether this represents a response to the culture environment or has any significance in vivo.

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References and Notes

1. L. Hayflick, *Exp. Cell Res.* **37**, 614 (1965); S. Goldstein, J. W. Littlefield, J. S. Soeldner, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 155 (1969); J. R. Smith and L. Hayflick, *J. Cell Biol.* **62**, 48 (1974).
2. C. A. Reznikoff, D. W. Brankow, C. Heidelberger, *Cancer Res.* **33**, 3231 (1973); T. Kakunaga, *Int. J. Cancer* **12**, 463 (1973); K.-Y. Lo and T. Kakunaga, *Cancer Res.* **42**, 2644 (1982).
3. A. Razin and A. D. Riggs, *Science* **210**, 604 (1980); M. Ehrlich and R. Y.-H. Wang, *ibid.* **212**, 1350 (1981).
4. M. Wigler, D. Levy, M. Perucho, *Cell* **24**, 33 (1981).
5. R. M. Harland, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2323 (1982); Y. Pollack, R. Stein, A. Razin, H. Cedar, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6463 (1980).
6. W. F. Benedict, N. Rucker, C. Mark, R. E. Kouri, *J. Natl. Cancer Inst.* **54**, 157 (1975).
7. S. Rasheed, W. A. Nelson-Rees, E. M. Toth, P. Arnstein, M. B. Gardner, *Cancer* **33**, 1027 (1975).
8. R. J. Shmookler Reis and S. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3949 (1982).
9. S. F. Wolf and B. R. Migeon, *Nature (London)* **295**, 667 (1982).
10. R. J. Shmookler Reis and S. Goldstein, *Cell* **21**, 739 (1980).
11. If one assumes that the highly repetitive DNA amounts to 10 percent of genomic DNA and contains twice the level of 5-methylcytosine per cytosine residues when compared to the rest of the DNA [see (*3*); T. L. Kautiainen and P. A. Jones, unpublished results], the level of methylation in the remaining 90 percent of the DNA is thus 3.45, 2.73, and 2.95 percent 5-methylcytosine in C3HME, SHE, and IMR 90 cells, respectively.
12. G. A. Romanov and B. F. Vanyushin, *Biochim. Biophys. Acta* **653**, 204 (1981).
13. M. Ehrlich, M. A. Gama-Sosa, L-H. Huang, R. M. Midgett, K. C. Lo, R. A. McCune, C. Gehrke, *Nucl. Acids Res.* **10**, 2709 (1982).
14. Low levels of ectopic protein production by normal fibroblasts in culture have been reported by S. W. Rosen, B. D. Weintraub, and S. A. Aaronson [*J. Clin. Metab.* **50**, 834 (1980)].
15. V. L. Wilson and P. A. Jones, *Cell* **32**, 239 (1983).
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Glucose Stimulation of the Antilipolytic Effect of Insulin in Humans

Abstract. *Dose-response studies of the inhibition of lipolysis by insulin in isolated human adipocytes were conducted with the use of a sensitive bioluminescent assay of glycerol release. The addition of glucose to the incubation medium was associated with an increase in insulin sensitivity and an increase in the maximum insulin effect. The results suggest that glucose plays an important role in regulating the antilipolytic action of insulin in humans.*

Insulin inhibits lipolysis and stimulates the cellular uptake and metabolism of glucose in adipose tissue. The antilipolytic effect of insulin in the rat is clearly independent of any action of the hormone on glucose metabolism (*1*). Dose-response studies of the inhibition of lipolysis by insulin in isolated rat fat cells indicate that although glucose does not influence the action of insulin on basal lipolysis (*2*), it may completely abolish the insulin inhibition of catecholamine-stimulated lipolysis (*3*). It is unclear whether glucose alters the antilipolytic effect of insulin in humans. In human fat cells insulin often fails to inhibit glycerol release (*4*). This may be due to the technical difficulties of estimating glycerol release, which is generally used as an index of the rate of lipolysis (*5*) and is about ten times more rapid from rat than from human adipocytes (*5, 6*). We recently developed a bioluminescent technique for the determination of glycerol release from fat cells. This technique is 100 times more sensitive than the commonly used enzymatic, fluorometric methods (*7*). In the present study we used the bioluminescent technique to determine whether there is an interaction between glucose and the antilipolytic effect of insulin in man. We prepared isolated human fat cells and measured the rate of glycerol release in vitro (*8*).

Figure 1 shows the dose-response curve for the effect of insulin on basal and isoprenaline-induced lipolysis. When either a submaximal effective or a maximal effective concentration of isoprenaline was used, we observed a dose-dependent influence of glucose on the antilipolytic effect of insulin. Furthermore, the two different isoprenaline concentrations produced almost identical results. When fat cells were incubated in a glucose-free medium, they were unresponsive to insulin added at physiological concentrations. A slight inhibition of lipolysis was observed in the presence of 2500 μU of insulin per milliliter. The addition of glucose (1 mg/ml) to the medium induced a shift to the left in the dose-response curve for insulin, but a plateau was never reached. A higher concentration of glucose (2 mg/ml) was associated with a further shift to the left. A maximum antilipolytic effect of insulin was obtained with the higher insulin concentrations tested. The concentration of insulin that produced a half-maximum effect (ED_{50}) in the presence of 2 mg of glucose per milliliter was about 5 μU/ml in both experiments with isoprenaline.

The addition of glucose to the incubation medium did not induce a shift to the left of the dose-response curve in the basal state (Fig. 1). The basal rate of lipolysis was inhibited by insulin at physiological concentrations, with and without the presence of glucose in the incubation medium. The ED_{50} for insulin was 2.5 μU/ml with 1 or 2 mg of glucose per milliliter. However, the amplitude of the dose-response curve was greater with than without glucose. This indicates that glucose may stimulate the maximum antilipolytic effect of insulin (insulin responsiveness) in the basal state. Further studies (*9*) showed that the addition of glucose (2 mg/ml) to the incubation medium was followed by an increase in insulin responsiveness from 40 to 56 percent ($P < .01$). Neither the isoprenaline-induced rate of glycerol release nor the basal rate were influenced by glucose (Fig. 1).

The interactions among glucose, insulin and lipolysis in human fat cells observed in the present study were markedly different from those described in rat adipocytes (*1–3*). In the rat the inhibitory effect of insulin may be either uninfluenced or abolished by the presence of glucose in the incubation medium. Our data indicate that, in man, glucose stimulates the antilipolytic effect of insulin. Furthermore, the present data suggest that glucose may regulate the antilipolytic effect of insulin in human adipocytes in two ways. When lipolysis is maximally or submaximally stimulated by catecholamines, insulin sensitivity is increased by glucose. In the basal state glucose does not influence insulin sensi-