Vol. 87, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 13, 1979 Pages 698-705

5-METHYLCYTOSINE CONTENT OF NUCLEAR DNA DURING CHEMICAL HEPATOCARCINOGENESIS AND IN CARCINOMAS WHICH RESULT

Jean-Numa Lapeyre and Frederick F. Becker[1]

Section of Experimental Pathology, M. D. Anderson Hospital and Tumor Institute, The University of Texas System Cancer Center, Texas Medical Center, Houston, Texas 77030

Received February 22, 1979

SUMMARY: The 5-methylcytosine content of nuclear DNA from nuclear hepatocellular tissues was determined during various phases of hepatic regeneration and carcinogenesis. DNA from premalignant nodules and primary hepatocellular carcinomas induced by exposure to acetylaminofluorene, as well as PHC induced by diethylnitrosamine was undermethylated by 20%, 45%, and 32.5% respectively. Since a 12.5% hypomethylation occurred during the DNA synthetic phase of hepatic regeneration, the effect of cell proliferation on DNA-methylation in malignancies was examined in transplantable hepatocellular carcinomas. The DNA from two transplantable hepatocellular carcinoma lines was less methylated than predicted rates of cell division in these tumors. This finding suggested that an aberration in endogenous DNA methylation may occur during neoplastic transformation.

The DNA of eukaryotes is modified exclusively at cytosine residues by DNA methyltransferases which form the methylated base 5-methylcytosine (m^5C)[2] (1, 2). This process occurs subsequent to DNA replication (3) and DNA repair (4, 5) and, therefore, undermethylated tracts of DNA exist for a period of time ranging from several minutes to hours after strand synthesis (3,4,6).

In non-dividing cells, there are differences in the level of m^5C among various DNA Cot classes (5,7,8) between satellite and main-band DNA sequences (9,10) and among DNAs of various tissues in the same organism (11,12). Alterations in the content of m^5C have been demonstrated during development (13,14), during aging (15), and in different physiological states induced by hormones (16,17) or by exogenous compounds that influence the metabolism of SAM (18,19). From these diverse findings, many proposals as to the function of DNA methyla-

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: m^5C, 5-methylcytosine; PHC, primary hepatocellular carcinoma; AAF, acetyl aminofluorene; DEN, diethylnitrosamine; THC, transplantable hepatocellular carcinoma; and, SAM, S-adenosyl methionine

0006-291X/79/070698-08$01.00/0

tion have been forthcoming, including its potential role in DNA replication (20,21), transcription (22), and cellular differentiation and development (23-25).

Numerous investigations seeking a functional relationship between nucleic acid methylation and the malignant phenotype have been conducted. For the most part, these experiments have focussed on RNA methylases and methylation, especially as related to tRNA function (26). In general, a variety of alterations in the level or activity of tRNA methylases and in the site-specific methylation of bases have been reported to take place in malignant cells.

In contrast, the information regarding such alterations in terms of DNA methylation is very limited and often contradictory. Levels of DNA methylases and m^5C content have been reported to be increased in leukemic lymphocytes (27,28). Recently, the opposite finding has been reported in bovine lympholeukemia, where the DNA has been found to be undermethylated by approximately 20% (29). During ethionine carcinogenesis, nuclear DNA is undermethylated (30,31), possibly as a result of depletion of intracellular SAM (31). Gantt et al. (32) have reported that there is no change in the methylation of satellite versus main-band sequences in mouse embryo cells after transformation by selection on soft agar. Yet, cells transformed by adenovirus (2), polyoma virus (33), and Rous sarcoma virus (34) demonstrate elevated levels of m^5C in the host genome.

To examine the relationship between DNA methylation and malignancy, we have measured the m^5C content in nuclear DNA of normal liver and premalignant and malignant hepatic tissues induced by the chemical carcinogens AAF and DEN. We report hypomethylation in the nuclear DNA of PHC induced by these agents and in the DNA of THC.

MATERIALS AND METHODS

Animals. Male CFE Sprague-Dawley rats weighing 150 g were partially hepatectomized by removal of two thirds of the liver, as described previously (35).

AAF was mixed into a synthetic diet (#101, Bio-Serv, Frenchtown, N.J.) and fed to male CFE Sprague-Dawley rats, which weighed approximately 80 g. The details of the AAF feeding regimen and the induction and characterization

of premalignant nodules and malignant tumors have been reported elsewhere (36-38). DEN (Eastman) hepatocarcinogenesis resulted from the administration of 60 ppm in the drinking water for 10 weeks. The induction and characterization of DEN hepatic tumors will be reported elsewhere (39). Details on the growth characteristics of THC and 253 used in these experiments have been published (40).

DNA Purification and Base Analysis. Nuclei were isolated from the livers, PHC, and THC by the procedure of Blobel and Potter (41) modified to include 0.25% Nonidet-P-40 in the homogenizing steps. Nuclei were digested according to Gross-Bellard et al. (42) with proteinase K (Boehringer-Mannheim) and the DNA extracted with Sevag (chloroform isoamyl alcohol, 24:1, v/v) then banded in preparative CsCl density gradients. The DNA band was recovered and dialyzed against TE (0.01 M Tris-HCl, pH 7.9, 1 mM Na-EDTA) and further digested with preboiled RNase A and RNase T1 at 10 μg/ml and 5 units/ml, respectively, for 30 minutes at 37°C. The DNA was extracted with phenol-Sevag (1:1 v/v), ethanol precipitated and dried.

DNA was hydrolysed to bases with concentrated HF for 1.5 hours at 80°C (43), and analyzed by high pressure liquid chromatography on a Partisil 10 SCX column (0.6x25 cm, Whatman) eluted isocratically with 0.07 M ammonium formate, pH 3.2, at room temperature in a Glenco HPLC system. Bases were identified relative to the elution of authentic compounds and their quantity determined by measurement of the base peak area at 280 nm/base extinction coefficient at 280 nm at pH 3.2. The m^5C content of the samples was then determined in triplicate and this mean was used in the calculation of the mean and standard deviation (σ) of the sample populations. Statistical significance of the results were examined by unpaired t-tests and analysis of variance (F-test).

RESULTS

Baseline measurements of the m^5C content of the DNA from normal non-dividing liver and the DNA isolated during different phases of hepatic regeneration are tabulated in Table 1 for comparison with hepatic malignant tissues. The m^5C content prior to operation and then 4 hours, 10 hours, 32 hours, and one and two weeks after 70% hepatectomy is quite stable (3.60-3.76%). During the peak of DNA synthesis (S-period), which occurs 20 to 21 hours after 70% hepatectomy (44), the m^5C content declines by 12.5% to approximately 3.23% ($P<<0.01, t=4.76$). Therefore, this decline in m^5C content, which is related to the proportion of cells undergoing DNA synthesis, is a factor that must be considered when comparing DNA methylation in replicating and non-replicating cells.

To determine the m^5C level in PHC, five malignant lesions induced by DEN and 11 induced by AAF were analyzed. As seen in Table 2, the m^5C content of the former had a mean value of 2.88 ± 0.19 and the latter, 1.98 ± 0.21 - hypomethylation of 20% and 45% when compared to normal liver.

Table 1. 5-Methylcytosine content of rat nuclear DNA during liver regeneration

Time after partial hepatectomy	Mean $m^5C/m^5C + C \times 100 \pm \sigma$ (n)
0 hrs.	3.60 ± 0.24 (4)
4 hrs.	3.68 ± 0.28 (4)
10 hrs.	3.72 ± 0.15 (5)
21 hrs.	3.23 ± 0.09 (5)*
33 hrs.	3.76 ± 0.14 (5)
1 wk.	3.66 ± 0.29 (2)
2 wks.	3.70 ± 0.34 (4)

*t=4.76 ($P<<0.01$)

Table 2. 5-Methylcytosine content of nuclear DNA from carcinogen-induced hepatic nodules and hepatocellular carcinomas

Regimen	Mean $m^5C/m^5C + C \times 100 \pm \sigma$ (n)
DEN 60 ppm x 10 weeks/individual PHC	2.88 ± 0.19 (5)
AAF 4x3 cycles; individual nodules	2.43 ± 0.37 (19)
AAF 4x3 cycles; individual PHC	1.98 ± 0.21 (11)
THC 253	2.65 ± 0.23 (3)
THC 252	2.44 ± 0.19 (4)

To determine if a similar hypomethylation existed during chemical carcinogenesis, hepatic nodules were isolated from livers that had been exposed to four cycles of AAF. These nodules have been characterized extensively and are considered to represent a putative premalignant lesion (36-39), since they are at a substantial risk to result in PHC despite cessation of feeding. The m^5C content of the nuclear DNAs of 19 of these nodules was 2.43% or an hypomethylation of 32.5% when compared to normal livers. Data derived from these regenerating livers and hepatic nodules indicate that the DNA polymerase-α level of

Table 3. Relative DNA methylation and synthesis in regenerating liver, 252 THC and 253 THC

Tissue	Relative m^5C	Relative DNA synthesis	Methylation relative to regenerating liver
Normal liver	1.00* (4)†	1.00‡	---
21-hour regenerating liver	0.88 (5)	9.4	1.00§
252 THC	0.72 (5)	6.0	0.51
253 THC	0.62 (8)	5.6	0.41

*Normalized to 3.62% $m^5C/m^5C + C$

†Experimental sample size

‡Normalized to specific activity of 18,000 cpm/mg DNA measured one hour after a subcutaneous dose of 1 μCi[^{3}H]-thymidine (NEN, 6.7 Ci/nmole) per gram body weight.

§Direct proportionality between decline in methylation and level of DNA synthesis assumed. Normalized to value obtained in 21-hour regenerating liver.

the former is approximately 40% greater than of the latter (Chan and Becker, unpublished results). This finding is in accord with results of labeling experiments which demonstrated that the DNA of regenerating liver replicated faster than that of nodules (35), and DEN and AAF tumors. If hypomethylation was due to cell proliferation alone, PHC and nodules would not be expected to show lower levels of methylation than S-phase regenerating liver.

However, to better determine the effect of cell proliferation on methylation, DNA synthesis in 21-hour regenerating rat liver was determined and compared to that in THC 252 and 253 (39). The rates of DNA synthesis of each tissue was derived from a pulse of [^{3}H]-thymidine given subcutaneously one hour before sacrifice and the specific activity of the same purified DNA which was used for base analysis was determined. As shown in Table 3, DNA synthesis, hence the rate of cell proliferation in the 21-hour regenerating liver, was

approximately 33% and 39% greater than in THC 252 and 253, respectively. Despite this, the THC demonstrated significantly less m^5C than the regenerating liver.

A one-way analysis of variance of the data reveals a statistically significant different level of methylation, $F_{4,28} = 78.03$ ($P<<0.01$) in the PHC and THC sample populations as compared to normal liver and S-phase regenerating liver. Considering that DNA methylation lags behind DNA replication (3, 4,6), increased DNA synthesis should lead to a corresponding increase in the amount of newly replicated and correspondingly undermethylated DNA. By assuming a model in which the degree of DNA hypomethylation is directly proportional to the amount of de novo synthesized DNA, one can assess whether there is a component of hypomethylation intrinsic to the tumors, beyond the contribution due to cell proliferation. Analysis of the data on THC 252 and 253, compared with that of regenerating liver, indicates a relative 49% and 59% hypomethylation in the THC DNA (Table 3).

DISCUSSION

In the regenerating rat liver, an elevated level of DNA synthesis was associated with a decreased m^5C content when compared with normal liver. The degree of hypomethylation in putative premalignant lesions, the hepatic nodules, PHCs and THCs is greater than that expected from the effect of DNA replication alone. The PHC tested in these experiments appear approximately 3-4 months after the cessation of AAF administration. This delay, along with the probable clonal origin of the tumors and the persistence of hypomethylation in THC, mitigate against the likelihood that hypomethylation in these malignant lesions or in the hepatic nodules is due to persistent binding of carcinogens (45). The distinctly differing mechanisms of DNA-interactions of the two carcinogens suggest that endogenous mechanism for DNA methylation are affected by their interaction with DNA. One consequence of the presence of aberrantly methylated DNA after the completion of DNA synthesis might be the persistent cell replication that is characteristic of these tissues.

This investigation was supported by Grant No. CA 20657 awarded by the National Institutes of Health, Department of Health, Education and Welfare.

REFERENCES

1. Wyatt, G.R. (1951) Biochem. J. 48,584-590.
2. Gunthert, U., Schweiger, M., Stupp, M., and Doerfler, W. (1976) Proc. Nat. Acad. Sci. U.S.A. 73, 3934-3937.
3. Adams, R.L.P. (1974) Biochim. Biophys. Acta 335, 365-373.
4. Low, M., Read, E.L., and Borek, E. (1976) Int. J. Radiat. Oncol. Biol. Phys. 1, 289-294.
5. Drahovsky, D., Lacko, I., and Wacker, A. (1976) Biochim. Biophys. Acta. 447, 139-143.
6. Kappler, J.W. (1971) J. Cell Physiol. 78, 33-36.
7. Solange, A., and Cedar, H. (1978) Biochemistry 17, 2934-2939.
8. Singer, J., Stellwagen, R.H., Robert-Ems, J., and Riggs, A.D. (1977) J. Biol. Chem. 252, 5509-5513.
9. Solomon, R., Kaye, A.M., and Horzberg, M. (1969) J. Mol. Biol. 43, 581-597.
10. Harbers, K., Harbers, B., and Spencer, J.H. (1975) Biochim. Biophys. Res. Commun. 66, 738-746.
11. Vanyushin, B.F., Maizin, A.L., Vasilyev, V.K., and Belozersky, A.N. (1973) Biochim. Biophys. Acta 299, 397-403.
12. Vanyushin, B.F., Tkacheva, S.G., and Belozersky, A.N. (1970) Nature 225, 948-949.
13. Berdyshev, G.D., Korataev, G.K., Boyarokikh, G.V., and Vanyushin, B.F. (1967) Biokhimiiya 32, 988-990.
14. Kappler, J.W. (1970) J. Cell Physiol. 75, 21-31.
15. Vanyushin, B.F., Nemirovsky, L.E., Klimenko, V.V., Vasilev, V.K., and Belerozersky, A.N. (1973) Gerontologia 19, 138-152.
16. Kudryashova, I.B., and Vanyushin, B.F. (1976) Biokhimiiya 41, 1106-1115.
17. Romanov, G.A., Kiryanov, G.I., Dvor'kin, V.M., and Vanyushin, B.F. (1976) Biokhimiiya 41, 1038-1043.
18. Lombardini, J.B., Coulter, A.W., and Talalay, P. (1971) Molec. Pharmacol. 6, 481-499.
19. Leboy, P.S., Glick, J.M., Steiner, F.G., Haney, S., and Borchardt, R.T. (1978) Biochim. Biophys. Acta 520, 153-163.
20. Raiez, A., Lawrence, F., Robert-Gero, M., Loche, M., and Cramer, R. (1976) FEBS Lett. 70, 622-628.
21. Friedman, J., and Razin, A. (1976) Nucleic Acids Res. 3, 3665-3675.
22. Vanyushin, B.F., Kiryanov, G.I., Kudryshova, I.B., and Belozershy, A.N. (1971) FEBS Lett. 15, 313-316.
23. Holliday, R., and Pugh, J.E. (1975) Science 187, 226-232.
24. Riggs, A.D. (1975) Cytogenet. Cell Genet. 14, 9-25.
25. Sager, R., and Kitchin, R. (1975) Science 189, 426-433.
26. Pegg, A.E. (1977) Adv. Cancer Res. 25, 195-269.
27. Silber, R., Berman, E., Goldstein, B., Stein, H., Farnharn, G., and Bertino, I.R. (1966) Biochim. Biophys. Acta 123, 638-640.
28. Federov, N.A., Kuzmichev, V.A., Kritskii, G.A., and Vinogradova, Y.E. (1977) Biokhimiiya 42, 791-793.
29. Burtseva, N.N., Azizov, Y.M., Itkin, B.Z., and Vanyushin, B.F. (1977) Biokhimiiya 42, 1331-1335.
30. Sneider, T.W., and Potter, V.R. (1969) J. Mol. Biol. 42, 271-289.
31. Cox, R., and Irving, C.C. (1977) Cancer Res. 37, 222-225.
32. Gantt, R., Montes de Oca, G., and Evans, V.J. (1973) In Vitro 8, 288-294.
33. Rubery, E.D., and Newton, A.A. (1973) Biochim. Biophys. Acta 324, 24-36.
34. Nass, M.M.K. (1973) J. Mol. Biol. 80, 155-175.

35. Becker, F.F., and Klein, K.M. (1971) Cancer Res. 31, 169-173.
36. Teebor, G.W., and Becker, F.F. (1971) Cancer Res. 31, 1-3.
37. Farber, E. (1973) Methods Cancer Res. 7, 345-375.
38. Squire, R.A., and Levitt, M.H. (1975) Cancer Res. 35, 3214-3223.
39. Becker, F.F., and Sell, S. (1979) Cancer Res. In Press.
40. Becker, F.F., Klein, K.M., Wolman, S.R., Asofsky, R., and Sell, S. (1973) Cancer Res. 33, 3330-3338.
41. Blobel, G., and Potter, J.R. (1965) Science 154, 1662-1665.
42. Gross-Bellard, M., Oudet, P., and Chambon, P. (1973) Eur. J. Biochem. 36, 32-41.
43. Walker, M.S., and Mandel, M. (1978) J. Virol. 25, 500-509.
44. Becker, F.F., and Land, B.P. (1965) Am. J. Pathol. 47, 783-801.
45. Westra, J.G., Kriek, E., and Hittenhausen, H. (1976) Chem. Biol. Interact. 15, 149-164.