The Possible Role of Nucleic Acid Methylases in the Induction of Cancer

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The possible importance of tRNA methylases in carcinogenesis was suggested by Srinivasan and Borek (48, 49). This hypothesis has stimulated a considerable amount of subsequent work and is currently under investigation in several laboratories. The hypothesis was based originally on the observation by Bergquist and Mathews (3) that some tumors contained higher levels of methylated RNA than their corresponding normal tissues and on the reports by Farber and Magee (14, 31) that the potent hepatic carcinogens ethionine (13) and dimethylnitrosamine (29) methylated the RNA of the liver after administration to rats. Thus Borek (4) stated, "If, as it seems likely, these imposed alkylations by chemical carcinogens have a causal relation to the tumours they produce, then such chemical alkylations may find a counterpart in aberrant or excessive methylations of RNA or DNA by naturally occurring methylating enzymes." The hypothesis was extended to include viral carcinogenesis as follows: "We have postulated that, since all nucleic acid-methylating enzymes are species specific, an oncogenic virus might introduce a capacity for the synthesis of nucleic acid-methylating enzymes which are foreign to the host. Should this occur, then the host would harbor enzymes which might methylate its nucleic acids aberrantly, just as alkylating carcinogens do."

Subsequent work by Borek and his colleagues and by other workers has given support for this hypothesis. Increased levels of tRNA methylases were reported in human mammary tumor and rat hepatoma (57), in mouse hepatoma (20), in hamster tumors induced by SV40 virus (37, 40), and in liver tumors from chicks with Marek’s disease (19, 34). The work with hamsters by McFarlane and Shaw (37) is of particular interest because the tRNA methylase activity of the SV40 tumor cells was not only higher than that of liver, lung, kidney, spleen, and some other tissues, but no differences were found between the capacities of normal and tumor cell extracts to methylate DNA or ribosomal RNA. Increased urinary excretion of methylated purines was found in human subjects with leukemia (43), in tumor-bearing rats and mice (35), and in hamsters with tumors induced by SV40 virus (36). In contrast to these essentially confirmatory findings, however, Kaye and Leboy (22), using different conditions of enzyme assay (46), concluded that neither the rate, nor the extent, nor the pattern of methylation could be used to distinguish unequivocally between extracts from tumor and from normal organs. The essential difference in the conditions used was based on the observation that the rates and extents of methylation of tRNA by organ and tumor extracts could be considerably stimulated by the presence of ammonium ions. More recently, however, Stewart and Corrance (52), using assay procedures similar to those of Kaye and Leboy, have reported that levels of methylase activity with *Escherichia coli* tRNA as substrate were higher in extracts of malignant, newborn, and regenerating tissue than in extracts of normal tissue when comparison was made at optimum concentration of ammonium ions. When liver tRNA was used as substrate, there was no incorporation of methyl groups by various liver extracts, but a small amount of incorporation was observed with extracts from ethionine-induced liver tumors. The authors conclude that there are some differences between the complements of tRNA methylases in malignant and normal tissue (52). Gantt and Evans (18) have compared the RNA methylase capacity of an *in vitro* long-term nonneoplastic mouse embryo cell line with a neoplastic subline and also with tumor tissue arising from another subline or cells implanted into syngeneic animals. Cell-free extracts of the neoplastic tissues had much greater capacity to methylate *E. coli* soluble RNA than the cultured nonneoplastic control line, and the authors conclude that these results support the hypothesis that aberrant alkylation of nucleic acids may be a necessary event in some types of neoplasia.

The work discussed above gave evidence of increased methylase activity in a variety of tumors but did not give any indication of increased biosynthesis of the methylated bases in the intact animal during carcinogenesis. This aspect of the problem was investigated by Craddock (10), who studied methylation of tRNA and rRNA in normal animals and in animals fed carcinogenic diets containing dimethylnitrosamine, aflatoxin, and ethionine. The rats were given injections of methionine-14C, and the labeled nucleic acids were analyzed for the presence of labeled methylated bases. The level of labeling of each methylated base in tRNA was found to be increased by each carcinogen, and there appeared to be a change in composition of the major bases of tRNA during carcinogenesis by dimethylnitrosamine and by aflatoxin, giving support to the view that there is a change towards more highly methylated species of tRNA during carcinogenesis.

It seems, therefore, that the balance of evidence does favor some form of aberrant nucleic acid methylation during carcinogenesis as postulated by Borek and his colleagues, and an attempt will be made here to assess its significance and to compare these aberrant methylations caused by methylases with those induced by alkylating carcinogens.
The Role of Alkylation in the Induction of Cancer

Certain alkylation agents, including sulfur and nitrogen mustards, were reported to induce tumors in experimental animals some years ago, and the suggestion was made that carcinogenesis resulted from alkylation of some component or components of the cell. The question of carcinogenesis by alkylation agents has been discussed by Brookes and Lawley (6), who concluded that alkylation can result in carcinogenesis but that, in general, alkylation agents cannot be regarded as powerful carcinogens. With the discovery, of the carcinogenic action of dimethylnitrosamine by Magee and Barnes (29), however, and the subsequent very extensive studies on structure-activity relationships by Druckrey et al. (11), it appeared possible that alkylation, and in particular methylation, could, under some circumstances, be powerfully carcinogenic since dimethylnitrosamine and subsequently other nitrosamines were shown to act as alkylation agents in vivo (30-32). The evidence for alkylation by dimethylnitrosamine was based on the presence of methylated components of proteins and nucleic acids in the tissues of animals treated with the labeled nitrosamine, the methyl groups being derived from it. These presumably represented aberrant methylations either in excess of or at sites other than those utilized by the normal metabolic transfer of methyl groups from S-adenosylmethionine. Since, however, the nitrosocompounds appear to be very considerably more powerful carcinogens than the known alkylation agents, doubts were expressed as to whether alkylation could explain the whole or perhaps any of the carcinogenic activities of the nitrosamines. Since the original hypothesis of Borek was partly based on the reported capacities of dimethylnitrosamine and ethionine to alkylate in vivo, it is clearly important for this hypothesis to know whether the carcinogenic action of dimethylnitrosamine is mediated via alkylation.

An early objection to the alkylation hypothesis of nitrosamine carcinogenesis was based on the powerful tumor-inducing capacity of some cyclic nitrosamines, including N-nitrosomorpholine, N-nitrososopiperidine, and N-nitrosopyrrolidine, and their supposed inability to give rise to alkylating intermediates in vivo (11, 30). This question has been studied experimentally by Ljinsky and Ross (25), who failed to find evidence of alkylated bases in the liver DNA's after treatment of rats with tritium-labeled nitrosoazetidine, nitrosohexamethylenimine, nitrosomethylenamine, and nitrosomethylcyclohexylamine. Evidence for an alkylated base was found in liver RNA of rats given nitrosoazetidine but not after treatment with nitrosohexamethylenimine. On the other hand, nitrosomethylcyclohexylamine, although not a liver carcinogen, did give rise to an alkylated base in liver RNA, identified as 7-methylguanine by mass spectrometry. It is not clear whether all possible alkylated bases would have been detected by the methods used in this work. Nevertheless, some doubt is thrown on the alkylation hypothesis of nitrosamine carcinogenesis. It is possible, of course, that dimethylnitrosamine and other methylating carcinogens might be active by virtue of methylation in the cell while the cyclic compounds might act by some other mechanism, but this does not seem likely. Additional contrary evidence has recently been published by Krüger et al. (23), who could find no evidence of methylation of nucleic acids or proteins by dimethylnitrosamine in the rainbow trout, a species highly susceptible to liver tumor induction by this carcinogen.

If nitrosamine carcinogenesis was the only evidence for aberrant methylation as a mechanism of chemical carcinogenesis, the methylase hypothesis of Borek might be more readily open to challenge. There is now, however, evidence that, under some conditions, methylating and ethylating agents that are not nitroso compounds may show carcinogenic potencies approaching that of the latter. Repeated s.c. injections of dimethyl sulfate were shown some years ago to give rise to sarcomas at the injection site (12). These findings, however, to some extent lacked force because of the high susceptibility of rat s.c. tissue to carcinogenesis by a variety of compounds that do not induce tumors elsewhere. An indication, however, appeared in a brief report by Alexander and Connell (1) that the simple ethylating agent, ethylmethanesulfonate, could give rise to a high incidence of kidney tumors in mice when given in 3 relatively high separate doses. The same compound, under similar conditions of dosage, was shown by Swann and Magee (54) to induce in the rat an incidence of about 50% renal tumors, the histological structure of which was identical with those induced by dimethylnitrosamine. In comparable experiments, methylmethanesulfonate induced about a 10% incidence of tumors of the nervous system (54) and the same compound, administered to pregnant rats, has induced nervous tumors in their progeny (P. Kleihues, personal communication). Unless these alkylalkanesulfonates, which do alkylate in vivo (53, 55), owe their carcinogenic action to some mechanism other than alklylation, it appears that alkylation, and in particular aberrant methylation, can be a potent carcinogenic stimulus. The relatively weak carcinogenic actions of some alkylation agents reported in the past may have been explicable by their chemical reactivity with nonspecific body components and their failure to react at a sufficient intensity with crucial cellular receptors. These latter findings support the suggestion that methylation by aberration of normal metabolic processes might have the same result as those of externally administered methylating agents.

The Significance of Methylation of Nucleic Acids in Carcinogenesis

The observations discussed above suggest that methylation of some cellular constituent may result in carcinogenesis but do not in themselves give any indication of the nature of this component. Most authors agree that the crucial cellular target must be a macromolecule, either nucleic acid or protein, but there is no absolutely compelling evidence for this. It is now known that all the major groups of chemical carcinogens, including polycyclic hydrocarbons, aromatic amines, nitroso compounds, and alkylating agents, either react directly or give rise to chemically reactive intermediates (proximate and...
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ultimate carcinogens) that react with DNA, RNA, and proteins. A large part of the recent research effort in chemical carcinogenesis has been devoted to attempts to determine which one of these macromolecules is the crucial target for malignant transformation (38, 39). As pointed out by Miller and Miller (39), all carcinogenic agents must alter, directly or indirectly, the content and/or expression of heritable information, governing growth and replication of the cell destined to become neoplastic. Such an alteration could be brought about directly by a change in DNA or indirectly by a change in RNA or protein, as suggested by Pitot and Heidelberger (44). The original observations on interactions of carcinogens with cellular macromolecules involved proteins, but more recently increasing attention has been paid to nucleic acid interactions, with emphasis on possible relationships between carcinogenesis and mutagenesis (5, 24). Perhaps because of this, DNA has been favored as the most likely cellular target, partly because the cellular change must be heritable and partly on the basis of correlations between quantitative measurements of binding of carcinogens with nucleic acids and proteins. Brookes and Lawley (7), for example, found no positive correlation between the extent of binding of a series of polycyclic hydrocarbons to the RNA or proteins of mouse skin with their carcinogenic potency in this tissue, but the binding to DNA did show a strong positive correlation. In similar experiments, Colburn and Boutwell (9) compared the binding of β-propiolactone and some related alkylating agents to DNA, RNA, and protein of mouse skin with their capacity to initiate and promote the induction of tumors. Again there was a correlation between capacity to initiate tumors and the extent of binding to DNA but no such correlation with binding to RNA or protein. The recent work of Cleaver (8) and Reed et al. (45) on xeroderma pigmentosum gives support of another kind for DNA as the critical target for carcinogenesis.

On the other hand, reasons for preferring tRNA rather than DNA as the primary target for chemical carcinogens have been put forward in the papers of Weinstein (58) and Weinstein et al. (59). These include the lack of evidence for a change in DNA analogous to mutation and the apparent normality of tumor DNA together with the evidence that tumor cells can, in some circumstances, revert to normal. If carcinogenesis involves changes in the pattern of gene expression rather than in the genetic material itself, loss of tRNA or a change in its specificity might result in cancer. The hepatic carcinogen ethionine is of interest in this context since it has been clearly shown to ethylate RNA (2, 13, 15, 41, 42, 47, 51), while it has been claimed that DNA is ethylated only to a very small extent or not at all (16, 42). This difference between the behavior of ethionine and other alkylating carcinogens could clearly be of significance in determining the relative importance of cellular macromolecules as targets for carcinogens. In the experiments of Ortweth and Novelli (42), in which no significant labeling of DNA was seen, the radioactive ethionine was given in tracer doses but recent experiments by Swann, Pegg, Hawks, Farber, and Magee (56) have suggested that DNA may become labeled when the ethionine is given in larger doses. Tritium-labeled ethionine was administered to rats in doses of 500 mg/kg, and the liver DNA appeared to contain radioactivity that was confined to 7-ethylguanine. If this result can be substantiated it will support an earlier report by Stekol et al. (50, 51) that DNA was ethylated on the 7-position of guanine by ethionine in vivo (50, 51). Assessment of the significance of these findings must await further study. From the standpoint of Borek’s hypothesis, however, the important point arising from this ethionine work is that the tRNA is much more highly ethylated than other RNA species, but it should not be overlooked that nuclear proteins were also relatively highly labeled (15).

An interesting further conclusion from the studies of Ortweth and Novelli (42) is that ethylation of tRNA by ethionine in vivo may not entirely proceed in a manner similar to normal methylation because S-adenosylethionine does not seem to be the active intermediate. This conclusion receives support from recent unpublished work by Dr. A. E. Pegg at the Courtauld Institute of Biochemistry, London. He has compared the methylation and ethylation of E. coli tRNA in vitro after administration of methionine and ethionine. All the methylated and ethylated bases found in vivo were formed in the in vitro system with bacterial tRNA as an acceptor of alkyl groups, although the relative proportions were not exactly similar. In the in vitro experiments, 7-methylguanine and 7-ethylguanine both represented 6% of the total incorporation into guanine, but in vivo 7-methylguanine was 12% of the total incorporation whereas 7-ethylguanine represented about 24% of the total. One explanation of this result would be that another means of producing 7-ethylguanine in tRNA in vivo, not involving S-adenosylethionine as an intermediate, can take place.

The significance of alkylation of nucleic acids, and of reaction on the 7-position of guanine in particular, is far from clear. Under some circumstances 7-alkylguanine apparently may be transcribed as guanine. Ludlum (26) has shown that 7-alkylguanine in synthetic polynucleotide templates has no apparent effect on the incorporation of adenylcy acid by RNA polymerase but that the presence of 3-methylcytidylic acid leads to mispairing of uridylic acid (27, 28). Similar mispairing has recently been observed with polycytidylic acid templates treated with the potent carcinogens N-nitrosomethyl- and N-nitrosoureylene (D. B. Ludlum and P. N. Magee, unpublished results).

Although the circumstantial evidence discussed above tends to favor the importance of DNA alteration in carcinogenesis, there is no doubt that RNA must, in some situations at least, play an essential part since the Rous sarcoma virus and some other oncogenic viruses contain RNA but no DNA. In this context, the recent suggestion by Huebner and Todaro (21) that vertically transmitted oncogenes of RNA tumor viruses may be determinants of cancer is particularly interesting. Freeman et al. (17) have shown that morphological transformation of rat embryo cells in culture can be induced by the combined action of diethylaminoamine and murine leukemia viruses but not by either agent acting alone. Since diethylaminoamine is known to ethylate RNA in vivo (33, 55), it may be that alkylation can activate a latent RNA tumor
virus. if this is so, a similar activation might be mediated by an excessive or aberrant action of a cellular RNA methylase.

Conclusions

From the above consideration of carcinogenesis by alkylating carcinogens and by ethionine, it is concluded that the possibility of the induction of cancer by the aberrant action of nucleic acid methylases must be recognized. Apart from the greater degree of methylation of tRNA than of other types of RNA, however, there seems to be no strong reason, in the present rate of knowledge, for implicating aberrant tRNA in the initiation of cancer. The possibility of aberrant action by DNA methylases and the possibility that latent oncogenic viral RNA might be activated by methylase action should also be considered.

REFERENCES

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Dr. Gefter: Did you mention what the primary effect is, using chemical ethylation in DNA?

Dr. Magee: What do you mean by the primary effect?

Dr. Gefter: With er/zy/methanesulfonate.

Dr. Magee: 7-Ethylguanine

Dr. Gefter: With ethylmethanesulfonate.

Dr. Magee: Yes.

Dr. Gefter: Would you like to comment on its possible mutagenic effect. In bacteria, it is quite clear that it is a mutagen, and is clearly having an effect on DNA and producing a subsequent change.

Dr. Magee: Yes.

Dr. Gefter: So I don’t quite understand, then, the idea that it doesn’t have any effect on RNA synthesis or that it is copied like guanine by RNA polymerase. Clearly, it must have some effect on some nucleic acid.

Dr. Magee: That is true, but you see, there is ethylation at other sites as well. And it may be that these are important ones. But because they are quantitatively smaller, it doesn’t mean to say they are not the biologically significant ones. That is what I am suggesting.

Dr. Gefter: So with chemical ethylation, there are other compounds produced in DNA other than 7-ethylguanine.

Dr. Magee: Oh, yes, definitely. In fact, the slide that I showed of the distribution of bases produced in the intact animal by dimethylnitrosamine is very closely copied by the action of methylmethanesulfonate in vitro. This was shown by Brookes and Lawley. The proportions of the different methylated bases are very similar, indeed.

Dr. Mandel: Dr. Magee, could you comment on the appearance of the methylated bases in the urine following the administration of carcinogens? Do you find compounds other than 7-methylguanine, for example? Do you find them in DNA?

Dr. Magee: That is something we should do. We haven’t looked for them. We have looked for 7-methylguanine in the urine, and we find, when we give dimethylnitrosamine in toxic doses, that the level of 7-methylguanine in the urine goes up by a factor of about 2, 2.5, that’s all. But when we give it in carcinogenic doses for the liver, that is to say continued feeding over long periods, 50 mg/kg, then you can’t detect it.

Discussion

because there is a scatter, an increase in the urine of 7-methylguanine.

But equally, I know that under some circumstances the 7-methylguanine does go up with carcinogens. And I think this might indicate the action of the methylases because in the intact animal, 7-methylguanine put in by a chemical carcinogen like this is not very stable. It comes out very quickly. A large part of it comes out from both DNA and RNA in the first 24 hr after the application of the carcinogen.

Dr. Carbon: Unless I have made a miscalculation, the experiment in which you measured the amount of label incorporated from ethionine into DNA corresponded to 1 in 1,000,000 bases. This seems negligible.

Dr. Magee: I would think so, yes. But how can you tell? I don’t know.

Dr. Carbon: Well, DNA as we all prepare it, is not really 100% pure. This level of 1 base in 1,000,000 could easily be in RNA.

Dr. Borek: Is ethionine a mutagen? If it isn’t, this would be the first instance of a carcinogen which is not a mutagen. In view of its slight interaction with DNA, I would be surprised if it is a mutagen.
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