Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 150-155

# THE EFFECT OF 5-AZACYTIDINE ON MAMMARY GLAND DIFFERENTIATION IN VITRO

Franklyn F. Bolander, Jr.

Department of Biology
University of South Carolina
Columbia, South Carolina 29208

Received January 6, 1983

SUMMARY: 5-Azacytidine, a cytidine analog which leads to hypomethylation in DNA and usually induces differentiation, actually inhibits differentiation in mammary gland explants. Either the genes for casein and the components of lactose synthetase are inactivated by hypomethylation or, more likely, mammary differentiation is inhibited by regulatory gene(s) which must be inactivated by methylation before differentiation can commence.

Although there are many mechanisms by which gene expression can be controlled, the role of gene methylation is presently receiving wide attention. According to the prevailing hypothesis, a methylated gene is inactive, while hypomethylation is necessary for gene expression (1), although recent reports suggest that it may not be sufficient (2, 3). A useful probe in the study of methylation is 5-azacytidine (5-azaC)[1], a nucleoside analog of cytidine which becomes incorporated into DNA but cannot be methylated (1). Using this analog, other investigators have been able to induce differentiation both in vivo (4) and in vitro (5, 6). It was the purpose of this study to use 5-azaC to examine the role of methylation in mammary gland differentiation.

## MATERIALS AND METHODS

Ovine prolactin (o-PRL-15) was kindly provided by the Hormone Distribution Program, NIADDK, and crystalline porcine insulin (lot 615-08E-220) was a gift from Eli Lilly Co. Cortisol, triiodothyronine, Hepes, 5-azacytidine, cytidine, casein, rennet and UDP-galactose were purchased from Sigma Chemical Co. Medium 199 with Hanks' salts was obtained from Grand Island Biological Co. UDP-[6-$^{3}$H]galactose (16.3 Ci/mmol) was from Amersham Corp., while [5-$^{3}$H]uridine (26.4 Ci/mmol) and [$^{33}$P]orthophosphoric acid, carrier-free, were purchased from New England Nuclear Corp.

Mammary gland explants were prepared (7) from either virgin mice (C3H/HeN) or parous mice one month following the weaning of their first litter. The explants were cultured on siliconized lens paper in medium 199 containing 20 mM

[1]Abbreviations: 5-azaC, 5-azacytidine; I, insulin; F, cortisol; P, prolactin.

0006-291X/83/040150-06$01.50/0

Hepes (pH 7.5), insulin (1 μg/ml), cortisol (1 μg/ml), prolactin (1 μg/ml) and triiodothyronine (65 pg/ml) unless otherwise noted. The tissue was incubated under air at 37° and the medium was changed daily.

Casein was determined by calcium-rennin precipitation (7) after a 4-h pulse with [$^{33}$P]orthophosphoric acid (50 μCi/ml). Total RNA synthesis was measured by the incorporation of [$^{3}$H]uridine (2 μCi/ml) over 4 h (8) and lactose synthetase activity was assayed enzymatically (9).

## RESULTS AND DISCUSSION

Mammary differentiation, as defined by the expression of milk-specific components (e.g., casein or lactose synthetase activity), is a complex process in explants of the mouse mammary gland. For example, a minimum of three hormones - insulin (I), cortisol (F) and prolactin (P) - are required in short-term culture (10) and others may be essential for more prolonged incubations (11, 12). Assuming prima facie that 5-azaC alone would probably not be able to effect differentiation in such a complex system, it was first tested in the presence of I and F. Table 1 shows that not only was 5-azaC unable to replace P in culture but that it actually inhibited differentiation in the presence of all three hormones. Fig. 1 demonstrates that some inhibition could be seen both in virgins, whose glands had never undergone differentiation, and in parous mice, whose glands had lactated previously before involution and culture.

TABLE 1

Ability of 5-azacytidine to replace prolactin in mammary differentiation in explants from virgin mice

| Experimental group | Lactose synthetase activity (pmol lactose/mg wet tissue/30 min) |
|---|---|
| IF | 0 |
| IFP | 53.5 ± 9.5 |
| IF + 5-azaC | 0 |
| IFP + 5-azaC | 0 |

Explants from virgins were cultured with hormones and/or 5-azacytidine as indicated (1 μg/ml when present). After 3 d, lactose synthetase activity was determined; each value is the mean ± S.E. of three separate experiments.

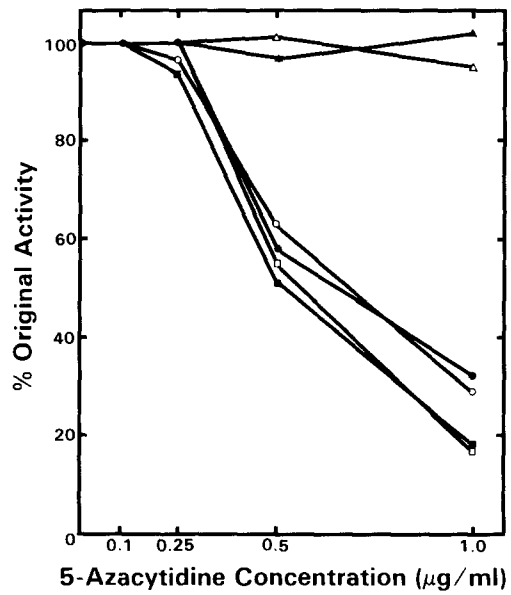


Figure 1. Effect of 5-azacytidine on mammary gland differentiation. Explants from virgin (o, □) or parous (•, ▪) mice were cultured with hormones (see Methods) and 5-azacytidine (as indicated). After 3 days lactose synthetase activity (□, ▪, △, ▲) and casein synthesis (o, •) were determined. Explants from virgins were also cultured with an excess of cytidine (10 μg/ml, ▲) or were cultured for 4 days in insulin and cortisol before prolactin and 5-azacytidine were added for an additional 3 days (△). Original activity is the amount of enzyme activity or casein synthesis in tissues from each of the above groups cultured in the presence of I, F and P (1 μg/ml for each) but without 5-azacytidine. Each point is the mean of three separate experiments per experimental group, except for those at the lower concentrations of 5-azacytidine where many of the points were fused. The S.E.M. varied between 9% and 13%.

Several experiments were performed in an attempt to determine if this inhibition were due to some nonspecific toxicity rather than hypomethylation. In order for 5-azaC to affect DNA methylation, it must be incorporated into this macromolecule during DNA synthesis. In the presence of an excess of cytidine, little 5-azaC should have been incorporated and there was no inhibition (Fig. 1). Furthermore, if 5-azaC were added at a time when there was no DNA synthesis, there should be no incorporation and no inhibition. Epithelial cells in mammary explants from virgins undergo a single round of DNA synthesis, which is complete after three to four days in culture (13); since this synthesis can occur in the absence of P, the cells remain undifferentiated. Fig. 1 shows that if these cells were subsequently exposed to IFP and 5-azaC, full differentiation ensued. The presence of excess

TABLE 2

Effect of 5-azacytidine on hormone-induced, total RNA synthesis in mammary gland explants from virgin mice.

| Experimental group | No hormone (cpm/mg) | IF (cpm/mg) | IFP (cpm/mg) |
|---|---|---|---|
| Control | 2346 ± 157 | 4174 ± 189 | 5767 ± 87 |
| 5-Azacytidine | 2899 ± 145 | 4449 1 92 | 5591 ± 157 |

Explants were cultured with hormones as indicated (1 μg/ml when present) and with or without 5-azacytidine (1 μg/ml). After 3 days the explants were pulsed with [$^3$H]uridine and total RNA synthesis determined; each value is the mean ± S.E. of three separate experiments.

cytidine or the use of a double incubation yielded similar results in tissue from parous mice (data not shown). Finally, 5-azaC had no effect on IF- or IFP-induced, total RNA synthesis in mammary explants (Table 2). Therefore, it appears that the inhibition of 5-azaC on mammary differentiation in vitro is dependent upon its incorporation into DNA and not upon any other nonspecific toxic effects.

There are two possible explanations for these results: (1) the genes for casein and the components of lactose synthetase, unlike all other known genes, are inactivated by hypomethylation, or (2) the process of differentiation is inhibited by regulatory gene(s) which must be inactivated (i.e., methylated) for the induction of casein synthesis and lactose synthetase activity to occur. Although the data do not allow the two possibilities to be distinguished, the latter hypothesis is more consistent with the known inhibitory role of methylation in other system (2-4). In this hypothesis, the presence of 5-azaC in the DNA would block the methylation and concommitant inactivation of the regulatory gene(s), resulting in the continued inhibition of mammary differentiation, even in the presence of an otherwise adequate complement of hormones. Furthermore, since both virgin and parous mice are sensitive to 5-azaC, it would appear that during involution, the regulatory gene(s) must be reactivated by hypomethylation.

It is noteworthy that not all of the effects of P are blocked by 5-azaC. Although casein synthesis and lactose synthetase activity are inhibited (Fig.

1), total RNA synthesis is unaffected (Table 2). This finding is reminiscent of the role of glucocorticoids in mouse mammary gland differentiation: the absence of glucocorticoids prevents the induction of lactose synthetase activity and casein mRNA accumulation, but these steroids are not required for P-induced, total RNA synthesis (14). Furthermore, colchicine administration to rabbits will block P-induced accumulation of casein mRNA but not P-induced, ribosomal RNA (15). Taken together, these data suggest that there are at least two pathways in P action: one pathway apparently leads to the induction of milk-specific components, while the other stimulates more ubiquitous compounds such as rRNAs and tRNAs.

Recent studies on the mechanism of action of steroid hormones have demonstated that the steroid receptor complex binds to DNA sequences in cloned, hormone-responsive genes (16, 17). The mechanism of action of peptide hormones is less clear, particularly for those for whom a second messenger has not been identified yet. The data in this report suggest that they or their intermediaries may act upon regulatory gene(s). This hypothesis is not meant to exclude an additional action at the structural genes themselves, but it is meant to call attention to the idea that hormones may have a greater repertoire of mechanisms for gene expression in eukaryotes than is commonly emphasized.

ACKNOWLEDGEMENTS

This research was supported in part by grant HD 16283 from the National Institute of Child Health and Human Development and a Biomedical Research Support Grant (2 SO-7RR07160). The author thanks Shoba Prakash for technical assistance.

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