Int. J. Dev. Biol. 43: 843-846 (1999)

Short Contribution

Changes in the placenta and in the rat embryo caused by the demethylating agent 5-azacytidine

MAJA VLAHOVIĆ[1]*, FLORIANA BULIĆ-JAKUŠ[1], GORDANA JURIĆ-LEKIĆ[2], ALEKSANDRA FUČIĆ[3], SVJETLANA MARIĆ[4] and DRAŠKO ŠERMAN[1]

[1]*Department of Biology,* [2]*Department of Hystology and Embryology, Medical Faculty, Zagreb,* [3]*Institute for Medical Research and Occupational Health, Zagreb and* [4]*Department of Biology, Medical Faculty, Osijek, Croatia*

ABSTRACT DNA methylation is an important mechanism for regulation of gene expression during vertebrate development. 5-azacytidine is used as an experimental tool for demethylation. In this work, a single dose of 5-azacytidine (5 mg/kg body weight) was administered to rats at different stages of development. After 5-azacytidine administration on the first or third day of pregnancy, no changes were detected. After administration on the fourth day of pregnancy or later, a reduction in growth was observed. After treatment on day five and on any other day till day eleven of pregnancy, no living fetuses were found. Of those treated on day twelve, 24% of fetuses survived, but forelimb and hindlimb malformations were present. Administered on day thirteen, 5-azacytidine did not interfere with survival, but malformations were still present. From day fourteen on, 5-azacytidine caused no gross external malformations. Placentas were also influenced by 5-azacytidine. They were significantly smaller and histological evaluation showed the labyrinthine part to be severely reduced. In contrast, trophoblast giant cells were more abundant than in controls.

KEY WORDS: *placenta, embryo, rat, methylation, 5azaC*

The DNA methylation pattern is dynamic and changes during development and cell differentiation (for review see Tajima and Suetake, 1998). Following implantation, there is a huge increase in methylation due to global *de novo* DNA methylation, affecting the whole embryo. At later stages of development, tissue specific genes are subjected to programmed demethylation in those cell types where they are expressed (Heby, 1995).

Nucleoside analog 5-azacytidine (5azaC) inhibits postreplication methylation of DNA. It is incorporated into DNA as well as into RNA and also inhibits RNA synthesis (Michalowsky and Jones, 1989). Its incorporation into DNA causes subsequent inhibition of DNA cytosine methyltransferase and loss of methylation followed by a change in gene expression, i.e. gene activation from their repressed state (Jones, 1984). 5azaC is also known to be an antileukemic agent and a useful tool to study the role of DNA methylation in cell differentiation and gene activation (Silverman *et al.*, 1993). *In vitro* experiments with 5azaC on gastrulating rat embryo showed changes in survival, growth and differentiation (Skreb *et al.*, 1993; Vlahovic *et al.*, 1994; Bulic-Jakus *et al.*, 1999). We have used 5azaC during pregnancy to elucidate the importance of DNA demethylation in the rat embryonic development involving also the later stages that were not investigated before (Cummings, 1994).

5azaC did not affect development at all when administered on the first or third day of pregnancy (Tables 1 and 2). On the contrary, after its administration to pregnant females between day 4 and day 16, we observed different types of disorders during embryonic development such as embryolethality, lower weight and presence of malformations.

The number of deaths was the highest after treatment between days 5 and 11 of pregnancy when no living fetuses were found (Table 1). In embryos treated on days 4 and 12, the survival rate was much lower than in those treated after day 12, when it was practically 100%. The administration of 5azaC on days 5-10 caused complete resorption of embryos, but on days 11 and 12 it caused partly complete resorption and partly intrauterine death. Intrauterine death was observed in the form of a necrotic embryo showing head, tail and limb buds in contrast to aforementioned resorptions where no embryonic tissue or shape was found either on day 17 or on day 20 of pregnancy.

In two embryos treated on day 12 of pregnancy and fixed on day 20, the shape of the embryo was preserved and histological evaluation showed numerous apoptotic changes (typical frag-

Abbreviations used in this paper: 5azaC, 5-azacytidine; PCNA, proliferating cell nuclear antigen.

***Address for reprints:** Department of Biology, Medical Faculty, Šalata 3, 10 000 Zagreb, Croatia. FAX: 385-1-4680-538. e-mail: majav@mef.hr

0214-6282/99/$15.00

Printed in Spain
www.ehu.es/ijdb

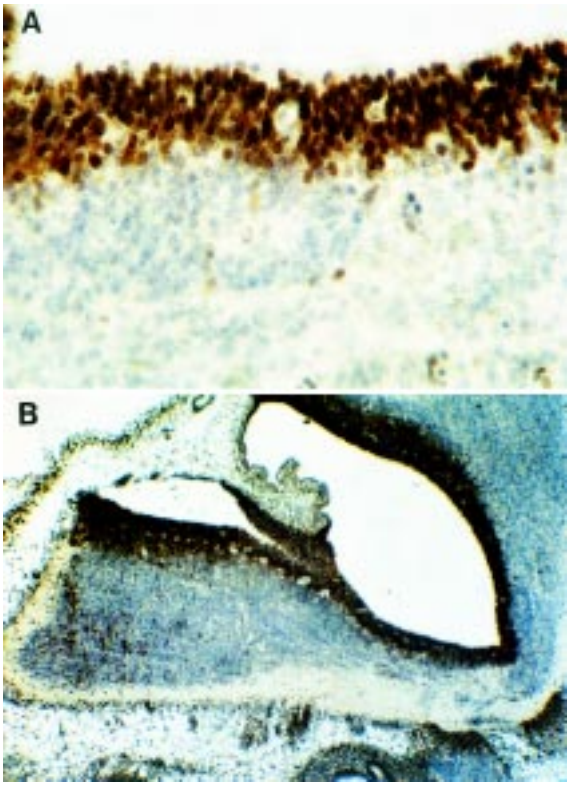

Fig. 1. Expression of PCNA (proliferating cell nuclear antigen) in developing rat brain treated with 5-azacytidine. *Immunohistochemical staining showed no differences in expression of endogenous proliferation marker (PCNA) in the brain between control and treated rats.* **(A)** *Control normal fetal rat brain (15th day of pregnancy) showing strong nuclear staining of proliferating cells around brain ventricule (DAB, hematoxylin, 200x).* **(B)** *Fetal rat brain (15th day of pregnancy) treated with 5azaC on 13th day of pregnancy showing strong nuclear staining of proliferating cells around brain ventricule (DAB, hematoxylin, 63x).*

mentations of nuclei), and we can suppose that resorptions caused by 5azaC probably begin with apoptotic changes in embryo. We observed apoptoses especially in the brain as was also shown in mouse (Hossain *et al.*, 1995). In developing neuronal cells, 5azaC promoted apoptoses more in proliferating than in terminally differentiated cells (Hossain *et al.*, 1997). When we administered 5-azacytidine on day 13 of pregnancy (when embryolethality was absent) and fixed the brain two days later, apoptoses were not present. Moreover, proliferation of the brain tissue was still normal, like in controls, as shown by expression of the endogenous proliferation marker PCNA (Bravo *et al.*, 1987; Sanders *et al.*, 1993) (Fig. 1).

Whenever living fetuses were found, a statistically significant difference in the mean body weight and crown-rump length was observed in comparison to controls (Table 2, Fig. 2). 5azaC sometimes also caused a statistically significant reduction of placental growth (Table 2). It has been known to be an antimitotic agent (Pfeifer *et al.*, 1989) and the effect upon growth can be explained simply by direct inhibiting action of 5azaC against the cellular synthesis of RNA and proteins (Cihak, 1974).

Histological analysis of control and experimental placentas demonstrated a difference in the width of placental layers, as shown in Figure 3. In experimental placentas, the labyrinth spread on approximately one half of the total width of the placenta and the basal region occupied the other half, in contrast to controls where the labyrinth normally occupied about 3/4 of the placenta and the basal region only 1/4. Recently, it has been found that rat trophoblast giant cells from the basal region during the process of their differentiation require modification of CpG islands by cytosine methylation (Ohgane *et al.*, 1998), and that multiple rounds of endoreplication are necessary for differentiation of those cells which contain aproximatelly 100 times more genomic DNA per nucleus than diploid cells. Our unpublished results show that 5azaC promotes numerous endomitoses in bone marrow of treated Fischer rats, which suggests that in our recent experiment, endoreplication of the trophoblastic cells could indeed be promoted by 5azaC.

Malformations in living fetuses were observed only when 5azaC was given on days 12 and 13 (Table 3). The malformation pattern appeared to be stage-specific and mostly consisted of defects in limb bones (Fig. 2) The malformed fetuses, however, did not survive more than one day after birth (own unpublished results). Limb malformations we have observed in rat are in accordance with the results obtained in mouse at corresponding gestation days, but in mouse the spectrum of malformations was wider, showing also e.g. cleft palate, fusion of sternebrae and tail anomalies (Schmahl *et al.*, 1984; Branch *et al.,* 1996). Limb formation depends on the interaction of numerous signals at the molecular level (Johnson and Tabin, 1997), and similar limb malformations were described in other species (Li *et al.*, 1996; Johnson *et al.*, 1998) in connection with changes in gene expression.

In conclusion, we can say that the methylation pattern disturbed by 5azaC in our experiments did not only affect the developing rat

Fig. 2. Growth and morphogenesis of 5azaC treated fetuses. *Pregnant females were treated with 5-azacytidine/kg body weight on day 13 of pregnancy and fetuses were examined on day 20 of pregnancy. Control* **(A)** *and treated* **(B)** *fetuses were stained with alizarin red S and alcian blue (cartilage-blue, bone-red) and photographed under the same magnification (6x). Note the hind limb oligodactily in smaller 5azaC treated fetus (B).*

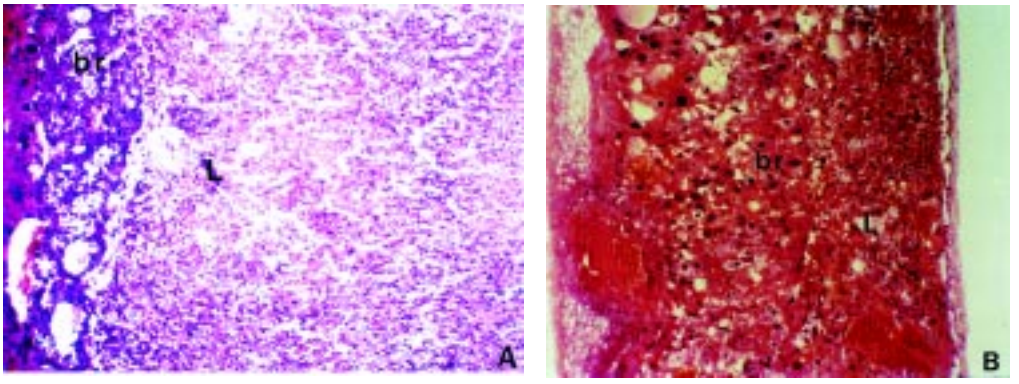

Fig. 3. Placenta in 5azaC treated fetus. (A) *Normal distribution of rat placental layers in control (br, basal region; L, labyrinth).* **(B)** *5azaC treated (on day 11 of pregnancy) placenta showing a reduced labyrinthine (L) and a larger basal region (br) than in controls (HE, 63x).*

embryo, but also had a major effect on both fetal and maternal parts of the placenta, which has not been previously described.

Experimental Procedures

Experiments were performed on pregnant Fischer female rats on different days of gestation. Adult (three-month-old) females were mated with males of the same age overnight. Vaginal plug designated day 0 of pregnancy.

5-azacytidine (Sigma) was dissolved in PBS and used at a concentration of 5 mg/kg body weight. Administration was by a single i.p. injection (1 ml) of either PBS (controls) or of 5-azacytidine solution. The animals were sacrificed in ether on day 20 or 17 of pregnancy. The weights of every single fetus and placenta as well as crown-rump lengths were determined. Malformations were observed under the dissecting microscope. Fixation was performed in Zenker's solution for routine histology. Embryos or different organs were embedded in paraffin, sectioned in 5 µm slices, deparaffinized and stained with hematoxylin and eosin.

Fixation was performed in St. Marie's solution (1% acetic acid and 96% ethanol for 24 h, +4°C) for immunohistochemical examination. Embryos were routinely processed and embedded in paraffin at 56°C. Five µm sections were dried for 2 h at 36°C, routinely deparafinized and processed with primary mouse monoclonal antibody against PCNA (proliferating cell nuclear antigen) clone PC 10 (M 0879- DAKO). Negative controls were treated with an unspecific antibody (V1617 mouse IgG_1, DAKO). Hydrogen peroxide block was applied for 5 min and slides were washed in buffer solution. 0.05M Tris HCl pH 7.6 containing 0.3M NaCl and 0.1% Tween 20 was used as wash and bath buffer. Primary antibody (1:50) was applied for

TABLE 1

SURVIVAL OF FETUSES AFTER TREATMENT WITH 5-AZACYTIDINE ON DIFFERENT DAYS OF PREGNANCY

Treatment on day	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Number of fetuses	19	20	30	7	27	17	9	21	9 13*	51	45	64	54	25	43
% of living fetuses	100	100	63.3	0	0	0	0	0	0 0*	0	24.4	98.4	100	100	100
% of complete resorptions	0	0	33.3	100	100	100	100	100	100 100*	70.6	51.1	1.6	0	0	0
% of i.u. deaths	0	0	3.3	0	0	0	0	0	0 0*	29.4	24.4	0	0	0	0

Pregnant females were treated with a single dose of 5mg 5-azacytidine/kg body weight on different days of pregnancy (between day 1 and day 16) and also with a single dose *2.5 mg/kg body weight on day 10 of pregnancy. Survival was determined on day 20 of pregnancy. Survival of 134 control fetuses was 100%. Note the stage specific lethality (days 5-10 of pregnancy: 100% lethality; days 1-3 and 13-16 almost 100% survival)

TABLE 2

GROWTH OF FETUSES AND PLACENTAS AFTER TREATMENT WITH 5-AZACYTIDINE ON DIFFERENT DAYS OF PREGNANCY

Treatment on day	**No of fetuses**	**Mean body weight (g ± SEM)**	**Crown-rump length (cm ± SEM)**	**Mean weight of placenta (g ± SEM)**
Controls	134	3.19 ± 0.03	3.13 ± 0.01	0.39 ± 0.01
1	19	3.19 ± 0.05	3.06 ± 0.04	0.42 ± 0.02
3	20	3.08 ± 0.05	3.12 ± 0.03	0.40 ± 0.01
4	30	2.57 ± 0.07*	2.90 ± 0.04*	0.50 ± 0.02*
5-11		Resorptions		
12	11	1.70 ± 0.09*	2.41 ± 0.06*	0.21 ± 0.02*
13	64	2.22 ± 0.04*	2.81 ± 0.03*	0.28 ± 0.01*
14	54	3.02 ± 0.06**	2.93 ± 0.02*	0.38 ± 0.01
15	25	2.57 ± 0.03*	2.69 ± 0.02*	0.34 ± 0.01*
16	43	2.69 ± 0.04*	2.74 ± 0.02*	0.40 ± 0.01

Pregnant females were treated with a single dose of 5 mg 5-azacytidine/kg body weight on different days of pregnancy and body weights, crown-rump lengths and weights of placenta were determined on day 20 of pregnancy. All values are denoted as means ± standard error of the mean and were compared to controls by Student's t-test.
*significantly different p<0,01 ** significantly different p<0,02

TABLE 3

FREQUENCY OF FETAL MALFORMATIONS AFTER TREATMENT WITH 5-AZACYTIDINE

Treatment on day	12	13	χ^2	p
Number of malformed fetuses/ Number of living fetuses (%)	8/11 (72.7%)	57/64 (90.00%)	0.23	>0,05
FRONT LIMBS:				
Micromelia	8 (72.73%)	0	44.75	<0.01
Oligodactyly	8 (72.73%)	41 (65%)	0.11	>0.05
Adactyly	4 (36.36%)	5 (8%)	10.20	<0.01
Brachydactyly	5 (45.45%)	22 (35%)	0.11	>0.05
HIND LIMBS:				
Micromelia	8 (72.73%)	0	44.75	<0.01
Syndactyly	4 (36.36%)	4 (6%)	12.37	<0.01
Oligodactyly	3 (27.27%)	55 (87%)	21.93	<0.01
Adactyly	4 (36.36%)	1 (2%)	0.42	<0.05
Brachydactyly	7 (63.64%)	34 (54%)	0.10	>0.05
HEMATOMAS:				
Limb	3 (27.27%)	8 (13%)	0.26	>0.05
Tail	0	5 (8%)	0.01	>0.05

Pregnant females were treated with a single dose of 5 mg 5-azacytidine/kg body weight on days 12 and 13 of pregnancy and malformations were determined on day 20 of pregnancy. Note the high percentage of malformed fetuses and specificity of malformations concerning only the limbs. Incidence of malformations was compared between days 12 and 13 of administration by χ^2-test and statistically significant differences were found. Note e.g. absence of micromelia in fetuses treated on day 13.

30 min and washed. A sensitive labeled streptavidin-biotin kit (DAKO, LSAB^R 2 Kit, Peroxidase) for use on rat tissue (K 609, DAKO) was used for detection of primary antibody. Link antibody consisting of secondary biotinylated anti-mouse and anti-rabbit antibody was applied for 30 min, washed in buffer, streptavidin-peroxidase complex was applied for 30 min and washed, sections were incubated with substrate-chromogen solution containing diaminobenzydine (DAB) for 2 min and finally briefly counterstained with hematoxylin, washed with water, mounted with glycerol and PBS (1:1).

For the differential staining of cartilage and bone, fetuses were skinned and eviscerated at the time of sacrificing, fixed in 95% ethanol for 5 days and placed in acetone for one day to remove fat. Fetuses were stained for 2 days in staining solution with alizarin red S and alcian blue and processed according to Inouye method (for details see Inouye, 1976). Cartilage was stained blue and ossified skeleton purple to red.

For statistical evaluation Student's t test and χ^2 were used.

Acknowledgments

We thank Miss Durtica Cesar and Mrs Jasna Ljubek for their skilled technical assistance. This research was supported by a grant number 108207 (Biology of the reproductive health) from the Ministry of Science and Technology of the Republic of Croatia.

References

BRANCH, S., FRANCIS, B.M., BROWNIE, C.F. and CHERNOFF, N. (1996). Teratogenic effects of the demethylating agent 5-aza-2'-deoxycytidine in the Swiss webster mouse. *Toxicology 112:* 37-43.

BRAVO, R., FRANK, R., BLUNDELL, P. and MacDONALD-BRAVO, H. (1987). Cyclin/PCNA is the auxilliary protein of DNA polymerase δ. *Nature 326:* 515-517.

BULIC-JAKUS, F., VLAHOVIC, M., JURIC-LEKIC, G., CRNEK, V. and SERMAN, D. (1999). Gastrulating rat embryo in a serum-free culture model: changes of development caused by teratogen 5-azacytidine. *ATLA 27:* 925-933.

CIHAK, A. (1974). Biological effects of 5-azacytidine in eukaryotes. *Oncology 30:* 405-422.

CUMMINGS, A.M. (1994). Effect of 5-azacytidine administration during very early pregnancy. *Fundam. Appl. Toxicol. 23:* 429-433.

HEBY, O. (1995). DNA methylation and polyamines in embryonic development and cancer. *Int. J. Dev. Biol. 39:* 737-757.

HOSSAIN, M.M., NAKAYAMA, H. and GOTO, N. (1995). Apoptosis in the central nervous system of developing mouse fetuses from 5-azacytidine-administred dams. *Toxicol. Pathol. 23:* 367-372.

HOSSAIN, M.M., NAKAYAMA, H., TAKASHIMA, A., GOTO, N. and DOI, K. (1997). 5-azacytidine (5Az) induces apoptosis in PC12 cells: a model for 5Az-induced apoptosis in developing neuronal cells. *Histol. Histopathol. 12:* 439-445.

INOUYE, M. (1976). Differential staining of cartilage and bone in fetal mouse skeleton by Alcian blue and Alizarin red S. *Cong. Anom. 16:* 171-173.

JOHNSON, K.R., SWEET, H.O., DONAHUE, L.R., WARD-BAILEY, P., BRONSON, R.T. and DAVISSON, M.T. (1998). A new spontaneous mouse mutation of Hoxd13 with a polyalanine expansion and phenotype similar to human synpolydactyly. *Hum. Mol. Genet. 7:* 1033-1038.

JOHNSON, R.L. and TABIN, C.J. (1997). Molecular models for vertebrate limb development. *Cell 90:* 979-990.

JONES, P.A. (1984). Gene activation by 5-azacytidine. In *DNA methylation. Biochemistry and Biological Significance* (Eds. A. Razin, H. Cedar and A.D. Riggs). Springer Verlag, New York, pp. 165-187.

LI, S., ANDERSON, R., REGINELLI, A.D. and MUNEOKA, K. (1996). FGF-2 influences cell movements and gene expression during limb development. *J. Exp. Zool. 274:* 234-247.

MICHALOWSKY, L.A. and JONES, P.A. (1989). DNA methylation and differentiation. *Environ. Health Perspect. 80:* 189-197.

OHGANE, J., AIKAWA, J., OGURA, A., HATTORI, N., OGAWA, T. and SHIOTA, K. (1998). Analysis of CpG islands of trophoblast giant cells by restriction landmark genomic scanning. *Dev. Genet. 22:* 132-140.

PFEIFER, G.P., STEIGERWALD, S.D. and GRUENWALD, S. (1989). The DNA methylation system in proliferating and differentiated cells. *Cell Biophys. 15:* 79-86.

SANDERS, E.J., VAREDI, M. and FRENCH, A.S. (1993). Cell proliferation in the gastrulating chick embryo: a study using BrdU incorporation and PCNA localization. *Development 118:* 389-399.

SCHMAHL, W., TOROK, P. and KRIEGEL, H. (1984). Embryotoxicity of 5-azacytidine in mice. *Arch. Toxicol. 55:* 143-147.

SILVERMAN, L.R., HOLLAND, J.F., WEINBERG, R.S., ALTER, B.P., DAVIS, R.B., ELLISON, R.R., DEMAKOS, E.P., WEINBERG, C. Jr., CAREY, R.W., SCHIFFER, C., FREI, E. III. and McINTYRE, O.R. (1993). Effects of treatment with 5-azacytidine on the *in vivo* and *in vitro* hematopoiesis in patients with myelodysplastic syndromes. *Leukemia (Baltimore) 7:* 21-29.

SKREB, N., BULIC-JAKUS, F., CRNEK, V., STIPIC, J. and VLAHOVIC, M. (1993). Differentiation and growth of rat egg-cylinders cultured *in vitro* in a serum-free and protein-free medium. *Int. J. Dev. Biol. 37:* 151-154.

TAJIMA, S. and SUETAKE, I. (1998). Regulation and function of DNA methylation in vertebrates. *J. Biochem. (Tokyo) 123:* 993-999.

VLAHOVIC, M., BULIC-JAKUS, F., CRNEK, V., SERMAN, D., HERMAN, S. and SKREB, N. (1994). 5-azacytidine can alter gene expression in cultivated mammalian embryos. *Period. Biol. 96:* 65-66.

Received: June 1999
Accepted for publication: October 1999