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Regulation of mouse trophoblast giant cell nucleus development in hatched mouse blastocysts by cyclic cytidine 3′,5′-monophosphate (cCMP)

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Summary. The dibutyryl analog of cCMP enlarged the nuclei of trophoblast gian cells and promoted blastocyst development. The result suggests that cCMP has a trophic effect on embryonic development, specifically by altering the size of the trophoblast cell nucleus but does not enhance trophoblast cell proliferation processes.
Key words. cCMP; cyclic nucleotides; blastocyst; trophoblast; mouse.

Mouse blastocysts cultured in tissue culture media will subsequently hatch out of the zona pellucida and attach to the substratum [1–4]. The attachment of the hatched blastocysts appear to require certain tissue culture conditions such as the presence of a collagen substratum [5], a serum source such as human cord serum [6], essential amino acids [7, 8] and glucose [9, 10]. The inner cell mass (ICM) differentiates into the ectoderm and endoderm layers after culturing the blastocysts for 96 h [11]. The stages of embryonic development have been reported [6, 12, 13]. Recently, a less well-known cyclic nucleotide, cCMP, found endogenously in cells [14] has been implicated in cell proliferation [15, 16], activation of protein kinases [17], initiation of hemoglobin synthesis [18] and is found at high intracellular levels in rapidly-growing hepatoma cells [19]. It has been shown that the mouse embryo is responsive to cCMP and that cCMP promotes the attachment process of the hatched blastocyst [20]. The purpose of the present study was to further define the action of cCMP in the trophoblast giant cells and determine if cCMP is involved in the regulation of cell proliferation in the implanted embryo.

Female Swiss-Webster mice were superovulated with an intraperitoneal injection (i.p.) of 5 IU pregnant mares serum gonadotropin (PMSG) followed by 5 IU i.p. of human chorionic gonadotropin (hCG) 48 h later. The animals were mated and the presence of a vaginal plug the following morning indicated successful copulation. Embryos at the blastocyst stage were flushed out of the uteri 90 h after hCG administration, and were placed into Falcon petri dishes (Falcon No. 3037) containing either 1 ml culture medium as control or 10 µM dibutyryl cCMP (dbcCMP; Sigma Chemical Co., Cat No. D-7392). The dibutyryl analog of cCMP was used in this study, instead of cCMP or cytosine because the dibutyryl group facilitates penetration into the cell [21] and is more resistant to phosphodiesterase degradation [22]. The cultures were incubated at 37 °C in a moist 5% CO_2 in air mixture.

The culture medium consisted of Ham's F-10 (with L-glutamine, 5.6 mM D-glucose; GIBCO, Grand Island, NY) supplemented with 2.1 g/l sodium bicarbonate, 245.2 mg/l calcium lactate, 75 mg/l penicillin G and 75 mg/l streptomycin [20, 23, 24]. In addition, the medium contained 4 mg/ml human serum albumin fraction V (HSA-V; Sigma, St. Louis, MO). The pH and osmolarity were adjusted to 7.2 and 290 mOsm respectively and the medium was filter-sterilized and equilibrated overnight in the incubator prior to use the following day. The blastocysts were examined under a Nikon Diaphot inverted microscope equipped with a microscope incubator on days 1, 3 and 5 after the start of incubation (day 0). Photomicrographs were taken at the appropriate stages of development for each embryo. On day 3 of culture, a portion of the implanted embryos in the treatment and control groups were fixed with methanol and stained with Giemsa and photomicrographs were taken of the preparations. Measurements of the photomicrographs of the trophoblast cells of implanted embryos (at the egg cylinder stage) were made using the Zeiss Videoplan computerized image analyzer equipped with statistical software. The area and perimeter of the trophoblast cell nuclei were measured. Measurements of individual trophoblast cells and ICM cells were not carried out because the borders between each cell were not clear and appeared to be fused into syncytiums. The experimental results were expressed as mean ± standard error of the mean (SEM). Categorical data were analyzed using the chi-square statistic. The area and perimeter measurements were analyzed using the Student's t-test statistics. A $p < 0.05$ value was considered significant.

After 3 days of culture (table 1) the mean area of the nucleus was significantly ($p < 0.05$) larger in the trophoblasts cells exposed to dbcCMP (1478.4 ± 519.9 µm²) when compared with the control (1110.9 ± 363.0 µm²). The mean perimeter of the nucleus which is a reflection of the nuclear area was also larger in the dbcCMP treatment group. There were no differences in the number of trophoblast giant cells associated with the inner cell mass in both treatment and control groups.

Table 1. Morphometric analysis of trophoblast giant cell nucleus development in the presence of cyclic cytidine 3′,5′-monophosphate (day 3)

Treatment	No. of cells examined	Mean area of cell nucleus (µm² ± SEM)	Mean perimeter of nucleus (µm ± SEM)
Control	74	1110.9 ± 363.0	138.1 ± 23.2
10 µM dbcCMP	49	1478.4 ± 519.9[a]	157.6 ± 27.1[a]

[a] Significantly different from control ($p < 0.05$).

Table 2. The effect of cyclic cytidine 3',5'-monophosphate on the development of mouse blastocysts in vitro on day 1 of culture

Treatment	Proportion of blastocysts		
	No. of implanted blastocysts (%)[a]	No. of hatching blastocysts (%)	No. of expanded blastocysts (%)[b]
Control	15/39 (38.5)	7/39 (18.0	14/39 (35.9)
10 μM dbcCMP	9/33 (27.3)	9/33 (27.3)	13/33 (39.4)

[a] The expanded blastocysts hatch out of the zona and grow on the petri dish as implanted blastocysts. [b] For categorical purposes, a blastocyst is considered expanded when the diameter of the blastocyst is equal to or exceeds 125 μm.

Table 3. The effect of cyclic cytidine 3',5'-monophosphate on the advanced development of mouse embryos in vitro (day 5)

Treatment	No. at embryonic* disc stage (%)	No. retarded at egg cylinder stage (%)
Control	8/16 (50.0)	7/16 (43.8)
10 μM dbcCMP	10/12 (83.3)	1/12 (8.3)[a]

* The embryonic disc stage is stage 10[6,13]. [a] Significantly different from control ($p < 0.05$).

The in vitro development of the blastocysts in the presence of dbcCMP was examined on days 1, 3 and 5. The observations on day 1 after the initiation of culture (table 2) indicated no significant differences in the percentages of expanded and implanted blastocysts in the dbcCMP group in comparison with the control. There was a significant retardation of further development of egg cylinders ($p < 0.05$) in the control group in comparison with embryos in the dbcCMP treatment group (table 3).

The trophoblast cells transformed into giant cells had significantly larger cell nuclei when these cells were exposed to dbcCMP in comparison with control cells. The number of blastocysts that advanced into the embryonic disc stage after 5 days of incubation in the presence of dbcCMP was numerically higher than the control. The control blastocysts were significantly retarded at the egg cylinder stage suggesting that cCMP may be an important factor for promoting the development of embryos past the egg cylinder stage[25] into more advanced embryonic stages.

There was no evidence of increased cell proliferation in the trophoblasts by dbcCMP. This is in contrast to reports that suggest a role of cCMP in the cell proliferation process[16,19]. A possible explanation is that the concentrations of dbcCMP chosen did not lie in the effective concentration range necessary to elicit cellular responses. Variations in the G1 phase between the trophoblast cells[26] and cancer cells may also partly explain the observed differences in response of the cells to dbcCMP.

Mouse blastocysts cultured in tissue culture medium hatched out of the zona pellucida layer and attached to the substratum[1-3]. Hatching of the blastocyst in vitro involved repeated contractions and expansions[27]. The data from the present study indicated that cCMP did not affect the ability of blastocysts to expand or hatch from the zona pellucida. Studies conducted at an earlier cell stage, the 8-cell stage, also indicated that cCMP is not involved in the hatching of the blastocysts[20]. An interesting observation is that the point of hatching always occurred at a mural trophoblasts site and that the extrusion of ICM cells followed closely behind the extruded trophoblast cells. The collagen substratum has been shown to be essential for hatched blastocyst attachment and trophoblast cell outgrowth[5]. The point of attachment always involved mural trophoblasts[28]. The use of HSA fraction V as the serum source and a culture medium containing essential amino acids such as histidine and cystine needed for the outgrowth of trophoblast cells[7,8] also permitted blastocyst attachment without restrictions.

In the present study, dbcCMP is shown to have a trophic effect on the development of the nuclei of in vitro attached trophoblast giant cells. The results showed that the addition of dbcCMP to blastocysts enhanced the nuclear size of the trophoblast cells of the implanted embryo and promoted further development in vitro. However, the cyclic CMP analog was not involved in the proliferation of the trophoblast cells.

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