Hatching and Distribution of Actin Filaments in Mouse Blastocysts Treated with Cytochalasin B

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Summary

The hatching rate and the distribution of actin filaments were observed in mouse blastocysts whose ability of actin polymerization had been suppressed by cytochalasin B (CB), in order to examine the role of actin filaments in blastocyst hatching.

The hatching rate of blastocysts developed from morulae in a medium containing 0.4 μg/ml CB was 9.7 %, which was significantly lower than the 78.1 % of control blastocysts developed from morulae in a medium without CB.

The fluorescence showing the presence of actin filaments was observed in the cytoplasm of trophectoderm and inner-cell-mass cells in blastocysts, and was especially strong in the peripheral cytoplasm where two trophectoderm cells adhering to each other. Also actin fluorescence was much brighter in protruded trophectoderm cells at the region of small hole or slit in the zona pellucida of hatching blastocysts. Such the distribution of actin filaments was similar in CB-treated and control blastocysts in each hatching period. On the other hand, the rate of CB-treated blastocysts in which most trophectoderm cells possess the fluorescence of actin filaments did not differ from that of control blastocysts in the post-hatching period, whereas such the rates of CB-treated blastocysts in the pre-hatching and hatching periods (34.1 and 46.5 %) were significantly lower than the 95.0 and 82.9 % of control blastocysts, respectively. The CB-treated blastocysts in the pre-hatching and hatching periods had many trophectoderm cells devoid of the fluorescence of actin filaments.

From these findings, it was suggested that the polymerization of actin is essential for blastocyst hatching, and that actin filament-mediated movements of trophectoderm cells contribute to the hatching by facilitating the protrusion of trophectoderm cells from a small hole in the zona pellucida and also by enlarging the protrusion for slit formation in the zona pellucida.

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It is generally accepted that the hatching of mouse blastocysts is accompanied by regional dissolution of the zona pellucida by a trypsin-like proteinase synthesized in trophectoderm cells (Perona and Wassarman, 1986; Hogan *et al*., 1994), and trophectoderm cells protrude from the dissolved hole of the zona pellucida (Orsini and McLaren, 1967; McLaren, 1970; Niimura and Fujii, 1997). Then a slit is formed in the zona pellucida from the hole by enlargement of the protruding trophectoderm cells (Orsini and McLaren, 1967; McLaren, 1970; Niimura and Fujii, 1997). During hatching, the blastocyst repeats contractions, leading to the enlargement of the slit, and then escapes from the zona pellucida (Orsini and McLaren, 1967; McLaren, 1970; Niimura and Fujii, 1997).

Generally, it is known that most animal cell motility is induced by actin filaments (Mitchison and Cramer, 1996; Lodish *et al*., 2008). In blastocysts also it is considered that trophectoderm cell motility is involved in hatching through the action of actin filaments (Cheon *et al*., 1999; Niimura and Wakasa, 2001). That is, actin filaments are distributed abundantly in trophectoderm cells of blastocysts during the hatching period compared to before and after hatching periods, and are densely distributed especially in the peripheral cytoplasm of trophectoderm cells protruding from the zona pellucida (Cheon *et al*., 1999). Furthermore, in mouse blastocysts treated with cytochalasin B (CB), an inhibitor of actin polymerization, the distribution of actin filaments changes (Cheon *et al*., 1999) and changes also occurr in contractions that increase the protrusion of trophectoderm cells from the zona pellucida and the protruded trophectoderm (Niimura and Wakasa, 2001). As a result, hatching in such the blastocysts was inhibited. From the results about the distribution of actin filaments in mouse blastocysts treated with CB, Cheon et al. (1999) suggested that dynamic polymerization of actin molecules in trophectoderm cells is essential for blastocyst hatching, and that actin filament-mediated movements of trophectoderm cells play an important role in the hatching process of mouse blastocysts. However, there have been no observations on the distribution of actin filaments in CB-treated blastocysts in the pre-hatching and post-hatching periods, in order to determine the role of actin filaments in hatching process.

In the present study, the hatching rate and the distribution of actin filaments were examined in mouse

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blastocysts whose ability of the polymerization of actin had been suppressed by CB (Lin *et al*., 1980; Lodish *et al*., 2008), and were compared with those in blastocysts cultured in a medium without CB, in order to determine the role of actin filaments in blastocyst hatching.

MATERIALS AND METHODS

Animals

Eighty female mature mice of ICR strain were used in the present study. They were housed in autoclaved metal cages and were given a standard chow (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room (24 ℃), under controlled-lighting conditions (14L/10D; L: 0400 h to 1800 h). They received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Niigata University Animal Care Committee). These females were intraperitoneally injected with 5 i.u. of PMSG (Serotropin®, Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan), and with 5 i.u. of hCG (Gonatropin®, Teikoku Hormone Manufacturing Co. Ltd.) 48 hrs later to induce superovulation. Immediately after the hCG injection, these females were mated with mature males of the same strain.

Observation of hatching rate in cultured blastocysts

In order to observe the hatching rate in blastocysts, morulae were collected from oviducts and uteri of superovulated and mated females 72 hrs after the hCG injection, and were cultured in M16 medium (Whittingham, 1971) without CB or in M16 medium with 0.4 μg/ml CB (Sigma Chemical Co., MO, USA). These morulae were cultured in a CO_2 incubator (5 % CO_2 in air) at 37 ℃ . The CB had previously been dissolved in DMSO. The morulae cultured in M16 medium containing 0.2 % DMSO, but devoid of CB were observed as controls.

Development of morulae to blastocysts and completion of hatching in resultant blastocysts were observed after 24 and 100 hrs of culture, respectively.

Demonstration of actin filaments in cultured blastocysts

In order to observe the distribution of actin filaments, morulae were collected 72 hrs after the hCG injection and cultured in M16 medium without CB or in M16 medium with 0.4 μg/ml CB. These morulae were cultured in a CO_2 incubator (5 % CO_2 in air) at 37 ℃ . After culture, blastocysts at the stages of expanded (pre-hatching), hatching and post-hatching were collected and fixed in PBS (pH 7.4) (Dulbecco and Vogt, 1954) containing 3.7 % formaldehyde at room temperature for 30 min. Rinsed in a PBS, they were immersed in a PBS containing 0.25 % Tween-20 (Bio-Rad Laboratories, NY, USA) for 5 min at room temperature. Again rinsed in a PBS, the blastocysts were immersed in 100 μl PBS containing 16.5 ng phallacidin (Molecular Probes Inc., CA, USA) at room temperature for 20 min. The treated blastocyst was placed in the center of 4 vaseline spots on a slide. A cover slip was then carefully placed on the vaseline spots and pressed gently to anchor the embryos in between the cover slip and the slide. Observation was carried out under a reflected-light fluorescing microscope (OPTIPHOT, Nikon Corporation, Tokyo, Japan).

Statistical analysis

The hatching rate of blastocysts and the incidence of blastocysts in which most trophectoderm cells possess the fluorescence of actin filaments were statistically analyzed by Chi-square test or Fisher's exact probability test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

The hatching rates of cultured blastocysts

When morulae were cultured in a medium containing 0.4 μg/ml CB, 91.2 % (31/34) of the embryos developed to blastocysts, showing no difference from the developmental rate (100 %; 32/32) of control morulae cultured in a medium without CB. On the other hand, the hatching rate of blastocysts developed from morulae in a medium containing CB was 9.7 % (3/31), which was significantly lower than the 78.1 % (25/32) in control blastocysts developed from morulae in a medium without CB. From these results, it was clarified that the concentration of CB at 0.4 μg/ml did not affect the development of morulae to blastocysts and had inhibitory effect on hatching of resultant blastocysts.

Distribution of actin filaments in cultured blastocysts

The fluorescence showing the presence of actin filaments was observed in the cytoplasm of trophectoderm and inner-cell-mass cells in blastocysts, and was especially strong in the peripheral cytoplasm where two trophectoderm cells adhering to each other (**Fig.1a-d**). Also actin fluorescence was much brighter in protruded trophectoderm cells at the region of small hole or slit in the zona pellucida of hatching blastocysts (**Fig.1b,c**). Such the distribution of actin filaments was similar in CB-treated and control blastocysts in each hatching period.

On the other hand, the rates of CB-treated blastocysts in which most trophectoderm cells possess the fluorescence of actin filaments were different from those of control blastocysts. As shown in **Table 1**, the rate of blastocysts in which most trophectoderm cells possess the fluorescence of actin filaments was similar for CB-treated (75.0 %) and control blastocysts (92.0 %) in the post-hatching period (Fig.1d). In the pre-hatching and hatching periods, however, such the rates of CB-treated blastocysts (34.1 and 46.5 %) were significantly lower than the 95.0 and 82.9 % of control blastocysts, respectively, and CB-treated blastocysts in those periods had many trophectoderm cells devoid of the fluorescence of actin filaments (**Fig.1c**).

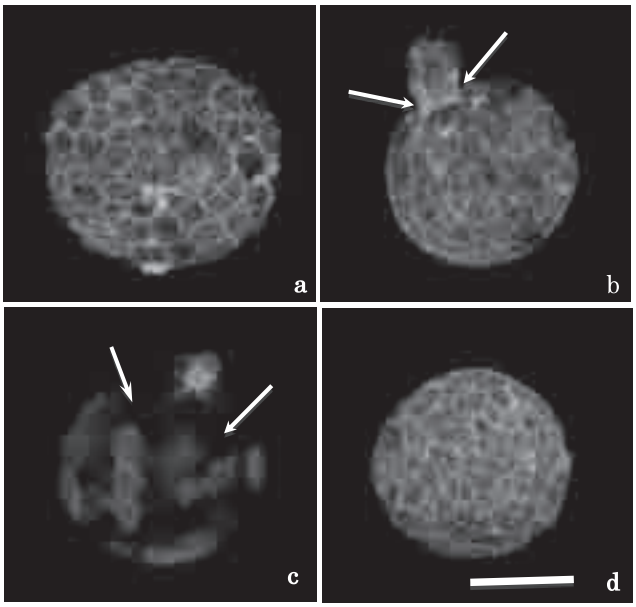

Fig.1 Fluorescent micrographs of mouse blastocysts developed from morulae in M16 medium without CB (a, b, d) and in M16 medium containing 0.4 μg/ml CB (c), and stained with phallacidin. Scale bar indicates 50 μm.

(a) Pre-hatching blastocyst. The fluorescence showing the presence of actin filaments is strong in the peripheral cytoplasm where two trophectoderm cells adhering to each other. (b) Hatching blastocyst. The fluorescence of actin filaments is much brighter in protruded trophectoderm cells (arrows) from zona pellucida. (c) Hatching blastocyst. The CB-treated blastocyst possesses many trophectoderm cells (arrows) devoid of the fluorescence of actin filaments. (d) Post-hatching blastocyst. The fluorescence of actin filaments is strong in the peripheral cytoplasm where two trophectoderm cells adhering to each other.

Table 1. The incidence of cultured mouse blastocysts in which most trophectoderm cells possess the fluorescence of actin filaments

Blastocysts	Periods of hatching		
	Pre-hatching	Hatching	Post-hatching
Control	95.0 (38/40)*[a]	82.9 (34/41)[a]	92.0 (23/25)[a]
CB-treated	34.1 (15/44)[b]	46.5 (20/43)[b]	75.0 (6/ 8)[a]

* The percentage of blastocysts with numbers in parentheses. Blastocysts observed were developed from morulae in M16 medium containing 0.4 μg/ml CB (CB-treated) or in M16 medium without CB (control).
Values with different superscripts in the same column are significantly different ($P<0.05$).

DISCUSSION

Recently, it has been suggested that actin filament-mediated movements of trophectoderm cells play an important role in the hatching process of mouse blastocysts (Cheon *et al*., 1999; Niimura and Wakasa, 2001). That is, Cheon et al. (1999) have reported that the number of actin filaments increased in blastocysts during hatching, compared with those before and after hatching, and the filaments were particularly densely localized in the cortical cytoplasm of trophectoderm cells that protruded from the zona pellucida, and that the blastocyst treated with CB had a different pattern of distribution of actin filaments. From these results, they suggested that dynamic polymerization of actin molecules in trophectoderm cells is essential for blastocyst hatching, and that actin filament-mediated movements of trophectoderm cells play a crucial role in the hatching process of mouse blastocysts. Nevertheless, there have been no observations on the distribution of actin filaments in CB-treated blastocysts in the pre-hatching and post-hatching periods, in order to determine the role of actin filaments in hatching process.

In the present study, it was clarified that the rate of CB-treated blastocysts completing hatching was significantly lower than that of control blastocysts, and that the rates of CB-treated blastocysts in which most trophectoderm cells possess the fluorescence of actin filaments were significantly lower than those of control blastocysts in pre-hatching and hatching periods. From these findings, it was considered that in blastocysts in which the polymerization of actin has been inhibited, actin filament-mediated movements of trophectoderm cells required to progress the hatching process were inhibited, and as a result, hatching could not be completed. Therefore, former findings with regards to the hatching process using CB-treated blastocysts (Niimura and Wakasa, 2001) and with regards to the distribution of actin filaments (Cheon *et al*., 1999), together with the results of the present study, strongly suggested that actin filament-mediated movements of trophectoderm cells contribute to the hatching by facilitating the protrusion of trophectoderm cells from a small hole in the zona pellucida and also by enlarging the protrusion for slit formation in the zona pellucida.

Cheon et al. (1999) have reported that mouse embryos at the stages from morula to blastocyst stopped developing when cultured with CB at 5 μg/ml for 12 or 24 hrs, but these embryos resumed development and escaped from the zona pellucida when they were transferred to and continuously cultured in a medium without CB. On the other hand, we have reported that the optimal concentration of CB was 0.4 μg/ml because CB did not affect the development of morulae to blastocysts and had its maximum inhibitory effect on hatching of resultant blastocysts at this concentration (Niimura and Wakasa, 2001). Therefore, the reason for no inhibition of the development of CB-treated morulae in the present study was thought to be that the concentration of CB used in the present study was suitable.

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サイトカラシンＢ処置したマウス胚盤胞におけるハッチングとアクチンフィラメントの分布

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要　約

サイトカラシンＢ（CB）で処置してアクチンの重合を抑制したマウス胚盤胞について、ハッチング能とアクチンフィラメントの分布を調べ、胚盤胞ハッチングに果たすアクチンフィラメントの役割を検討した。

0.4μg/mlのCBを含む培養液で桑実胚から発生した胚盤胞のハッチング率は9.7％であり、対照のCBを含まない培養液で発生した胚盤胞の78.1％に比べ、有意に低かった。

アクチンフィラメントの存在を示す特異蛍光は、栄養膜細胞と内細胞塊細胞の細胞質にみられ、栄養膜細胞同士が接する部位の細胞膜直下の細胞質で特に強かった。また、ハッチング中の胚盤胞において、この蛍光は透明帯の小孔から突出した栄養膜細胞および透明帯の裂け目付近の栄養膜細胞で特に強かった。このようなアクチンフィラメントの分布は、CB処置した胚盤胞と対照の胚盤胞の間で相違なかった。

また、ほとんどの栄養膜細胞がアクチンフィラメントの存在を示す特異蛍光を有している胚盤胞の割合は、ハッチング後では、CB処置したものと対照のものとの間で相違なく、それぞれ75.0％と92.0％であった。一方、ハッチング前とハッチング中の期間において、ほとんどの栄養膜細胞がアクチンフィラメントの存在を示す特異蛍光を有しているCB処置胚盤胞の割合は、それぞれ34.1および46.5％であり、対照の胚盤胞の95.0および82.9％に比べて有意に低かった。

以上のように、CBで処置してアクチンの重合を抑制したマウス胚盤胞では、ハッチング前とハッチング中にアクチンフィラメントを欠く栄養膜細胞が多数出現したために、このような胚盤胞ではハッチングの開始と完了に不可欠な透明帯での小孔形成と裂け目形成に必要な栄養膜細胞の運動が阻害され、結果としてハッチング能が低下したものと思われた。従って、アクチンフィラメントを介した栄養膜細胞の運動は、胚盤胞のハッチング過程の進行に重要な役割を果たしていることが考えられた。

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キーワード：アクチンフィラメント、胚盤胞ハッチング、サイトカラシンB、ファラシジン

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