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The Cytopathic Effects of Leptospira and Leptonema spp. on Various Tissue Cultured Cells

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Abstract The interaction of 18 strains of *Leptospira* (2 species, 9 serovars) and a strain of *Leptonema illini* with 6 tissue cultured cell lines (3 epithelial and 3 fibroblastic) during leptospiral replication was investigated.

Cytopathic effects were observed not only on fibroblastic cells but also on epithelial cells infected with leptospires. In addition to most strains of *Leptospira interrogans, Leptospira biflexa* and *L. illini* produced cytopathic effects. Thus the cytopathic effect caused by leptospires on cultured cells *in vitro* is not necessarily associated with the virulence *in vivo*. The leptospires of *L. interrogans* serovar *icterohaemorrhagiae* strain RGA produced cytopathic effect without entering the cells. On the other hand, strain Pyrogenes of *L. interrogans* serovar *pyrogenes* and strain Akiyami A of serovar *autumnalis* efficiently attached to and entered Sf1Ep cells, although they did not produce any cytopathic effect. These results suggest that the cytopathic effects of some pathogenic leptospires are caused by extracellular toxic substances produced by the microorganisms.

Key Words : *Leptospira, Leptonema,* Spirochetes, Tissue culture, Cytopathic effect

Introduction

Leptospirosis is an acute febrile disease caused by bacteria of the genus *Leptospira,* and is primarily classified as a zoonosis. The disease exhibits worldwide distribution and presents medical and veterinary problems.

Pathogenic leptospires produced deleterious effects on various tissues *in vivo* in man and animals[1,2]. Since cell cultures have been found to be useful in studying the interaction between pathogenic bacteria and tissue cells, several investigators have applied this technique to leptospires. Miller et al.[3] reported that a virulent strain of *Leptospira interrogans* serovar *pomona* produced a much greater cytopathic effect on mouse fibroblastic L cells than an avirulent strain of the same serovar. Cytopathic factors were found in the culture supernatant of a virulent strain of *pomona*[4] and of a virulent strain of *L. interrogans* serovar *copenhageni*[5]. Miller et al.[3] also described that the saprophytic *Leptospira biflexa* never produced cytopathic effects on L cells. There are contradictory reports, however, that the saprophytic leptospires also produced cytopathic effects on cultured cells[6-8]. Thus the significance of cytopathic effects of saprophytic *L. biflexa* has remained to be clarified. In addition, there is an apparent contradiction on the nature of cells which is attacked by leptospires. Miller et al.[9] reported that the cytopathic effect of a virulent strain of *pomona* occured

only on fibroblastic cells but not on fetal bovine kidney epithelial cells. Similarly, Knight et al.[10] reported a factor which is toxic to fibroblastic L cells but not to epithelial HeLa cells in the plasma of hamsters infected with leptospires. On the other hand, *in vivo* experiments of Marshall[11] showed that degeneration and necrosis of epithelial cells occured in the proximal tubules in the sheep kidney infected with leptospires.

In this paper, I describe the cytopathic effect of 18 strains of *Leptospira* and 1 strain of *Leptonema* on 6 cultured cell lines including 3 epithelial cell lines. The aim of this study was to determine i) whether only pathogenic strains produce a cytopathic effect on cultured cells, ii) whether only fibroblastic cells were attacked by leptospires, and iii) whether leptospires need to attach to and enter the cells for producing a cytopathic effect.

Materials and Methods

Leptospiral strains. Strains of *Leptospira interrogans* were provided by J. Sugiyama, Denka Seiken, Co. LTD., Tokyo. Among them, serovar *autumnalis* strain I192 and I201 and serovar *hebdomadis* strain I203 were isolated in Okinawa. All strains of *L. interrogans* primarily isolated as pathogenic species and kept by successive passages in Korthof medium for more than 10 years might have become avirulent, since the leptospires are known to lose virulence under such conditions. *Leptospira biflexa* strain Urawa was obtained from National Institute of Health, Tokyo, and *Leptonema illini*, former *Leptospira illini*[12], strain 3055 was provided by R. Yanagawa, Hokkaido University, Sapporo. The latter two strains are saprophytic and non-pathogenic. All the organisms were grown in Korthof medium at 30℃ for 7 days.

Cells. Epithelial cell lines Sf1Ep (a cottontail rabbit epidermis cell line, ATCC CCL-68) and Chang liver (an adult human liver cell line, ATCC CCL-13) were obtained from Flow Laboratories, Inc., Mclean, Va., USA., and C2 (a rat liver carcinoma cell line) from T. Fukumoto, Department of Anatomy, Yamaguchi University School of Medicine, Ube. Fibroblastic cell lines BHK-21 (a baby hamster kidney cell line) and Balb/C (a Balb/3T3 mouse cell line) were provided by S. Hotta, Kanazawa Medical School, Kanazawa, and HEL (a human embryonic lung cell line) by A. Iwasaki, Yamaguchi Prefectural Research Institute of Health, Yamaguchi. Cells were cultivated in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) in an atomosphere of 5% CO_2 in air at 34℃. For the passage, confluent monolayers were dispersed with 0.1% trypsin and 0.02% versene in calcium- and magnesium-free phosphate buffered saline (PBS). No antibiotics were added to the culture.

Cultivation of leptospires on cell monolayers and examination of cytopathic effects. To obtain confluent monolayers, $4x10^4$ to $8x10^4$ cells were seeded in a well of a tissue culture tray (16 mm in diameter, Becton Dickinson and Company, New Jersey, USA) and incubated for 2 to 5 days. Cell monolayers washed three times with Eagle's MEM were inoculated with approximately 10^6 leptospires, and incubated in an atomosphere of 5% CO_2 in air at 34℃. The growth of leptospires was determined 3, 6 and 14 days after inoculation by counting the organisms in culture fluid with a Thoma-haemocytometer with a 0.02 mm-depth chamber under dark field microscopy. Controls were handled similarly without cultured cells. For determination of cytopathic effects, cells were challenged with 0.1 ml of the inoculum containing approximately $2x10^7$ leptospires grown in Korthof medium, and incubated in CO_2 incubator (5% CO_2 in air, 34℃) for 10 days. The cytopathic effects on cultured cells were examined every day under phase contrast microscopy.

Attachment of leptospires to Sf1Ep cells. Attachment of leptospires to Sf1Ep cells was determined according to Tsuchimoto et al.[13]. In brief, Sf1Ep cells were grown on glass cover slips (15 mm in diameter) in wells of tissue culture trays to approximately 30% confluence and washed three times with Eagle's MEM. Each culture received 1 ml of a leptospiral suspension containing $2x10^7$ cells per ml in Eagle's MEM with 10% FCS and incubated for 1hr in 5% CO_2 in air at 34℃. Cover slips were taken from the wells, gently rinsed 10 times with PBS to remove non-adhering leptospires, and placed on a glass slide with the cell side downward. Preparation were examined under dark field microscopy. The number of adherent leptospires was determined by counting 20 cells.

Leptospires within Sf1Ep cells. Leptospires

were inoculated on Sf1Ep cell monolayers on cover slips (approximately $2x10^7$ organisms/well) and incubated for 3 or 9 days. After extensive washing of cover slips, cells were fixed in 10% formalin, stained by the modified Steiner and Steiner technique[14], and the number of intracellular organisms was determined by counting 20 cells, using a bright field and oil immersion x100 objective.

Electron microscopy. Sf1Ep cells which were grown on cover slips treated with Teflon were cocultivated with leptospires for 3 days as described above. The cells were observed under electron microscopy by the methods of vertical ultra-thin sectioning as described by Konishi et al.[15]

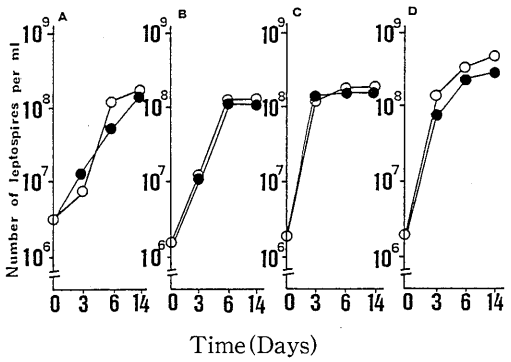

Fig. 1. Growth of leptospires cocultivated with (○) or without (●) Sf1Ep cells. A ; *L. interrogans* serovar *copenhageni* strain Shibaura, B ; *L. interrogans* serovar *canicola* strain Hond Utrecht IV, C ; *L. biflexa* strain Urawa, D ; *L. illini* strain 3055.

Results

Growth of Leptospires with Cultured Cells

The growth of leptospires cocultivated with or without Sf1Ep cells was investigated (Fig.1). *L. interrogans* serovar *copenhageni* strain Shibaura and serovar *canicola* strain Hond Utrecht IV reached to the maximal growth of approximately 10^8 organisms per ml after 6 days. On the other hand, saprophytic strains, *L. biflexa* strain Urawa and *L. illini* strain 3055, reached to the maximal growth of 10^8 organisms per ml within 3 days. Other 15 strains of *L. interrogans* showed the growth comparable to that of Shibaura and Hond Utrecht IV.

Cytopathic Effects of Leptospires on Cultured Cells

The interaction of *Leptospira interrogans* (17 strains), *L. biflexa* (one strain) and *L. illini* (one strain) with 6 tissue cultured cell lines during leptospiral replication was investigated (Table 1).

Most leptospires cocultivated with Sf1Ep cells showed a normal spiral shape and moved freely in the culture medium, whereas some organisms appeared to attach to the surface or exist within cells. When the incubation progressed, monolayer cells started to degenerate and eventually detached from culture plates. Because nonspecific degeneration was observed after 10 days of incubation on the BHK-21 cells and Balb/C cells unchallenged with leptospires, the cytopathic effect was determined after 7 days cocultivation on these cell lines. In contrast, unchallenged cells of other 4 cell lines remained intact during 10 days incubation. Therefore the cytopathic effects on these cell lines were determined after cocultivation for 10 days.

Cytopathic effects of leptospires on cultured cells varied in leptospiral strains and cell lines. Among the 19 strains tested, strain Hond Utrecht IV of *L. interrogans* serovar *canicola*, and saprophytic leptospires, *L. biflexa* and *L. illini*, produced the strongest cytopathic effects on all cell lines. In particular, *L. biflexa* and *L. illini* produced the cytopathic effects as early as 3 days after inoculation, whereas strain Hond Utrecht IV produced the cytopathic effect after 7 days of incubation (data not shown). Strain Shibaura of *L. interrogans* serovar *copenhageni* also produced a strong cytopathic effect on most of the cell lines. On the other hand, strain I201 of *L. interrogans* serovar *autumnalis* did not produce a significant cytopathic effect on either cell lines.

Cytopathic effects of leptospires were observed not only on fibroblastic cell lines but also on epithelial cell lines. Among 6 cell lines, epithelial C2 cells were most susceptible to leptospires, being affected by 16 strains out of 19 leptospiral strains. Epithelial

Table 1 The interaction between leptospires and cultured cells.

Leptospiral strain	CPE[a]						Attachment to	Intracellularity to Sf1Ep cells[c]	
	Epithelial			Fibroblastic					
	Sf1Ep	Chang liver	C2	BHK-21*	HEL	Balb/C*	Sf1Ep cells[b]	Normal spiral form	Granular form
L. interrogans									
icterohaemorrhagiae									
Uchida	-	-	2+	-	-	-	-	-	-
Yokohama	-	-	2+	-	-	-	1+	1+	1+
Mikawashima	-	-	3+	-	-	3+	-	1+	-
Mano	1+~4+	2+	4+	-	3+	-	1+	1+	-
Endoh	-	-	1+	1+	-	3+	1+	-	1+
RGA	4+	-	3+	1+	1+	3+	1+	-	-
copenhageni									
M20	1+	-	4+	-	-	-	1+	1+	-
Shibaura	4+	3+	1+~4+	4+	2+	1+	1+	2+	-
canicola									
Hond Utrecht IV	4+	4+	4+	4+	4+	4+	-	1+	2+
autumnalis									
Akiyami A	-	-	2+	-	-	1+	1+	2+	2+
I192	-	-	-	-	-	3+	-	1+	2+
I201	-	-	-	-	-	-	-	1+	-
hebdomadis									
Akiyami B	-	-	1+	-	-	-	1+	1+	1+
I203	-	-	-	-	-	1+	1+	1+	1+
australis									
Akiyami C	-	1+	3+	-	-	2+	-	1+	1+
bataviae									
Bataviae	-	-	1+	-	-	-	-	1+	1+~2+
pyrogenes									
Pyrogenes	-	-	1+	-	-	-	2+	2+	2+
L. biflexa									
Urawa	4+	4+	4+	4+	4+	4+	2+	2+	2+
L. illini									
3055	4+	4+	4+	4+	4+	4+	2+	2+	2+

a) The cytopathic effect (CPE) was determined after cocultivation for 7* or 10 days. - : less than 25% of cells were damaged, + : 25 to 50% of cells were damaged, 3+ : more than 75% of cells were damaged, 4+ : all of cells were damaged.

b) The attachment was determined after incubation for 1hr. - : less than one leptospire attached to a cell, 1+ : one to ten leptospires attached to a cell, 2+ : more than ten leptospires attached to a cell.

c) The intracellularity was determined after cocultivation for 9 days. - : less than one leptospire entered a cell, 1+ : one to ten leptospires entered a cell, 2+ : more than ten leptospires entered a cell.

Sf1Ep and Chang liver cells as well as fibroblastic HEL cells were affected by 6 or 7 leptospiral strains which showed cytopathic effects on the rest of cell lines. The infected Sf1Ep cells were swollen and detached from the culture plate (Fig. 2). Similar cytopathic effects were observed on fibroblastic cells.

Attachment to Sf1Ep Cells

Sf1Ep cells were used to determine the relationship between the cytotoxicity and attachment of individual strains (Table 1). After incubation for 1hr, the leptospires of *L. interrogans* serovar *pyrogenes*, *L. biflexa*, and *L. illini* attached efficiently to the cells. Strain Pyrogenes did not produce any cytopathic effect, whereas the saprophytic leptospires produced strong cytopathic effects. Leptospires of strain Yokohama, Mano, Endoh, and RGA of serovar *icterohaemorrhagiae*, strain M20 and Shibaura of serovar *copenhageni*, strain Akiyami A of serovar *autumnalis*, strain Akiyami B and I203 of serovar *hebdomadis* also attached but

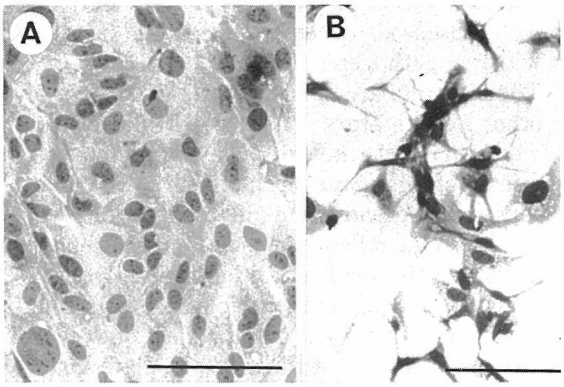

Fig. 2. (A) Control Sf1Ep cells showing a typical confluent monolayer. (B) Sf1Ep cells at 7 days after cocultivation with strain Shibaura of *L. interrogans* serovar *copenhageni* showing cytopathic effect. Giemsa stain. Bar 100μm.

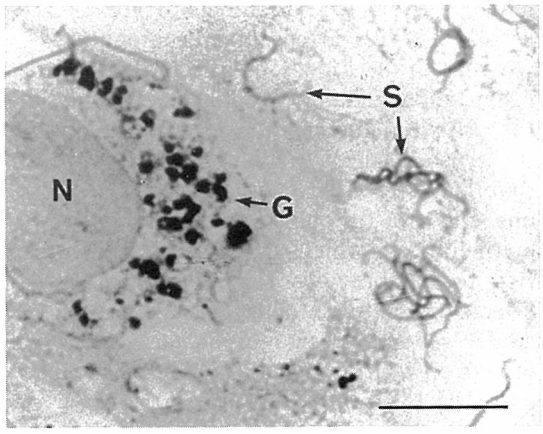

Fig. 3. Two types of leptospires found in the cytoplasm of a Sf1Ep cell at 9 days after cocultivation with strain Pyrogenes of *L. interrogans* serovar *pyrogenes*. Notice normal spiral form (S) and granular form (G) of leptospires around the nucleus (N). Silver stain. Bar 10μm.

less efficiently. Among these, strain Mano, RGA, and Shibaura produced strong cytopathic effects, whereas strains Yokohama, Akiyami A, Akiyami B and I203 did not produce any cytopathic effect. On the other hand, Strain Hond Utrecht IV of serovar *canicola* did not show attachment, although it produced a strong cytopathic

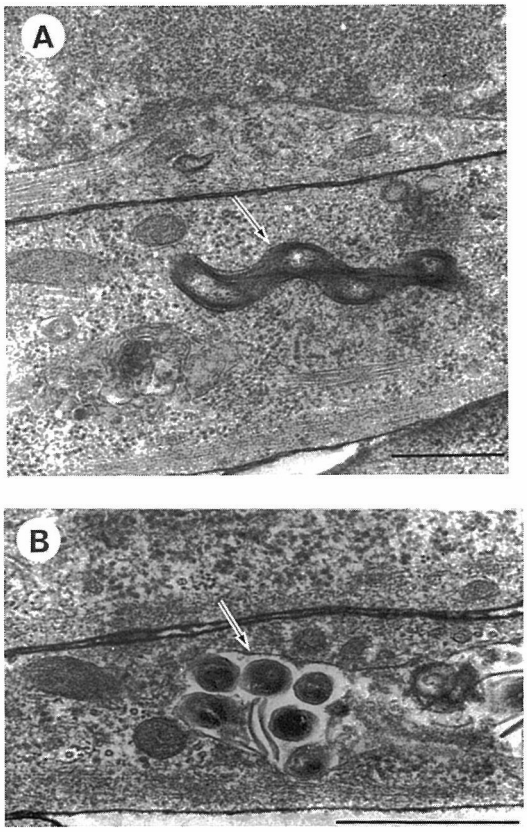

Fig. 4. Electron micrograph showing two types of leptospires (arrow) in the cytoplasm of the Sf1Ep cells at 3 days after cocultivation with strain 3055 of *L. illini*. (A) Normal spiral form. (B) Granular form. Bar 0.5μm.

effect. Other leptospires neither attached to the cells nor produced any cytopathic effect under the conditions tested.

Leptospires within the Sf1Ep Cells

Fig.3 and 4 are typical of what was observed in specimens. They show that leptospires existed within the cells, and that the intracellular organisms displayed two forms in morphology. One was "normal spiral form" (Fig. 3 ; S, Fig, 4-A), and the other was the " granular form" in which the organisms were folded (Fig. 3 ; G, Fig. 4-B). All the strains except strains Uchida and RGA of *L. interrogans* serovar *icterohaemorrhagiae* showed internalization to Sf1Ep cells after 9 days of cocultivation (Table 1).

The ratio of intracellular leptospires of the "normal spiral form" and the "granular form" after 9 days of incubation was varied in leptospiral strains (Table 1). Numbers of the "normal spiral form" and the "granular form" were approximately equal in strain Akiyami A of serovar *autumnalis* and the strains of serovar *pyrogenes, L. biflexa*, and *L. illini*. In contrast, the "normal spiral form" was predominant in strain Shibaura of serovar *copenhageni*, whereas the "granular form" was predominant in strain Hond Utrecht IV of serovar *canicola*. It should be noted that the intracellular leptospires of the "normal spiral form" of strain Shibaura of serovar *copenhageni* and strain Pyrogenes of serovar *pyrogenes* appeared as early as 3 days after inoculation (data not shown). Saprophytic leptospires, *L. biflexa* and *L. illini*, also appeared within the cells after 3 days. At this time, the leptospires reached to the maximal growth and showed cytopathic effects.

The leptospires of serovar *pyrogenes* strain Pyrogenes and serovar *autumnalis* strain Akiyami A attached to and entered into Sf1Ep cells, although it did not produce any cytopathic effect. On the other hand, the organisms of strain Hond Utrecht IV did not attach to the Sf1Ep cells, although it entered and produced a significant cytopathic effect. Among 17 strains of *L. interrogans*, only strain Shibaura of serovar *copenhageni* showed strong cytotoxicity associated with the ability of attachment and intracellularity.

Discussion

In leptospirosis, liver and kidney are the common targets of leptospires. However, the relationship between the pathological changes of the organs *in vivo* and the cytotoxicity to cultured cells is not fully understood. I examined the interaction of 18 strains of *Leptospira* and a strain of *Leptonema* with 6 cell lines.

In the present study, several strains of *L. interrogans*, which have become avirulent by successive passages *in vitro*, produced various degrees of cytopathic effects. Even in the same serovar of *L. interrogans*, some strains caused cytopathic effects whereas others did not. In addition, significant cytopathic effects were observed on the cultured cells infected with nonpathogenic *L. biflexa* and *L. illini*. These results are in accordance with those of other investigators[6,7,16] except Miller et al.[3] who reported the absence of cytopathic effects of *L. biflexa*. Such a discrepancy may represent a variation in cytotoxicity of strains of *L. biflexa*. Several factors seem to involve in the virulence of leptospires *in vivo*. These findings of cytopathic effects on tissue cultured cells *in vitro* might reflect one of the factors which is not directly associated with the establishment of infection.

In human and animals suffered from leptospirosis remarkable pathological changes are seen in liver and kidney consisting of focal necrosis, separation of hepatocytes from hepatic cords, and interstitial nephritis[17]. In the liver of infected hamsters, leptospires exist between hepatocytes or are associated with degenerated hepatocytes[17,18], whereas in the kidney of infected sheep, the organisms are mainly around the periphery of the proximal tubules and intermingled with the brush border[11,17]. These findings suggest that leptospires probably have the affinity not only to fibroblastic cells but also to epithelial cells which is in apparent contradiction to the previous reports on cytopathic effects to cultured cells[9,10]. In the present study, several strains produced cytopathic effects on both fibroblastic (BHK-21, Balb/C and HEL) and epithelial (Sf1Ep, Chang liver and C2) cells. These results suggest that the cytopathic effects of pathogenic strains play an important role in pathological changes of epithelial cells *in vivo*.

Previous studies on the leptospiral attachment to cultured cells have shown that virulent strains of *L. interrogans* attached more efficiently than avirulent strains[7,13,19], whereas the saprophytic *L. biflexa* attached not only to cultured cells but also to glass surfaces[13]. The relationship between leptospiral attachment and cytopathic effects, however, has not been established. In the present study, leptospires of strain Hond Utrecht IV of serovar *canicola* produced strong cytopathic effects without any attachment, whereas those of strain of Pyrogenes of

serovar *pyrogenes* attached efficiently without showing any cytopathic effect. *L. biflexa* and *L. illini* attached efficiently to Sf1Ep cells and produced extensive cytopathic effects. Thus cytopathic effects and attachment of *L. interrogans* were not associated with each other.

After several days of cocultivation, leptospires were observed in cultured cells with two types of morphology. The "granular form" might be in the stage of latency within the cell vacuole as suggested by Czekalowski et al.[20]. The "normal spiral form" may be in the active stage, since it showed active movement within the cytoplasm. Fitzgerald et al.[21] observed that the ability of attachment of *Treponema pallidum* to cultured cells correlated to intracellularity of the organisms. Such correlation appears to be not the case in leptospires. Internalization of leptospires of serovar *icterohaemorrhagiae* strain RGA was not observed, although the organisms efficiently attached to the cultured cells. On the other hand, the leptospires of serovar *canicola* strain Hond Utrecht IV, serovar *autumnalis* strain I192, and serovar *bataviae* strain Bataviae showed efficient internalization without attachment.

Several factors have been reported to be involved in the cytopathic effects of leptospires. They include extracellular products produced by virulent strains of pathogenic leptospires (cytotoxic factors)[4,5], common components of leptospires including saprophytic leptospires[8], and direct trauma to the cells by the active movement of intracellular leptospires[22]. The cytopathic effects of pathogenic strains to cultured cells could not be attributable simply to physical trauma, since leptospires such as strain Pyrogenes of serovar *pyrogenes* entered the cells efficiently without showing any cytopathic effect, whereas strain RGA of serovar *icterohaemorrhagiae* produced a significant cytopathic effect without internalization. Similarly, participation of common leptospiral components reported by Vinh et al.[8] appears to be unlikely, since cytopathic effects on cultured cells varied in leptospiral strains. In addition, it is known that some strains of leptospires produce hemolysin which may contribute to the virulence *in vivo*[23]. Hemolysin may contribute also to the cytotoxicity on the cultured cells reported in the present study.

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