TECHNICAL NOTE

The value of LYM-1 cells for examining vacuole formation and loss of cell viability induced by culture supernates of *Helicobacter pylori*

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Some strains of *Helicobacter pylori* are known to produce an extracellular cytotoxin that causes vacuolation in cultured mammalian cells. Screening for such strains makes use of HeLa cells which may not be sensitive enough to detect minimal changes. The aim of this study was to develop a more sensitive cell line. Vacuole formation was examined in HeLa cells, as well as four other cell lines established in this laboratory by ammonium chloride induction. Among five cell lines tested, LYM-1 cells were most sensitive for the detection of intracellular vacuolation with this agent. Loss of cell viability of LYM-1 and HeLa cells induced by *H. pylori* culture supernates was also examined: LYM-1 were more sensitive than HeLa cells. Cell death was not always accompanied by vacuole formation. This suggests that the mechanism whereby cell death occurs must be different from that for vacuole formation. LYM-1 cells may be useful when measuring vacuole formation and cell death of the cultured cells induced by culture supernates of clinical isolates of *H. pylori*.

Introduction

Some strains of *Helicobacter pylori* are known to produce an extracellular cytotoxin that causes vacuolation in various mammalian cells [1, 2]. Recently, a protein isolated from *H. pylori* culture supernates has been shown to cause vacuolation *in vivo* [3]. This activity of *H. pylori* may play a major role in the development of gastritis and peptic ulcer disease [4]. The gene encoding this protein (*vacA*) [2, 3, 5] and the cytotoxin-associated protein (*cagA*) [6, 7] have been cloned and sequenced, although the involvement of *cagA* in vacuolation is still under discussion [8, 9]. Vacuole formation induced by *H. pylori* supernates is activated *via* an autophagic mechanism [10, 11]. HeLa cells are often used for examining vacuolating cytotoxin activity, but may not be sensitive enough to detect minimal changes.

In this study, a more sensitive cell line was developed to examine the vacuolating cytotoxin activity of *H. pylori*, and quantify cell death of the cultured cells induced by clinical isolates of *H. pylori*.

Materials and methods

Cells

The following cell lines were used: HeLa (derived from human cervical carcinoma), HuL-1 (derived from normal fetal human liver), M (derived from normal rat liver), MK (derived from cynomolgus monkey kidney) and LYM-1 (derived from rat lymph nodes).

Measurement of vacuole formation and cell death of cultured cells

For cell culture, 24-well microtitration plates were used. The cells were cultured together with various concentrations of ammonium chloride or the *H. pylori* culture supernates. Cells were incubated for 5 days at 37°C in air with CO₂ 5%. The cultured cells were then fixed with methanol, Giemsa stained, and were observed histologically.

The extent of cell death was classified into two groups in the screening cell line, with ammonium chloride, (–, 100% alive; +, presence of cell death) and into four groups when examining *H. pylori* culture supernates (–, 100% alive; +, 25% dead; 2+, 26–49% dead; 3+, > 50% dead).

The severity of vacuole formation was classified into

two groups. (–, no vacuole formation; and +, vacuole formation present).

Tissue culture and reagents

The growth medium was DM-201 supplemented with heat inactivated fetal bovine serum (Gibco, NY, USA) 2%. Ammonium chloride (WAKO, Osaka, Japan) was used to induce vacuole formation. Various solutions of ammonium chloride were prepared after filter sterilisation of a stock solution (50 mg/ml) stored for the various experiments.

H. pylori culture supernates

All *H. pylori* strains were isolated from gastric biopsy samples taken from patients at Jichi Medical School

Hospital. *H. pylori* was cultured on Brucella agar (Difco, Detroit, USA) and then transferred to Brucella Broth (Difco) containing heat inactivated horse serum (JRH, Tokyo, Japan) and incubated at 37°C for up to 3 days in a micro-aerobic atmosphere. The *H. pylori* culture supernates were collected by centrifugation and sterilised by filtration (0.22 µm pores). The supernate of a *vac A* gene-positive strain of *H. pylori* was selected for vacuolation experiments and was used at final concentrations of 1 in 4, 8, 16 and 32. DM-201 medium was used for making dilutions of the supernates.

DNA preparation and PCR detection the vacA gene

After culture in Brucella broth, *H. pylori* cells were collected by centrifugation. The pellet was suspended

Fig. 1. Ammonium chloride-induced vacuole formation in LYM-1 cells. **A**, control; **B**, 3 h after addition of ammonium chloride 500 µg/ml. Bars, 20 µm.

in TE buffer (pH 8.0) and digested with proteinase K (Gibco) and sodium dodecyl sulphate (SDS) 0.5% for 1 h at 37°C. The solution was then treated with NaCl. DNA was purified by phenol, phenol–chloroform extraction and ethanol precipitation, and resuspended in 100 µl of double distilled H₂O; 10 µl of DNA samples were used for PCR. The primers for amplification of the *vacA* gene were synthesised according to the method of Telford *et al.* [3]. The primers used had the following sequences: 5'-CCGAAGAAGCCAA-TAAAACCCAG-3' and 5'-CAAAGTCAAAACCG-TAGAGCTGGC-3'. The amplification cycle consisted of an initial denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and extension at 72°C for 1 min. The final cycle included an extension of the product. Samples were amplified through 20 consecutive cycles with Vent DNA polymerase (New England Biolabs, Inc., MA, USA). Samples (10 µl) of the PCR products were analysed by electrophoresis in agarose 1.4% gels (Funakoshi Co. Ltd., Tokyo, Japan) containing ethidium bromide 1 ml/L.

Results

Sensitivity of the cultured cells for the detection of vacuole formation induced by ammonium chloride

Five-day-old cell cultures were examined microscopically to measure the sensitivity of five cell lines for the detection of vacuole formation by ammonium chloride. Vacuole formation was observed only in LYM-1 cells with NH₄Cl 500 µg/ml (Fig. 1).

*Vacuole formation induced by *H. pylori* culture supernates*

Vacuole formation induced by *H. pylori* culture supernates with HeLa cells and LYM-1 cells was compared. When culture supernates of 10 strains of *H. pylori* shown to be positive for the *vacA* gene by PCR were tested at a final dilution of 1 in 4, vacuole formation in HeLa cells was induced by 20% of the *H. pylori* culture supernates. On the other hand, vacuole formation in LYM-1 cells was induced by all 10 supernates. In terms of vacuole formation, each *H. pylori* culture supernate showed different degrees of activity with these cells. The supernates could be classified into two groups (Table 1) where: (1) vacuole formation was found even at a dilution of 1 in 16; (2) vacuole formation was found only at a concentration of 1 in 4.

*Cell death induced by *H. pylori* culture supernates*

Cell death occurred in each cell line but to a much lesser extent in HeLa cells, and no clear differences

Table 1. Vacuole formation and loss of viability of HeLa cells and LYM-1 cells at various concentrations of *H. pylori* culture supernates

Isolate No.	Dilution of supernates	HeLa		LYM-1	
		Loss of viability*	Vacuolation†	Loss of viability*	Vacuolation†
1	1 in 4	+	—	3+	+
	1 in 8	+	—	3+	+
	1 in 16	—	—	+	—
	1 in 32	—	—	+	—
2	1 in 4	2+	—	3+	+
	1 in 8	+	—	2+	+
	1 in 16	+	—	+	—
	1 in 32	—	—	+	—
3	1 in 4	+	—	3+	+
	1 in 8	+	—	2+	+
	1 in 16	+	—	+	—
	1 in 32	—	—	+	—
4	1 in 4	+	—	3+	+
	1 in 8	+	—	2+	+
	1 in 16	+	—	+	—
	1 in 32	+	—	+	—
5	1 in 4	+	+	2+	+
	1 in 8	+	—	+	—
	1 in 16	—	—	—	—
	1 in 32	—	—	—	—
6	1 in 4	+	—	—	+
	1 in 8	+	—	—	—
	1 in 16	—	—	—	—
	1 in 32	—	—	—	—
7	1 in 4	+	—	—	+
	1 in 8	+	—	—	—
	1 in 16	+	—	—	—
	1 in 32	—	—	—	—
8	1 in 4	+	—	3+	+
	1 in 8	+	—	3+	+
	1 in 16	+	—	3+	+
	1 in 32	+	—	3+	+
9	1 in 4	+	—	+	+
	1 in 8	—	—	—	—
	1 in 16	—	—	—	—
	1 in 32	—	—	—	—
10	1 in 4	2+	+	3+	+
	1 in 8	+	—	3+	+
	1 in 16	—	—	3+	+
	1 in 32	—	—	3+	+

* —, 100% alive; +, 25% dead; 2+, 26–50% dead; 3+, >50% dead.

† —, absence of vacuole formation; +, presence of vacuole formation.

were found among the 10 *H. pylori* culture supernates. On the other hand, the degree of cell death in LYM-1 cells differed among the culture supernates of *H. pylori*. Regarding cell death of the LYM-1 cells, the supernates could be classified into three groups (Table 1) where: (1) no cell death was found even by the addition of supernate at 1 in 4 dilution; (2) some cell death was found depending on the concentration of the supernates; and (3) extensive cell death was found even at a 1 in 16 dilution.

For two strains (nos. 6 and 7), the results of cell death were discordant with those for vacuole formation (vacuole formation without cell death) (Fig. 2).

Fig. 2. Effect of *H. pylori* culture supernates on cell death and vacuole formation in LYM-1 cells at a final dilution of 1 in 4 of *H. pylori* culture supernates. **A**, no cell death, vacuole formation present; **B**, cell death, little vacuole formation. Bars, 20 μ m.

Discussion

Toxigenic strains of *H. pylori* cause progressive vacuolation in mammalian cells, such as HeLa cells [1, 2, 13]. It has been speculated that the phenomenon of vacuole formation is associated with the gastric pathologies, such as gastric ulcer and gastritis induced by *H. pylori* infection. *H. pylori* produces ammonia by a potent urease enzyme and thus increases the pH of the surrounding mucus, facilitating colonisation by *H. pylori*. Ammonia produced by the urease of *H. pylori* [14] induces damage to the gastric mucosa. Moreover, vacuolating activity may be potentiated by urease-mediated ammonia production [15].

Leunk *et al.* [13] reported that, *in vitro*, broth culture filtrates of *H. pylori* induced cytopathic effects in seven of nine mammalian cell lines. Of the cells tested, Int 407 and HeLa cells were the most responsive. However, HeLa cells are not sufficiently sensitive to detect minimal differences in vacuole formation amongst different *H. pylori* culture supernates. Therefore, this study tried to identify a more sensitive cell line.

Four cell lines established in our laboratory were tested as well as HeLa cells, and it was found that LYM-1 cells were the most sensitive to damage by ammonium chloride. LYM-1 cells seemed to

be suitable for the screening of vacuole-inducing *H. pylori*. The responses of HeLa cells and LYM-1 cells were compared by means of culture supernates from 10 strains of *H. pylori* in which the *vacA* gene had been identified by PCR. At a final dilution of 1 in 4, *H. pylori* culture supernates, only 20% of HeLa cells displayed vacuole formation and 100% in LYM-1 cells. Although there were exceptions, when cell culture was prolonged for up to 5 days, LYM-1 cells showed clear evidence of vacuolation (strain nos. 8 and 10 in Table 1), even when the culture supernates were diluted to 1 in 16. For detection of cytotoxic activity in HeLa cells, the filtrates had to be concentrated before assay [13]; but in LYM-1 cells, concentration of the culture supernates was not required. This is a simple and effective method for the screening and characterisation of clinical isolates of *H. pylori*. The use of LYM-1 cells permitted the detection of minimal vacuolation activity.

When screening *H. pylori* culture supernates, two phenomena were observed – vacuolation, and loss of cell viability. With regard to vacuole formation, the *H. pylori* culture supernates showed different properties in LYM-1 cells. There are significant differences in *vacA* sequences between toxin-positive and toxin-negative *H. pylori*, and the mutations of the *vacA* gene result in the absence of cytotoxin production. In the cytotoxin-negative/*vacA*-positive strains, there are variations in the length of the *vacA* gene [2, 3, 5]. The detailed mechanism of the regulation of the *vacA* gene remains obscure. We suppose that the amount of cytotoxin produced may be influenced by phenotypic differences in *H. pylori* strains or the occurrence of a mutation in the *vacA* gene. The degree of cell death in LYM-1 cells varied amongst the different *H. pylori* supernates. Vacuole formation was not necessarily correlated with cell death in LYM-1 cells. This result suggests that the factor responsible for vacuole formation may be different from that for cell death.

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