

# Detection of Apoptotic Cells in Vero Cell Cultures Inoculated With Samples Derived from Fatal Cases of Sarawak Acute Childhood Viral Infection

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## Summary

Infectious agent(s) causing the fatal Sarawak acute childhood viral infection (SACVI) has not been identified. In the present study, results indicating that inocula prepared from the fatal cases of SACVI induced apoptosis in Vero cell cultures are presented. These findings suggest the possible involvement of apoptotic cellular responses in SACVI.

**Key Words:** Apoptosis, Childhood, Infection, Sarawak, TUNEL, Viral

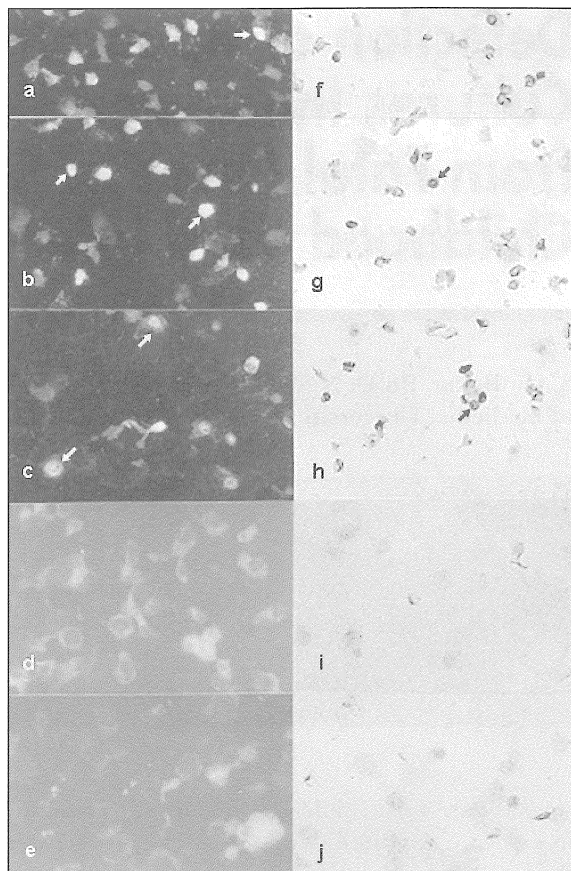
An outbreak of acute childhood viral infection resulting in sudden unexplained deaths of 31 children between the ages of 5 months to 6 years old occurred in Sarawak, Malaysia, in mid 1997. Initial reports indicated that the patients died due to acute viral myocarditis possibly associated with the hand, foot, and mouth disease (HFMD)<sup>1</sup>. In most instances it was reported that the patients' conditions deteriorated rapidly and most succumbed to the infection within a short period following admission to the hospitals. To date, the etiologic agent(s) of the infection and the actual cause of death are still unknown. In the present investigation, the possible involvement of apoptotic cellular responses in SACVI was investigated.

The Vero cells used in this study was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and propagated as previously described<sup>2</sup>. Patients' samples provided by Professor Lam Sai Kit (Department of Medical Microbiology, Universiti Malaya, Kuala Lumpur, Malaysia) were used to inoculate freshly prepared Vero cell cultures and virus inocula were prepared after the infected cells have shown

approximately 70% cytopathologic effects. The infected cells were lysed by rapid freeze-thawing and the supernatants were sequentially centrifuged at 10,000 and 40,000 X g. Supernatants after the final centrifugation were filtered through 0.22 µm syringe filters and used as inocula. Inocula were similarly prepared from mock-treated cell cultures and cultures treated with the non-fatal SACVI cases, which did not show any cytopathic effects after 6 days post-infection. In a typical experiment, 100 µl of the inoculum was added to semi-confluent Vero cells cultured on glass coverslips in 1 ml growth medium. After 2 hours incubation at 37°C, the inoculum was removed and fresh growth medium was added. Cells were fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton-X 100 after six days post-infection. Apoptotic cells were labelled using TdT-mediated dUTP (Promega, Madison, WI, USA) or dNTP (Oncogene Research Products, Amersham International plc, England) nick end-labeling (TUNEL) of fragmented DNA. The labeling was performed according to the protocols provided by the apoptotic detection kits' manufacturers.

Cerebrospinal fluid, sera, throat and vesicle swab samples of nine patients who succumbed to the infection and two non fatal suspected SACVI patients were evaluated. Inocula derived from all nine available fatal SACVI patients' samples were found to induce significant ( $P < 0.05$ , T-test) presence of apoptotic cells in the treated Vero cell cultures in comparison to the mock-treated controls or those treated with samples of the suspected but non fatal SACVI. TUNEL results using the two staining protocols for three of the fatal cases, one non fatal suspected SACVI case and the mock-treated control are shown in Figure 1 (a - j). Labeling using FITC-conjugated dUTP gave rise to intense fluorescent green-stained apoptotic cells nuclei when viewed under an ultraviolet light microscope (Figure 1; a, b, and c), whereas, the FragEL staining procedures utilizing the biotinylated dNTP and horse-radish peroxidase-conjugated streptavidin gave rise to dark coloured apoptotic cells (Figure 1; f, g, and h). Apoptotic cells were detectable using both methods in samples treated with inocula derived from sera of SACVI patients SKV 5 (MY 1/1), SKV 6 (MY 15/3) and SKV 8 (MY 2/1) (Figure 1; a & f, b & g, and c & h, respectively). Approximately 31.6 (s.d.  $\pm 6.6$ ), 30.0 (s.d.  $\pm 9.4$ ), and 18 (s.d.  $\pm 4.7$ ) percent of the infected cells, respectively were apoptotic. In contrast,  $< 1.5\%$  of the cell cultures treated with inoculum of a non-fatal SACVI (SKV 4b; MY 17/2) and mock-treated cells (Figure 1; d & i and e & j) were apoptotic.

Apoptosis or programmed cell death is initially described as "chromatolysis" by Flemming in 1885 to characterize cells with nuclear fragmentation observed in epithelial lining of regressing ovarian follicles<sup>3</sup>. Kerr and colleagues<sup>4</sup> later coined the terms "apoptosis" and "programmed cell death" to describe the non necrotic cell death in atrophying rat liver. It is now recognized that apoptosis is an important integral component in normal maturation of the immune systems, ageing, homeostasis and as one of the possible defence mechanisms evoked by virus-infected cells to prevent replication and spread of the virus<sup>5</sup>. Since, results presented in this investigation suggest that there are apoptotic inducing factor(s) present in the inocula of patients who succumbed to the SACVI, it is not surprising that the SACVI-associated virus is difficult to be isolated from patients samples. It is possible that



**Fig. 1:**

**SACVI inocula-induced apoptosis detected using TUNEL. Vero cells cultured on glass coverslips were infected with SACVI inocula. After 6 days post-infection cells were fixed, permeabilized and labeled with FITC-conjugated dUTP (a - e) or biotinylated dNTP (f - j). The biotinylated dNTP-labeled DNA fragments were detected using horseradish peroxidase-conjugated streptavidin and diaminobenzamide as substrate. SACVI inocula derived from fatal cases (a, b, c, f, g, and h) showed the presence of apoptotic cells (arrows) in contrast to those treated with samples derived from the non fatal SACVI (d and i) or mock-treated controls (e and j).**

during the initial infection, the virus replicates and synthesizes or stimulates Vero cells to synthesize factors, which activate apoptotic cellular responses, which in turn limit or inhibit virus replication. However, since the amount of fatal SACVI patients' samples was scarce it was not possible to ascertain if similar phenomenon could be observed in other cell culture systems. Nonetheless, judging from results presented here, better suited cell culture systems are needed in order to efficiently propagate the virus.

The relationship between the ability to induce apoptosis and manifestation of the disease if any could not be determined. It is suggested that in fatal cases a sudden yet massive apoptotic responses affecting vital functions including the heart and brain occurred, whereas, in non-fatal cases induction of apoptosis occurred in non-vital tissues resulting in transient clinical effects if any and patients recover uneventfully. In surviving patients, it is expected that no permanent tissue damages can be detected since in apoptotic cell death no significant inflammatory responses occurred<sup>4</sup>. Further investigations are presently being conducted to examine this possibility.

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