

Programmed Cell Death in HEp-2 Cells Infected with Human Adenovirus Isolate

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SUMMARY

During the course of adenovirus infection, a number of viral proteins stimulate or inhibit programmed cell death (PCD). In this study, adenovirus was isolated from stool sample of a child. Adenoviral antigens which were determined with Latex Agglutination (LA) test were propagated in HEp-2 cell lines. Adenovirus inoculated in cell culture was confirmed by direct immunofluorescent assay (DFA). Programmed cell death (PCD) determinations were made by nuclear staining with DAPI according to morphological criteria. Cytopathogenic effects of adenovirus were followed for 36 hours of post infection (h.p.i). Determination of da DAPI within 24 h.p.i. showed that PCD in HEp - 2 cells was induced by adenovirus isolate .

Key words: Adenovirus, apoptosis, HEp-2 cell lines, infection

ÖZET

İnsan Adenovirus İzolatı ile İnfekte HEp-2 Hücrelerinde Programlanmış Hücre Ölümü

Adenovirus enfeksiyonu süresince , çeşitli viral proteinler programlanmış hücre ölümünü (PHÖ) stimule veya inhibe ederler. Bu çalışmada, bir çocuğun gaita numunesinden adenovirus izole edilmiştir. Lateks Aglutinasyon (LA) testi ile tanımlanan adenoviral antijenler, HEp-2 hücre kültürlerinde üretilmiştir. Hücre kültürüne inokule edilen adenovirus, direkt floresans (DFA) testi ile doğrulanmıştır. PHÖ' nün belirlenmesi amacıyla D.A.PI ile nükleer boyama yöntemi uyarınca morfolojik kriterlere göre yapılmıştır. Adenovirusun sitopatojenik etkileri, enfeksiyondan sonraki 36 saat süresince izlenmiştir. Enfeksiyon sonrası 24 saatte DAPI belirlemeleri, adenovirus izolatının HEp-2 hücrelerinde PHÖ'nü indüklediğini göstermiştir.

Anahtar kelimeler: Adenovirus, apoptosis, HEp-2 hücre kültürü, enfeksiyon

INTRODUCTION

Human adenoviruses (HadV) that are a member of the adenoviridae family contain a linear double stranded DNA (1). HadVs are now classified into six subgroups (A-F) based on their several biochemical and antigenic characteristics (1-6). HadVs able to infect a variety of organs and systems containing respiratory tract, gastrointestinal tract, urinary tract, ocular system and liver, have choice for terminally differentiated epithelial cells (2,4). HEp-2 cell line is originated terminal differentiated cell which is human laryngeal epithelial carcinoma cell line and a

low level expression of wild type p53 protein (7).

Programmed cell death (PCD) also known as “apoptosis” is the process whereby individual cells of multicellular organisms undergo systemic self-destructive in response to a wide variety of stimuli. Apoptosis is genetically controlled, preprogrammed event and many viral proteins may stimulate and inhibit apoptosis. Human adenoviruses have both virus encoding apoptosis-inhibiting proteins involved E1B-19K, E1B-55K, E3-14.7K, E3-10.4K/14.5K and apoptosis inducing proteins involved E1A, E4, E3 (8-10) .

Thus in the case of virus-infected cells apoptosis represents a very efficient mechanism by which the vi-

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rus can induce cell death and disseminate progeny while limiting induction of inflammatory and immune responses. In spite of the virus can inhibit cell death since replication stage (11). As a result, viruses regulate the PCD by encoding various proteins according to replication timing. Therefore; new information on the induction and suppression of apoptosis by viral products as well as to propose how this knowledge may provide insights into basic cell biology and offer the potential of new therapeutic application on infection disease and cancer.

The present study was undertaken (i) to examine the probable role of apoptosis in the infection pathway of adenovirus in an epithelial like (HEp-2) cell line and (ii) to question its probable role in the viral progeny

MATERIAL AND METHODS

Cell lines. Human laryngeal epithelial carcinoma cell line (HEp-2) was grown in Eagle's minimal essential medium (EMEM, Sigma GmbH, Germany) with 5% fetal calf serum (FCS, Sigma GmbH, Germany) and 1% penicillin/ streptomycin (Biochrome, KG). The cells were maintained in 5% CO₂ atmosphere with 95 % humidity at 37 °C.

Preparation of stool samples. Stool samples from of human infants were treated with 1 ml of chloroform and 9 ml of PBS and then completed in sterile screw capped tubes containing small beads. This mixture was shaken for 20 minutes at room temperature. Following this process the mixture was centrifuged at 1500 rpm for 20 minutes. Supernatant was transferred to another tube and centrifuged again at 3000 rpm for 20 minutes. The last supernatant was divided into 2 ml Eppendorf tubes and aliquots were stored at -20°C for further use. All infectious procedures were processed in laminar air flow cabinet (Holten safe 2010 class 2, Denmark) and biosafety micro centrifuge (Eppendorf 5402, Germany).

Virus isolation procedure. Enteric adenoviruses were isolated from stools of human infants with diarrhea. Viral transport medium which was non-inhibitory to adenoviruses and the tissue culture cells were used for viral isolation. Isolated viruses were

propagated in HEp-2 cells and were monitored with microscopic examination until special cytopathogenic effects (CPE) were reached 80-100 % by an inverted microscope (Nixon TE, 2000S, Japan). Infected cells were frozen and thawed twice and aliquots of 1, 5 mL amount were stored at -20°C for further use.

Latex Agglutination for detection of adenovirus. The stool sample was inoculated onto HEp-2 cells and incubated in 5% CO₂ atmosphere with 95% humidity at 37° C. Adenoviral antigens were detected by latex agglutination test (Adenolex, Orion, Diagnostica, Helsinki, Finland) On test card, 50 µl of inoculum and 50 µl of latex reagent, which were containing anti-human-adenovirus antibodies, were mixed in the block circle. If the adenoviral antigen was present, agglutination was observed in this circle in five minutes at room temperature. The performance of latex agglutination was checked with the human adenovirus antigen positive and negative control sera at the same time in each experiment

Direct Fluorescent Antibody Test for confirmation of adenovirus. "Latex Test Positive" samples were confirmed by direct fluorescent antibody (DFA) test. HEp-2 cells were propagated in eight-well chamber slide (Nunclon, Germany) for DFA test which was culture confirmation. 50 µl of adenovirus positive isolate was inoculated onto monolayer cultured cell. The infected slide was incubated in 5% CO₂ atmosphere with 95% humidity at 37 °C. After 18 hours, the medium was removed and infected cells were fixed with cold acetone for 5 minutes. Slide was washed twice with Tween-20 and air dry completely, then 30 µl of adenovirus/FITC antibody was added to each well. The chamber slide was incubated at 37° C for 30 minutes; at last the slide was rinsed gently and washed three times with Tween-20. The slide was examined with a fluorescent microscope (Olympus BHZ, RFCA microscope with C-35AD-4 photograph attachment, Japan) at x100-200 for cells exhibiting an apple-green fluorescence of FITC (Detailed examination can be carried out of x40).

DAPI staining and criteria for apoptotic cells sco-

ring. For this purpose, HEp-2 cells were propagated in the plates with 24 wells (Greiner, Germany). On to the each well 100µl of adenovirus isolate inoculated, after 18 hours following viral infection, HEp-2 cells were fixed with cold 96% ethanol for 5 minutes. It was washed three times with PBS (pH 7, 4); cells were stained with 0, 5 µg/µl DAPI (Serva, Germany) for 5 minutes at 37° C. The slide was rinsed and then mounted on to glass slide using glycerol. The slide was examined by fluorescence microscopy set and photographed in x40 magnification.

Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries. Late apoptotic cells exhibited nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane.

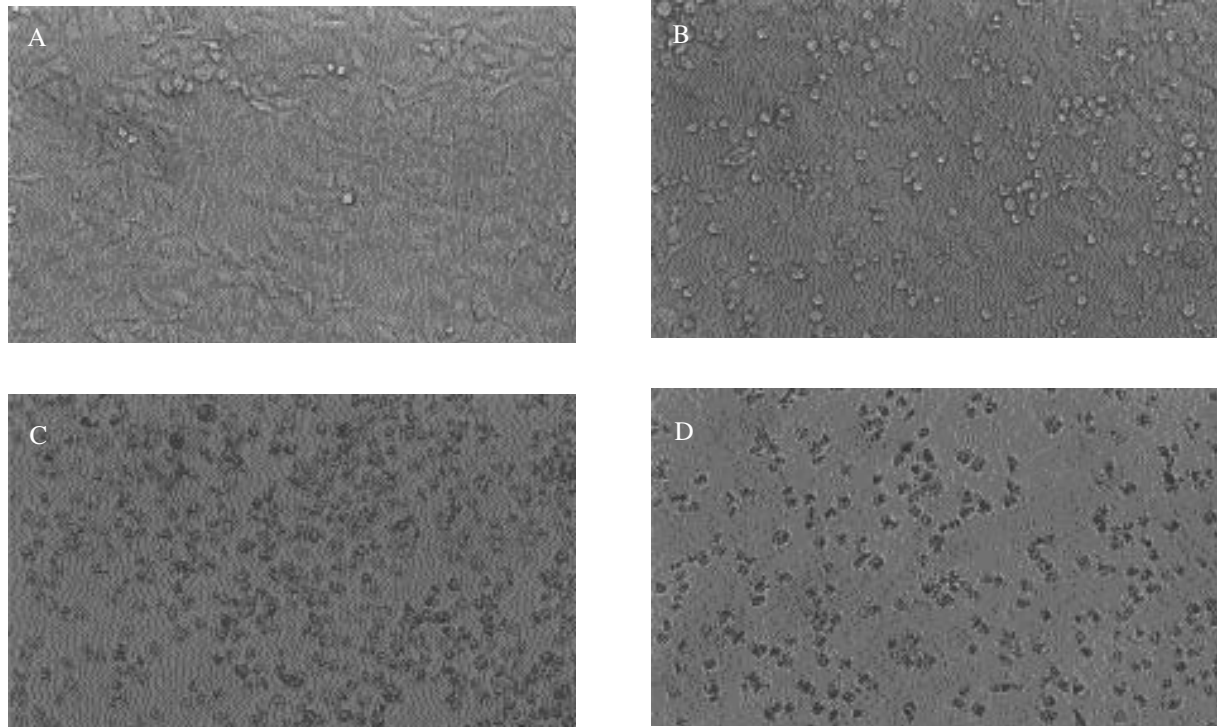
RESULTS

One of its advantage is adenovirus exhibit a broad

host ranges in vitro with high infectivity. Our studies demonstrated that more than 90% of HEp-2 cells could be infected by clinical isolates of adenovirus at 36 h.p.i. Characteristic cytopathogenic effects (CPE) consists of grapelike clusters of rounded and ballooning cells which appear at the 36 th hour with isolated enteric adenovirus.(Figure 1D). Initial CPEs were started from the periphery of monolayer cell line at 20 h.p. i. (.Figure 1C). The degree of CPE is 100% at the 36th hour. Latex agglutination test showed the presence of enteric adenovirus in clinical sample and DFA test confirmed the presence of enteric adenovirus in HEp-2 cell lines. Following the 24 hours of the post infection period, adenovirus infected cells were green, noninfected cells were red (Figure 2).

Effects of clinical isolates of adenoviruses on apoptosis in later stages of infection in HEp-2 cell lines: HEp-2 cells, which had a low level expression if wild-type p53 protein, were infected with HAdV and

Figure 1. Characteristic cytopathogenic effects (CPE) of clinical isolates of enteric adenoviruses. (A)Time course of HAdVs CPE; HEp-2 cell lines were uninfected or (B)infected for 12 h.p.i, (C) 22 h.p.i and (D)36 h.p.i. CPE was evaluated by using inverted microscopy (Cells should have been evaluated at 36 hours due to extensive viral cytopathic effects).



then incubated for 24 hours to allow later stage effects. A representative experiment is shown in Figure 3. Effects of later stage of infection on HEp-2 cells showed clear apoptotic morphology, with markedly shrunken, chromatin condensation and fragmented nuclei (Figure 3A). In contrast, cells which were uninfected had a healthy morphology (Figure 3B).

DISCUSSION

There is increasing evidence that many animal viruses encode proteins which interact intimately with the biochemical pathways regulating apoptosis (13-

Figure 2. HEp-2 cell culture for isolation of adenovirus confirmed by DFA. The positive cells showed its exhibiting apple-green fluorescence of FITC with a fluorescent microscope. The white head of arrows shown the HAdV antigen positive cells (x40)

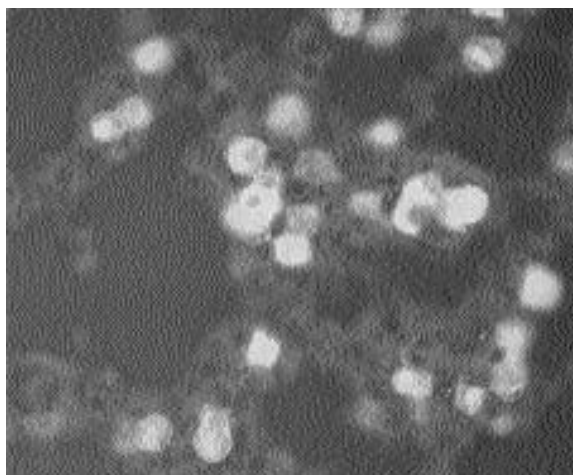
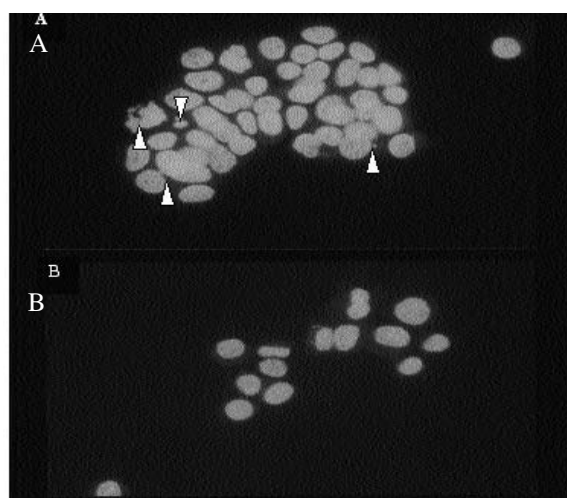


Figure 3. HEp-2 cell culture for isolation of adenovirus confirmed by DFA. The positive cells showed its exhibiting apple-green fluorescence of FITC with a fluorescent microscope. The white head of arrows shown the HAdV antigen positive cells (x40)



19). As these processes are understood in more detail, opportunities for the development of new drugs could promote the early death of infected cells, inhibit virus release or in the case of latent viruses, manipulate the lytic-latency switch to minimize the effects of infection (13).

Adenoviruses may also encode proteins which function as inducers of apoptosis at later stages of infection. Adenovirus was originally believed to originate from the E1A protein which induces accumulation of p53 and p53-dependent apoptosis (20). However, our studies clearly showed that to induce apoptosis in HEp-2 cells which had a low level expression of p53 protein and this activity are p53-independent apoptosis. During later stage of infection, encoded proteins induce apoptosis and effects of these proteins may help virus for viral progeny to spread to neighboring cells.

Finally, new information about viruses and apoptosis may be useful to develop new therapies for human diseases. Many current cancer treatments actually work by including p53-dependent apoptosis and thus p53 null or low level expressed tumor cells or particularly resistant to killing. In this respect, a p53 – independent strategy may become more important in the future. Furthermore, just such a strategy is being tested using a recombinant adenoviral vectors gene which is combination gene therapy strategy for laryngeal cancer to inhibit cancer cell growth and induce apoptosis (20). However many current studies neglect the combinative effect of adenovirus encoding proteins and transducer genes in to tumor cells due to apoptosis (21). Thus, infection of adenovirus in low level expressed p53 tumors could preferentially kill the cancer cells while having little effect on normal tissues. Viral death inducers could also be adapted to kill such cancer cells directly through a process. Then, continued investment in virus research may once again prove to be highly justified.

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