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ONCOLYTIC POTENTIAL OF E1B 55 kDa-DELETED YKL-1 RECOMBINANT ADENOVIRUS: CORRELATION WITH p53 FUNCTIONAL STATUS

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YKL-1, E1B 55 kDa-deleted recombinant adenovirus vector, capable of harboring a transgene casette of up to 4.9 kb, was newly constructed by reintroducing E1A and E1B 19 kDa into E1/E3-deleted adenoviral vector with a homologous recombination in *E. coli*. Virus replication and cytotoxicity were dramatically attenuated in all 3 different types of normal human cells. In contrast, YKL-1 efficiently replicated and induced cytotoxicity in most cancer cells, especially Hep3B and C33A cells with an inactivating p53 mutation. However, both H460 and HepG2 exhibited intermediate sensitivity to YKL-1, which was between that of Hep3B or C33A and normal human cells. The YKL-1 and DNA damaging agent, camptothecin effectively induced p53 in H460 and HepG2 as well as in normal cells. Furthermore, YKL-1 effectively prohibited both Hep3B and C33A tumor growth in *nu/nu* mice in a dose-dependent manner. H/E staining and TUNEL assay indicated a largely distributed necrotic area and apoptosis on its periphery. This study, therefore, indicates that YKL-1, possesses promising potential as an oncolytic adenoviral vector, which acts partially in a p53-dependent manner. *Int. J. Cancer* 88:454–463, 2000.

E1/E3-deleted replication-defective adenovirus derived from human adenovirus type 5 (had5) has been widely used for cancer gene therapy because it offers, in contrast to other vectors, much higher gene transfer efficiency and transgene expression in a broad spectrum of cell types (Jolly, 1994; Verma and Somia, 1997). Therapeutic genes are generally inserted in place of the E1 gene, which is essential for viral replication (Graham *et al*., 1987; Yee and Perricaudet, 1997; Hitt *et al*., 1994). A variety of antitumoral genes including cytokines, immune-costimulatory factors, suicide genes and tumor suppressor genes have been applied, which has resulted in cancer cell death *in vitro* and eventual tumor regression *in vivo* (Paillard, 1998; Roth and Cristiano, 1997; Runnebaum, 1997). However, the therapeutic efficacy of E1-deleted replication-incompetent adenovirus is limited by its inability to spread in and infect neighboring cancer cells subsequent to the initial infection event.

Bischoff *et al*. in 1996 reported that E1B 55 kDa-attenuated recombinant adenovirus (ONYX-015) preferentially replicated in and killed cells lacking functional p53, including the majority of human cancer cells. Subsequent study by Heise *et al*. demonstrated ONYX-015 to be an effective antitumoral agent (oncolytic viral agent) *in vitro* and *in vivo* (Heise *et al*., 1997). Furthermore, Kirn *et al*. (1998) reported encouraging clinical data by administering ONYX-015 intratumorally to patients with recurrent head and neck cancer. E1 gene, one of the adenovirus early genes, encodes multiple open reading frames, including E1A and E1B 55 kDa (Graham *et al*., 1987; Shenk, 1996). E1A gene product, a transcription factor, associates with pRB, p300 and other proteins, and is largely responsible for driving infected cells into the S phase to allow the synthesis of viral genome (Shenk, 1996; Shenk and Flint, 1991). E1A expression and unexpected foreign DNA synthesis triggers the expression/activation of p53 (Lowe and Ruley, 1993; Nakajima *et al*., 1998). In contrast, E1B 55 kDa physically associates with and inactivates p53 (Yew and Berk, 1992; Joseph and Vogt, 1996). By using this mechanism, wild-type adenovirus can manipulate host cells to provide the optimal conditions for effective virus replication and production. Therefore, E1B 55 kDa attenuated recombinant adenovirus should not be able to replicate in normal human cells, whereas it would be able to replicate in cells lacking functional p53.

p53 tumor suppressor gene is mutated in roughly 50% of human cancers, including non-small cell lung, colon, breast, head and neck, and ovarian cancers (Brennan *et al*., 1995; Bergh *et al*., 1995; Perkins and Steern, 1997). Furthermore, in many other human cancers encoding wild-type p53, p53 is not functional by the overexpression of mdm2 (Leach *et al*., 1993; Marchetti *et al*., 1995), human papilloma virus (HPV) infection (Scheffner *et al*., 1990; Joseph and Vogt, 1996) or other unknown mechanisms (Chang *et al*., 1995). Loss of functional p53 is well-correlated with the later stages of most human cancers, such as poor prognosis and resistance to conventional cancer therapy (Harris and Holstein, 1993; Kirsch and Kastan, 1998). However, the potential of E1B 55 kDa deleted adenovirus as an antitumoral reagent has been challenged by several reports, which has questioned its mechanistic action against human cancer cells, especially with respect to p53 (Hall *et al*., 1998; Goldsmith *et al*., 1998; Goodrum and Ornelles, 1998; Rothmann *et al*., 1998; Harada and Berk, 1999; Hay *et al*., 1999; Turnell et al., 1999).

In our study, we generated E1B 55 kDa-deleted recombinant adenovirus (YKL-1) by using a different strategy from ONYX-015 and evaluated its feasibility as an antitumoral agent *in vitro* and *in vivo*. The oncolytic potential of YKL-1 was investigated by examining virus replication and cytotoxicity in 3 different types of normal human cells as well as a variety of human cancer cells. The correlation of p53 status and the conditionally replicating capability of YKL-1 was evaluated by examining endogenous p53 transcription activity and the induction rate of p53 by DNA-damaging agents and YKL-1 infection. Finally, the therapeutic efficacy of YKL-1 was analyzed in a *nu/nu* mouse model bearing C33A and Hep3B xenografts.

MATERIAL AND METHODS

Cell lines

All the cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (50 μg/ml); 293, FHs 738Lu, FHs 173We, SK-Hep1, HepG2, Hep3B, C33A, Hela and H460 cells were purchased from the American Type Culture Collection (ATCC). Normal human fibroblast was recovered from the ascites of patients with liver cirrhosis.

Construction of recombinant adenoviruses

To generate E1B 55 kDa-deleted recombinant adenovirus (YKL-1), the E1 region from nucleotide 343 to 2,270 in the had5

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genome was amplified using the polymerase chain reaction (PCR) and pXC1 containing an E1 region (Microbix; Ontario, Canada) as a template. Primers used were upstream primer, TTATTGGATC-CTTTGTCTAGGGCCGCGGG, and downstream primer, TCTT-GGATCCAGATCTATACAGTTAAGCCACCTATACAAC. The resulting PCR fragment, which included E1A, E1B 19 kDa and a part of E1B 55 kDa, contained the BamHI site at each 5′ and 3′ end, BglII at the 3′ end for further cloning and premature translation stop codons in the E1B 55 kDa open reading frame by substitution *of* bases 2,253 (C to T) and 2,262 (G to T). The fragment was digested with BamH1 and cloned into pCA14 shuttle vector (Microbix; Ontario, Canada), predigested with BglII to generate pCA14-E1A/E1B 19kDa, and then the sequence was verified using an ABI PRISM377 automatic DNA sequencer. pCA14-E1A/E1B 19 kDa shuttle vector and adenovirus vector pTG-CMV containing the had5 genome, absent in the E1 region (1341–3535 in nucleotide sequence) and the E3 region (28593–30469; obtained from Dr. Verca at the University of Fribourgh, Switzerland) were linealized with XmnI and ClaI, respectively and cotransformed into BJ5183 *E. coli* (Chartier *et al*., 1996). To verify the proper homologous recombinants, DNAs were purified from overnight cultures, digested with HindIII and the digestion pattern was analyzed. The proper homologous recombinant DNA was digested with PacI and transfected into 293 cells to generate E1B 55 kDa-deleted recombinant adenovirus, YKL-1. Replication-defective counter parts (ad-ΔE1, ad-ΔE1/β-galactosidase) and replication-competent wild-type recombinant adenovirus ad-XJ were generated by similar manipulation using different shuttle vectors, pCA14, pΔE1sp1A/β-galactosidase, and pXC1, respectively. All the viruses were plaque-purified, propagated and purified in 293 cells using a standard method (Hitt *et al*., 1994).

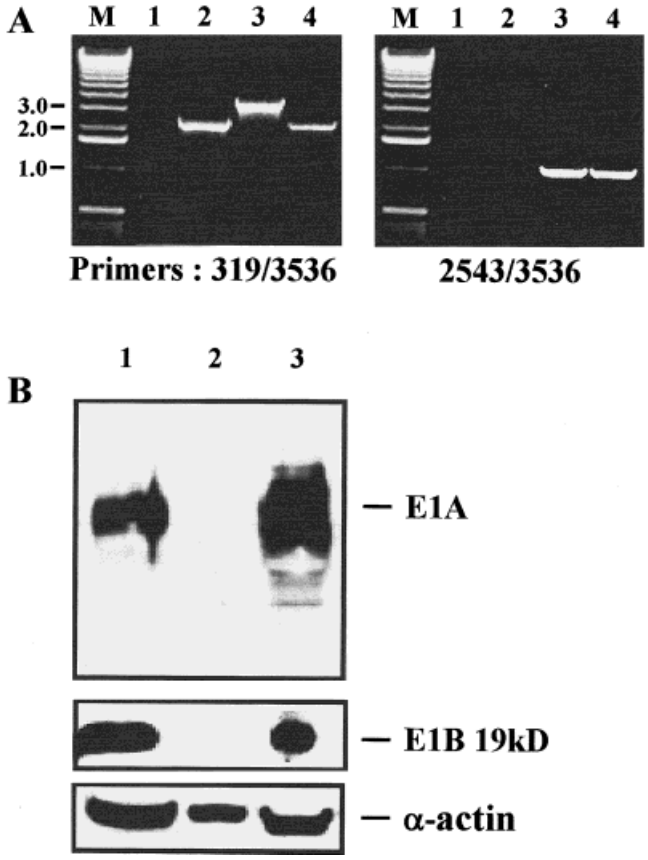

FIGURE 1 – Characterization of YKL-1. (*a*) PCR analysis of YKL-1. Two days post-infection, virus genome was recovered from C33A cells and PCR was performed using primers corresponding to the upstream sequence of E1A and the downstream sequence of E1B55 kDa (left panel) or primers corresponding to E1B 55 kDa (right panel). PCR products were visualized using 1% agarose electrophoresis and EtBr staining. lane 1, mock infected; lane 2, YKL-1 infected; lane 3, ad-XJ infected; lane 4, pCA-E1A/E1B 19 kDa (left panel), pXC1 (right panel) as a positive control. (*b*) Expression analysis of adenovirus E1A and E1B 19 kDa protein. Infected cells were harvested and lysed with cell lysis buffer. Reducing SDS-PAGE was then performed and E1A and E1B19 kDa were visualized by immunoblotting. lane 1, 293 cells as a positive control; lane 2, mock infected; lane 3, YKL-1 infected. A similar level of α-actin expression indicated that an equal amount of protein was loaded in each lane.

Isolation of viral DNA and PCR analysis

After 2 days postinfection, virus DNA was isolated with a Qiagen genomic isolation kit (Santa Clarita, CA) from C33A cervical cancer cells infected with adenovirus at a multiplicity of infection (MOI) of 10. Five microliters of virus DNA was used for PCR amplification by using 5′ primer, which corresponds to the upstream sequence of E1A or the upstream sequence of E1B 55 kDa, and 3′ primer, which corresponds to downstream sequence of E1B 55 kDa.

Immunoblotting analysis

Cells were harvested and lysed with lysis buffer (50 mM HEPES containing 0.15 M NaCl, 0.5% Nonidet P-40 and protease inhibitors: PMSF, TLCK and TPCK). Pre-cleared lysates corresponding to 10^5 cells were resolved by 8 or 12% reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membrane filters (RPN 303F, Amersham, Arlington Heights, IL). Immunodetection was performed using the Pierce enhanced-chemiluminescence system (RPN 2108, Amersham) with 1-–5 μg of mouse monoclonal primary antibody, specifically recognizing adenovirus E1A (sc-430; Santa Cruz, USA) , E1B 19 kDa (DP17; Calbiochem, San Diego, CA), p53 (MS-186-P1; Neomarkers, USA) or α-actin (sc-1615; Santa Cruz) proteins.

TABLE I – SUMMARY OF p53 STATUS OF HUMAN CELL LINES USED IN THE STUDY

Cell line	Cell type	p53 mutation	Comments	Reference
FHs 173We	Fetus lung cells	Wild type	Normal cells	–
FHs 738Lu	Embryonic cells	Wild type	Normal cells	–
Human fibroblast	Primary fibroblast	Wild type	Normal cells	–
SK-Hep1	Hepatocellular carcinoma	A partial deletion or rearrangement		Bressac B *et al.* (1990)
HepG2	Hepatoblastoma	Wild type	Wild type p53	Bressac B *et al.* (1990)
Hep3B	Hepatocellular carcinoma	Homozygous deletion	Homozygous deletion	Bressac B *et al.* (1990)
C33A	Cervical carcinoma	R273C	Mutation in DNA-binding domain of p53	Scheffner *et al.* (1991)
Hela	Cervical carcinoma	Wild type	HPV positive	Scheffner *et al.* (1991)
H460	Large cell lung carcinoma	Wild type	Wild type p53	Takahashi *et al.* (1989)

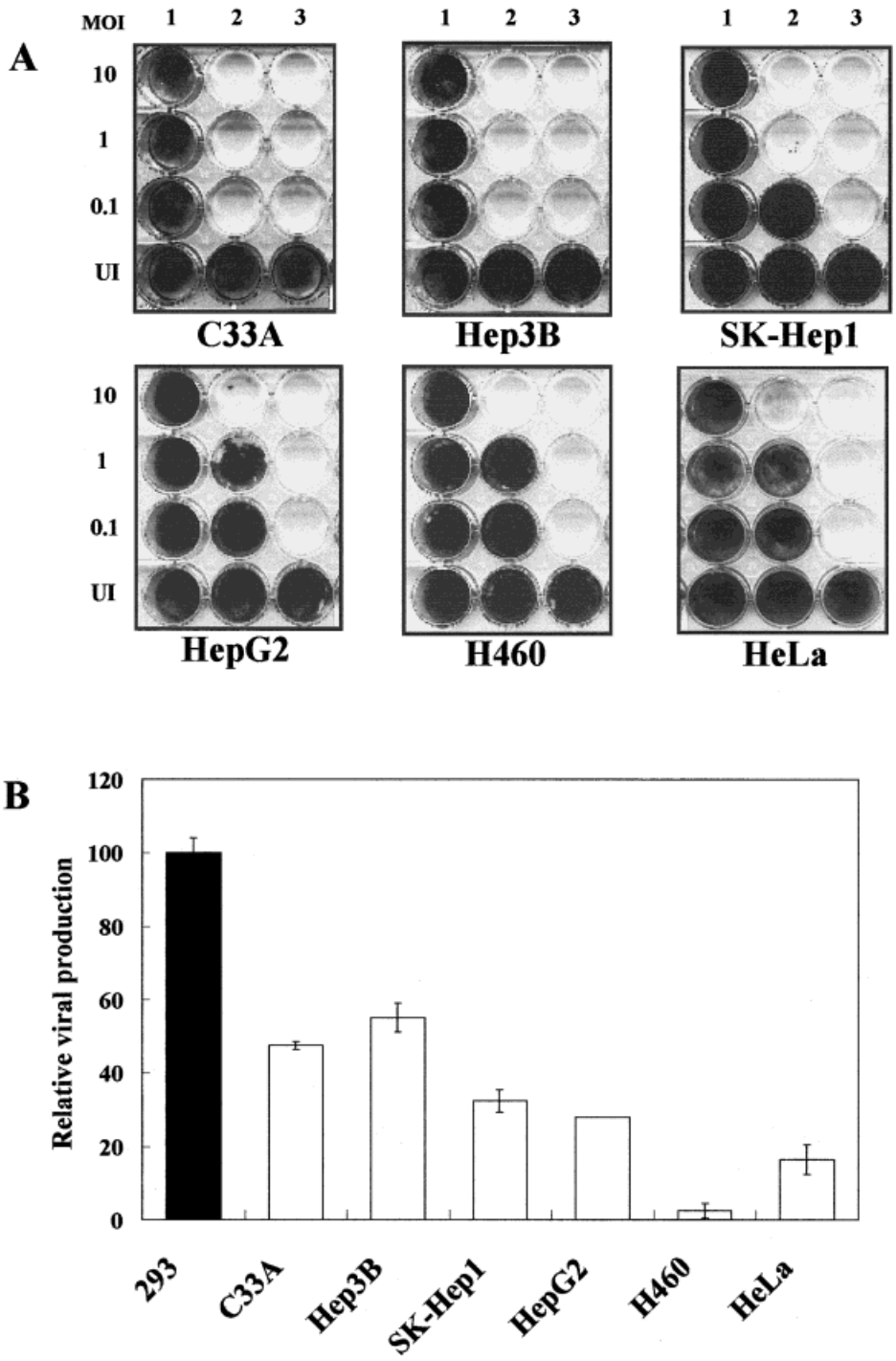

Figure 2 – Virus replication and the cytopathic effect of YKL-1 on a variety of human cancer cells. (*a*) Cytotoxicity of YKL-1 in human cancer cells. C33A, Hep3B, SK-Hep1, HepG2, H460 and Hela on 24-well plates were infected with YKL-1, ad-XJ, ad-ΔE1 or uninfected (UI) at MOIs of 10, 1 or 0.1. When the cells infected with ad-XJ at an MOI of 0.1 exhibited complete cytolysis, the cells were fixed and stained with crystal violet. (*b*) The virus replicating capability of YKL-1 in human cancer cells. Cells were infected with YKL-1 or ad-XJ at an MOI of 10. Four days post-infection, the virus was recovered and the virus titer was determined by a limiting dilution assay on 293 cells. The amount of YKL-1 produced was normalized against the amount of ad-XJ produced in the same cells. Values shown represent the mean values of at least 3 independent experiments.

Luciferase reporter gene assay

Luciferase assays were performed as described previously (Lee *et al*., 1999). Briefly, cells in 6-well plates were transfected with p53-luc, which contains a luciferase expression cassette derived from a p53-responsive element, or pGL-enhancer, which contains a luciferase expression cassette derived from SV40 promoter, together with pcDNA-LacZ (β-galactosidase; Invitrogen), in which LacZ is driven by CMV promoter, by using Lipofectamine (Gibco BRL) as described in the manufacturer's manual. Two days post-transfection, the cells were

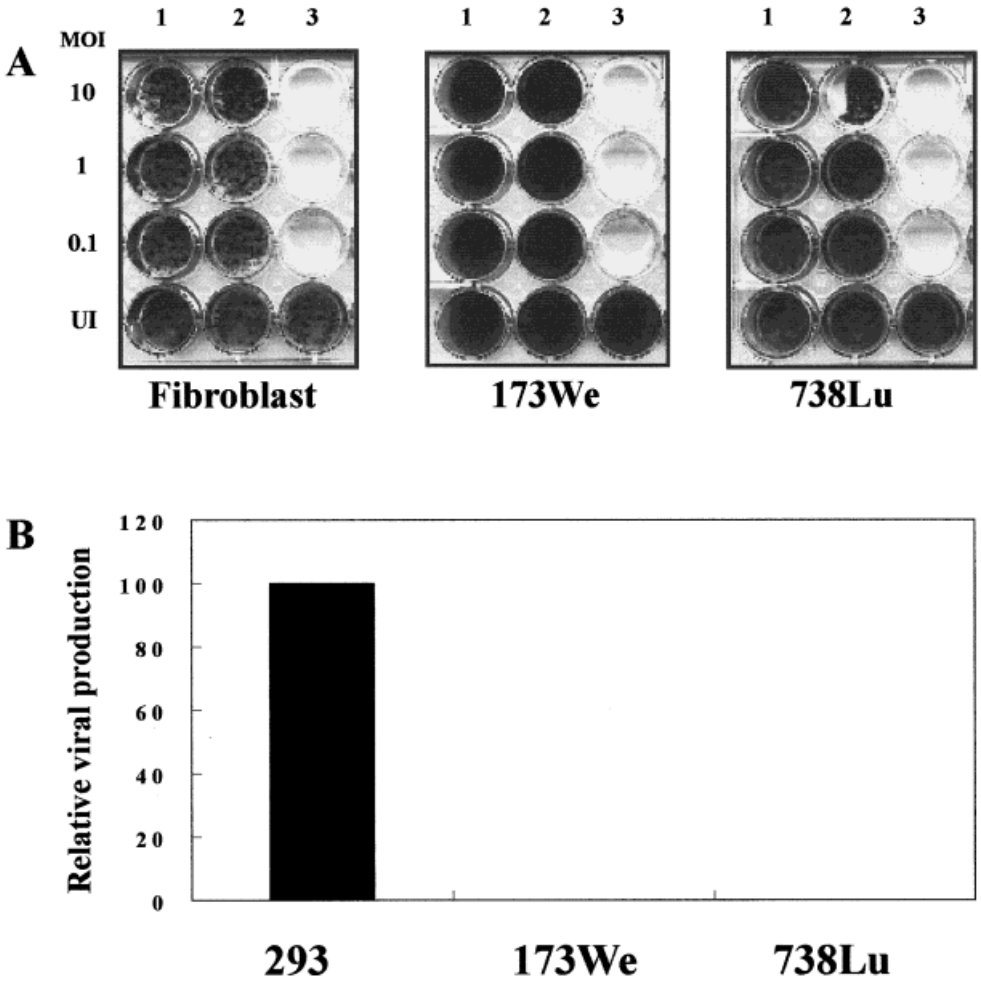

FIGURE 3 – Reduced virus replication and the cytopathic effect of YKL-1 in normal human cells. Cells were infected with YKL-1, ad-XJ or ad-ΔE1. Then virus production and cytopathic effects were examined, as described in Figure 2. (*a*) Cytopathic effect of YKL-1 in normal human cells. (*b*) Replication capability of YKL-1 in 173We and 738We cells. The amount of YKL-1 produced was compared with the amount of ad-XJ produced in the same cells. Values represent the means of at least 3 independent experiments.

washed once with phosphate-buffered saline and lysed in 200 μl of reporter lysis buffer (Luciferase assay kit; Promega, Madison, WI) and luciferase activity was determined as described in the manufacturer's manual with a Luminometer. Values were normalized to β-galactosidase activity.

Cytotoxicity and virus replication assay

To investigate their cytopathic effect, 2–3 × 10^4 cells in 24-well plates (60–80% confluency) at the moment of infection were infected with YKL-1, ad-XJ or ad-ΔE1 at an MOI of 10, 1 and 0.1. They were monitored daily for a cytopathic effect, stained with 0.5% crystal violet in 50% methanol, and analyzed when the cells infected with ad-XJ at an MOI of 0.1 were essentially cytolysed. To quantitate virus replication, cells in 6-well plates were infected with YKL-1 or ad-XJ at an MOI of 10. After 2 or 3 hr, the virus inoculum was thoroughly washed away and the cells were incubated at 37–C in an incubator for another 3 days. The cell pellets and supernatants were harvested, viruses were liberated by 3 cycles of freezing/thawing and the virus titer was determined by limiting dilution assay on 293 cells (Hitt *et al*., 1994). The relative virus replication efficiency was calculated by the following equation as described previously (Heise *et al*., 1997). (Virus titer in cells infected with YKL-1/virus titer in cells infected with ad-XJ) × (virus titer in 293 infected with ad-XJ/virus titer in cells infected with YKL-1) × 100.

Tumor growth studies in human cancer cell xenograft

Male athymic nu/nu mice were obtained at 5–6 weeks of age. All animals were housed and handled in accordance with the Animal Research Committee's Guidelines at Yonsei University. After being quarantined for 1–3 weeks, 1 × 10^7 cells of C33A cervical cancer or Hep3B hepatoma cells/200 μl of PBS were injected into the flanks and allowed to grow to 70–80 mm^3 (5–8 mm maximal diameter) in tumor volume. The tumors were injected 3 times with 1 × 10^7 to 10^9 of live or UV-inactivated CsCl-purified YKL-1 suspended in 50 μl PBS once every other day. UV inactivation was completed by exposing 50 μl aliquot of the purified virus to 3 consecutive cycles of 120,000 microjoules in a UV Startalinker (Stratagene, La Jolla, CA). Tumor size was measured twice weekly and the survival rate was determined. Tumor size was measured by the following equation:

$$(\text{minor axis})^2 \times \text{major axis} \times 0.523.$$

To examine the histology, C33A cervical cancer cell xenografts were intratumorally injected 3 times with 1 × 10^8 of live or UV-inactivated YKL-1 once every other day. Tumors were collected from the 20 day post-infected animals, formalin-fixed and paraffin-embedded.

H/E staining and TUNEL assay

H/E staining was performed in 6 μm formalin-fixed, paraffin-embedded tumor sections. Samples were processed according to

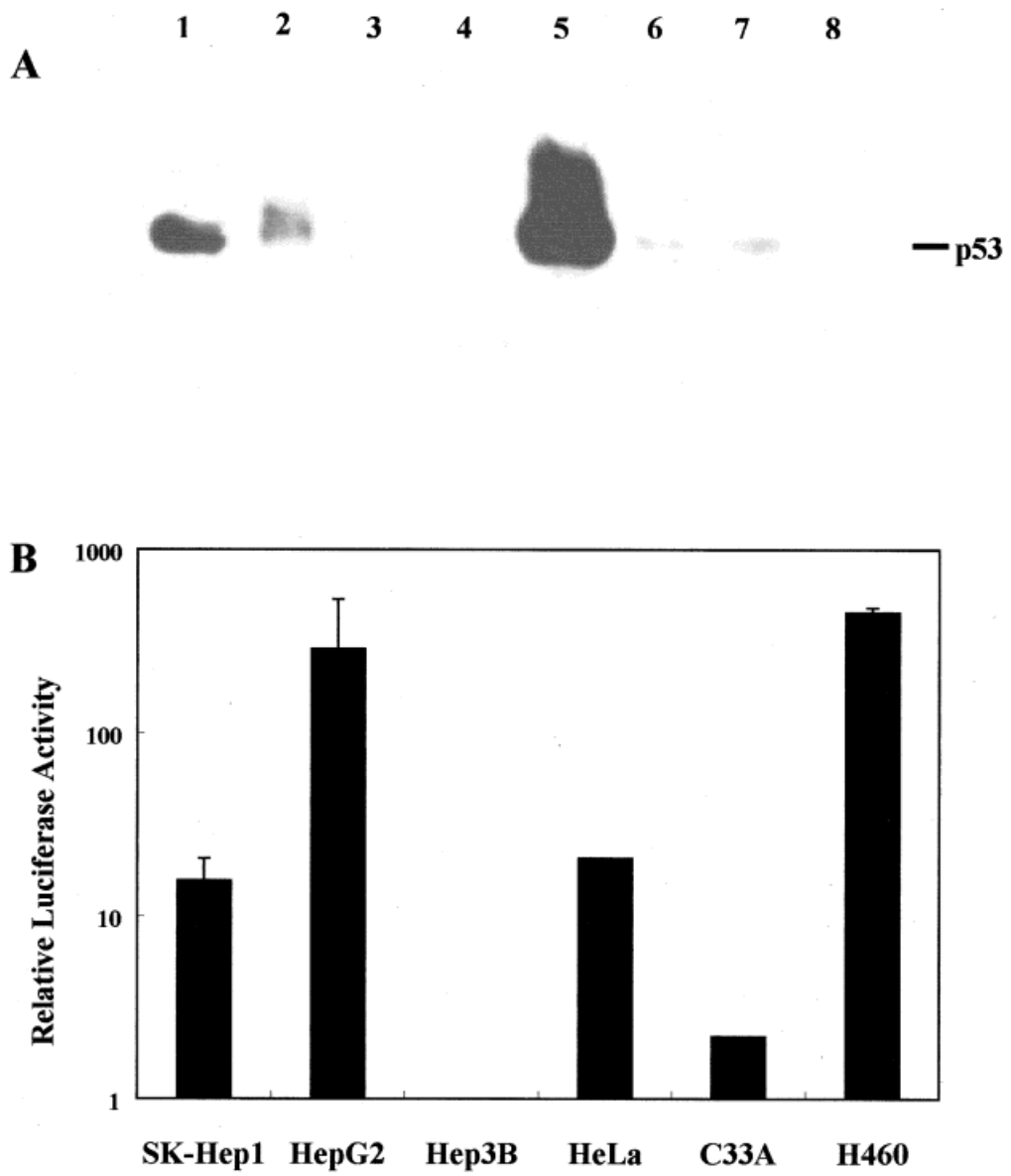

FIGURE 4 – Endogenous p53 expression/activity in human normal and cancer cells. (*a*) p53 expression in normal human cells and cancer cells. Equal amounts of cell lysates were loaded into SDS-PAGE and evaluated by immunoblotting using an antibody to p53. (*b*) Endogenous p53 transcriptional activity in human cancer cells. Cells were transiently transfected with p53-luc or pGL2-enhancer together with pcDNA-βgal, which is an indicative of transfection efficiency. Luciferase activity was determined and normalized on the basis of β-galactosidase activities. The fold increase represents the relative activity of p53-luc *vs.* pGL2-enhancer in the transfected cells. The values are the averages of at least 3 independent experiments.

the instructions of the ApopTag Kit (Oncor, Inc., Gaithersburg, MD) for the detection of cleaved deoxyribonucleic acid in situ, using the terminal deoxynucleotidyl transferase-mediated deoxyuridine 5′-triphosphate-biotin nick end labeling (TUNEL) method.

RESULTS

Construction and characterization of YKL-1 (E1B 55 kDa-deleted adenovirus)

YKL-1, E1B 55 kDa-deleted recombinant adenovirus was constructed as described in Material and Methods. To confirm the genotype and phenotype of YKL-1, the presence of E1A and E1B 19 kDa and the absence of E1B 55 kDa were examined by PCR and immunoblotting analysis (Fig. 1). Two days after infection, the virus genome or cell lysates were recovered from C33A cervical cancer cells infected with YKL-1 or wild-type ad-XJ at an MOI of 10, by PCR using primers (319/3536), which cover the entire E1 gene. A 2.0 and 3.2 kb fragments was generated with YKL-1 or ad-XJ virus genome as templates, respectively (Fig. 1*a*, left panel). The 2.0 kb fragment was also detected with pCA-E1A/E1B 19 kDa shuttle vector under identical conditions. Furthermore, when the primers covering the E1B 55 kDa gene (2543/3536) were used, a 1.0 kb fragment was amplified with the ad-XJ virus genome, but no PCR product with the YKL-1, indicating that YKL-1 retained only E1A and E1B 19 kDa in sequence (Fig. 1*a*, right panel). In addition, Figure 1*b* shows that E1A and E1B 19 kDa were readily detected by immunoblotting when C33A cells were infected with YKL-1 to a similar level as that of 293 cells expressing E1 gene products. These results indicated that YKL-1 was E1B 55 kDa-deleted recombinant adenovirus.

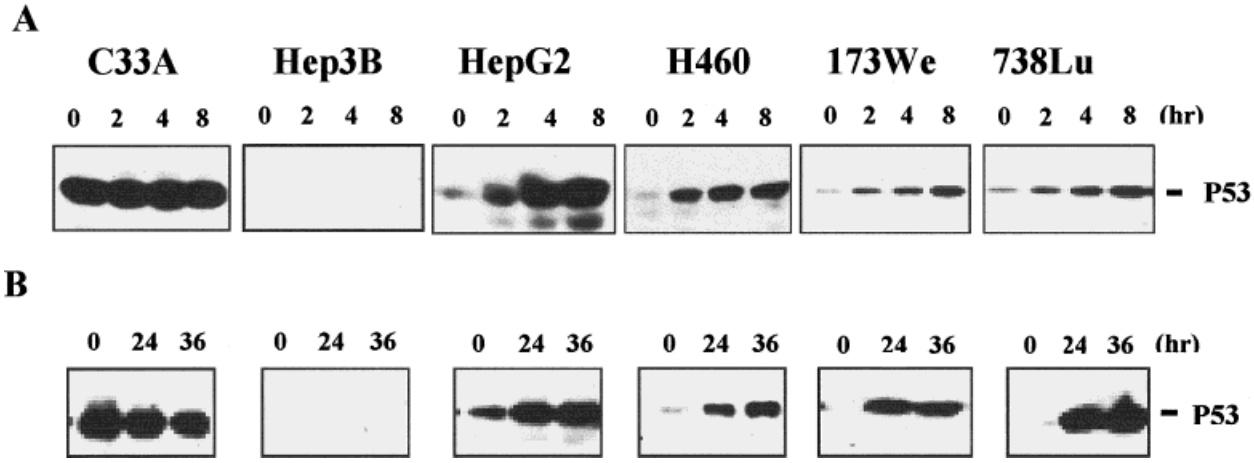

FIGURE 5 – P53 induction by DNA-damaging agent or YKL-1 infection. C33A, Hep3B, HepG2, H460, 173We and 738Lu were treated with camptothecin or YKL-1 for variable times as shown above. Cells were harvested and lysed with cell lysis buffer. 10% reducing SDS-PAGE was performed and p53 was visualized by immunoblotting. Equal amounts of protein were loaded in each lane, which was confirmed by immunoblotting to the identical blot with α-actin (data not shown). (*a*) Expression of p53 by 1 μM camptothecin. lane 1, 0 hr; lane 2, 2 hr; lane 3, 4 hr; and lane 4, 8 hr treatment. (*b*) Expression of p53 by YKL-1 treatment. lane 1, 0 hr; lane 2, 24 hr, and lane 3, 36 hr infection.

Virus replication and cytopathic effects of YKL-1 in a variety of human cancer cells

The characteristics of the cell lines used in our study are summarized in Table I. All 3 different types of normal human cells are expected to contain wild type p53 in genotype with normal functional activity, whereas human cancer cells employed in our study exhibited p53 with variable status in the genotype.

To investigate whether YKL-1 is able to replicate and induce cytopathic effects in human cancer cells, human cancer cell lines of lung, liver or cervical origin, as shown in Table I, were infected with YKL-1, wild-type ad-XJ or replication-deficient counterpart ad-ΔE1. Virus production and cytopathic effect were examined by comparison with an ad-XJ infection. Figure 2 shows that C33A or Hep3B containing inactivated p53 caused by point mutation or homozygous deletion were readily cytolysed after infection by both ad-XJ and YKL-1 with a minor reduction in virus production (55% and 47.5% relative to ad-XJ). In addition, SK-Hep1 with a rearranged p53 in sequence was found to be relatively sensitive to YKL-1, with less than one log reduction cytotoxicity and 37% in virus production. On the contrary, HepG2, Hela and H460 with wild-type p53 in sequence exhibited a relatively resistant phenotype with approximately a 2 log reduction cytotoxicity to YKL-1, compared with C33A or Hep3B (Fig. 2*a*). The virus replication rate of YKL-1 was 30.2%, 16.5% and 2.5% in HepG2, Hela and H460, respectively (Fig. 2*b*). In addition, the sensitivity of cancer cells to YKL-1 was nearly identical to that of ONYX-015 (data not shown).

Severe attenuation of virus replication and the cytopathic effect of YKL-1 in normal human cells

To investigate the capability of virus replication and cytolysis of YKL-1 in normal human cells, FHs 738Lu, FHs 173We and primary human fibroblast (Table I) were infected separately with YKL-1, ad-XJ and adΔE1, and virus replication and cytotoxicity was examined as described in Figure 2. All 3 normal human cells infected with YKL-1, even at the highest MOI of 10, exhibited no significant cytopathic effects until more than 12 days after infection, whereas the identical cells underwent complete cytolysis after infection with ad-XJ within 8 to 12 days at an MOI as low as 0.1 (Fig. 3*a*). In addition, virus production sharply decreased in the cells infected with YKL-1, only reaching 0.1 to 1% of ad-XJ in all 3 normal human cells (Fig. 3*b*). These results, therefore, indicated that virus production and subsequent cytolysis after infection with YKL-1 was dramatically attenuated in all 3 normal human cells. Cytotoxicity and virus production rate of YKL-1 in normal human cells were substantially lower than that of all the cancer cells investigated in our study (Figs. 2,3). Attenuated sensitivity of normal human cells to YKL-1 was nearly comparable to that of ONYX-015 (data not shown). Therefore, these results showed that all the human cancer cells examined are more sensitive to YKL-1 with a broad spectrum of sensitivity than normal human cells.

p53 functional status in normal human cells and cancer cells

p53 functional status can be predicted by the endogenous p53 level and its transcription activity without any stimulation (Butz *et al*., 1995). To examine whether the status of p53 gene expression was relevant to the sensitivity of the cells to YKL-1, we investigated the endogenous expression of p53 by immunoblotting analysis (Fig. 4*a*). As expected, extremely low levels of p53 were detected in both normal human cells (FHs 173We and FHs 738Lu) and cancer cell lines, such as HepG2, Hela and H460, which contain the wild-type p53 in sequence. On the contrary, p53 was heavily detected in C33A, which contains an inactivating CGT-to-TGT (arginine-to-cysteine) mutation at codon 273, due to a markedly increased half-life of the inactivated mutant p53 (Butz *et al*., 1995; Vogelstein and Kinzler, 1992). A higher level of p53 in SK-Hep1 than in normal cells has been detected previously (Bressac *et al*., 1990), which is an indicative of abnormal p53 functional status. p53 could not be detected in Hep3B cells with a homozygous deletion of the p53 gene (Bressac *et al*., 1990). To measure the endogenous transcriptional activity of p53, transient transfection analysis was performed using the p53-responsive luciferase reporter plasmid (Fig. 4*b*). H460 and HepG2 expressed 453.5 and 288.2-fold inductions and Hela and SK-Hep1 resulted in 20.7- and 15.6-fold inductions of luciferase activity, while Hep3B and C33A exhibited only a 1- to 2-fold inductions. Hela is the cervical cancer cell line infected with human papilloma virus, which results in abnormal p53 function, which may be inferred by the lower transcription activity of endogenous p53, compared with H460 (20.7 *vs*. 435.5-fold inductions; Scheffner *et al*., 1990).

Next, we investigated the induction rate of p53 by DNA-damaging agents or YKL-1 infection (Fig. 5). The cells were treated with camptothecin or YKL-1 for variable times and p53 expression was visualized by immunoblotting. p53 was efficiently induced in all 3 normal cells, H460 and HepG2 as expected, whereas p53 levels retained high in C33A and were undetectable in Hep3B regardless of the type of the treatment. Taken together with Figure 4, Figure 5 suggested that the relative sensitivity of different tumor cells to YKL-1 is considerably correlated with the functionality of p53.

Antitumoral efficacy of KL-1 in nu/nu mice.

To demonstrate the antitumoral effects of YKL-1, tumor growth was determined after YKL-1 was injected intratumorally into C33A (Fig. 6) or Hep3B (Fig. 7) tumor xenografts in *nu/nu* mice. Significant inhibition of tumor growth in both C33A and Hep3B xenografts was observed in the experimental group with live YKL-1 compared with UV-inactivated YKL-1, which was used as the control group. As more live YKL-1 was injected, more profound growth inhibition was detected (Fig. 7*a*). In addition, an increased animal survival rate was observed in both xenografts, and 4 out of 6 animals that received injections of YKL-1 at 1X109 PFU in Hep3B xenograft were tumor-free for 2 months after complete tumor regression (data not shown). H/E staining clearly indicated the profound necrosis in C33A (Fig. 6*b*) and Hep3B (data not shown) tumor xenograft with live YKL-1. In addition, we did not detect any significant tissue damage caused by adenovirus infection in the surrounding normal tissue. TUNEL assay (Fig. 7*b*) showed that active apoptosis occurred in the surrounding necrotic area, implying that an apoptotic event was probably one of the reasons for the regression of the Hep3B xenograft by YKL-1 (Fig. 7*a*). Therefore, these results demonstrate that the antitumoral effect of E1B 55 kDa-deleted YKL-1 adenovirus is dose dependent and induced by tumor necrosis and apoptosis following intratumoral administration.

DISCUSSION

Preferentially replication-competent adenovirus in tumor cells, but not in normal cells, is a highly attractive option, because it is unlikely that cancer gene therapy based on replication-defective adenovirus would be effective against large tumor masses in most human cancer patients. In our study, we generated E1B 55 kDa-deleted adenovirus YKL-1, which was confirmed by DNA sequencing, restriction enzyme digestion, PCR and immunoblotting analysis, and then we investigated its potential as an oncolytic adenoviral vector.

The therapeutic efficacy of E1B-attenuated adenovirus ONYX-015 for cancer treatment has been investigated previously (Bischoff *et al*., 1996; Heise *et al*., 1997) and YKL-1 exhibited a similar pattern of therapeutic efficacy to ONYX-015 *in vitro* and *in vivo* in this investigation. The E1B 55 kDa-deleted adenovirus (YKL-1) we generated is distinct from ONYX-015 in construction strategy and genomic structure. Instead of deleting a portion of E1B 55 kDa, it was generated by reintroducing E1A and E1B 19

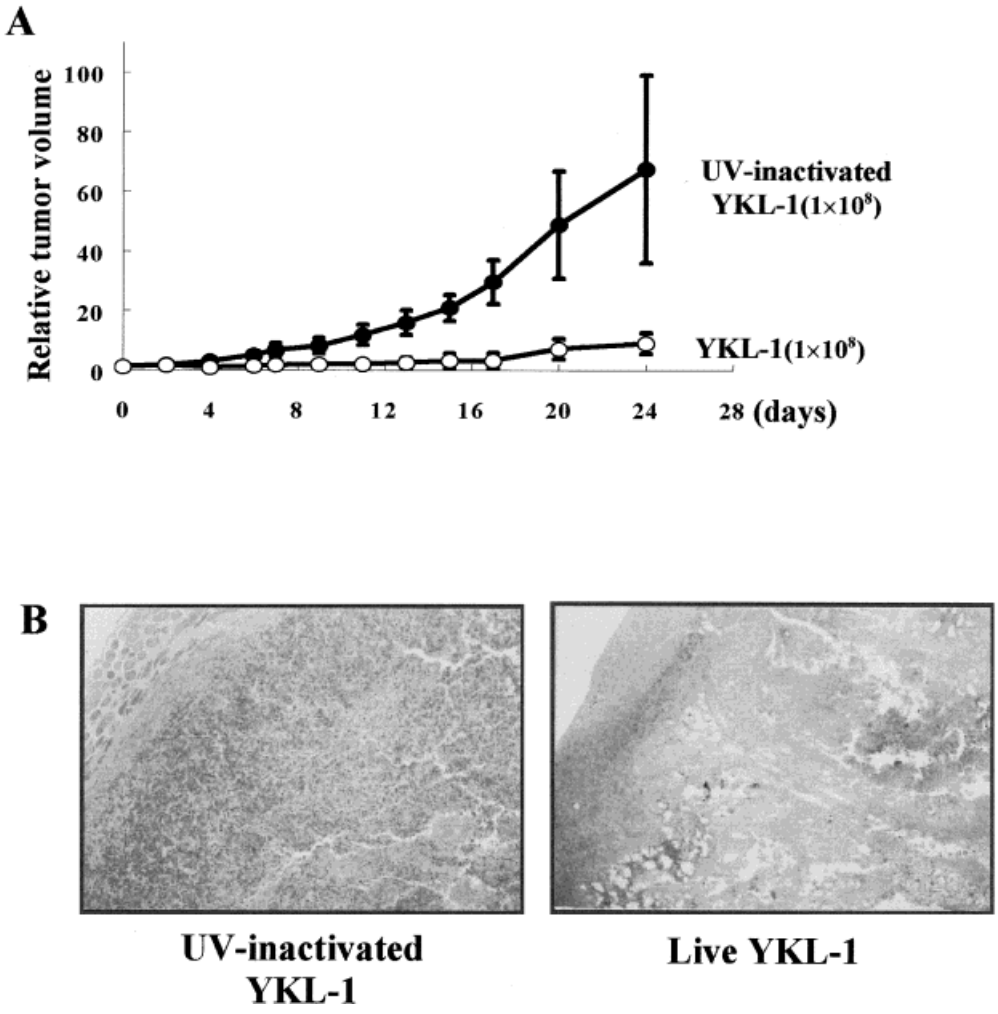

FIGURE 6 – Effect of YKL-1 on C33A xenograft. 1×10^7 cells were injected subcutaneously into the flanks of *nu/nu* mice. Once tumor volume reached about 80 μl in size, live or UV-inactivated YKL-1 was injected. Live or UV-inactivated YKL-1 of indicated PFU was injected 3 times intratumorally once every other day. Tumor growth was measured 3 times weekly by a caliper until the end of the study. Relative tumor growth was analyzed using the following equation: tumor volume at the time point of analysis/tumor volume at the time of the first virus injection. (*a*) Antitumoral effect of live YKL-1. (*b*) H/E staining. Fifteen days after the injection of live or UV-inactivated YKL-1, the animals were sacrificed and H/E staining was carried out in formalin-fixed paraffin-embedded tumor sections. Tumor specimens injected with UV-inactivated YKL-1 (left panel) demonstrated active proliferation of tumor cells and the specimen injected with live YKL-1 (right panel) exhibited tumor necrosis.

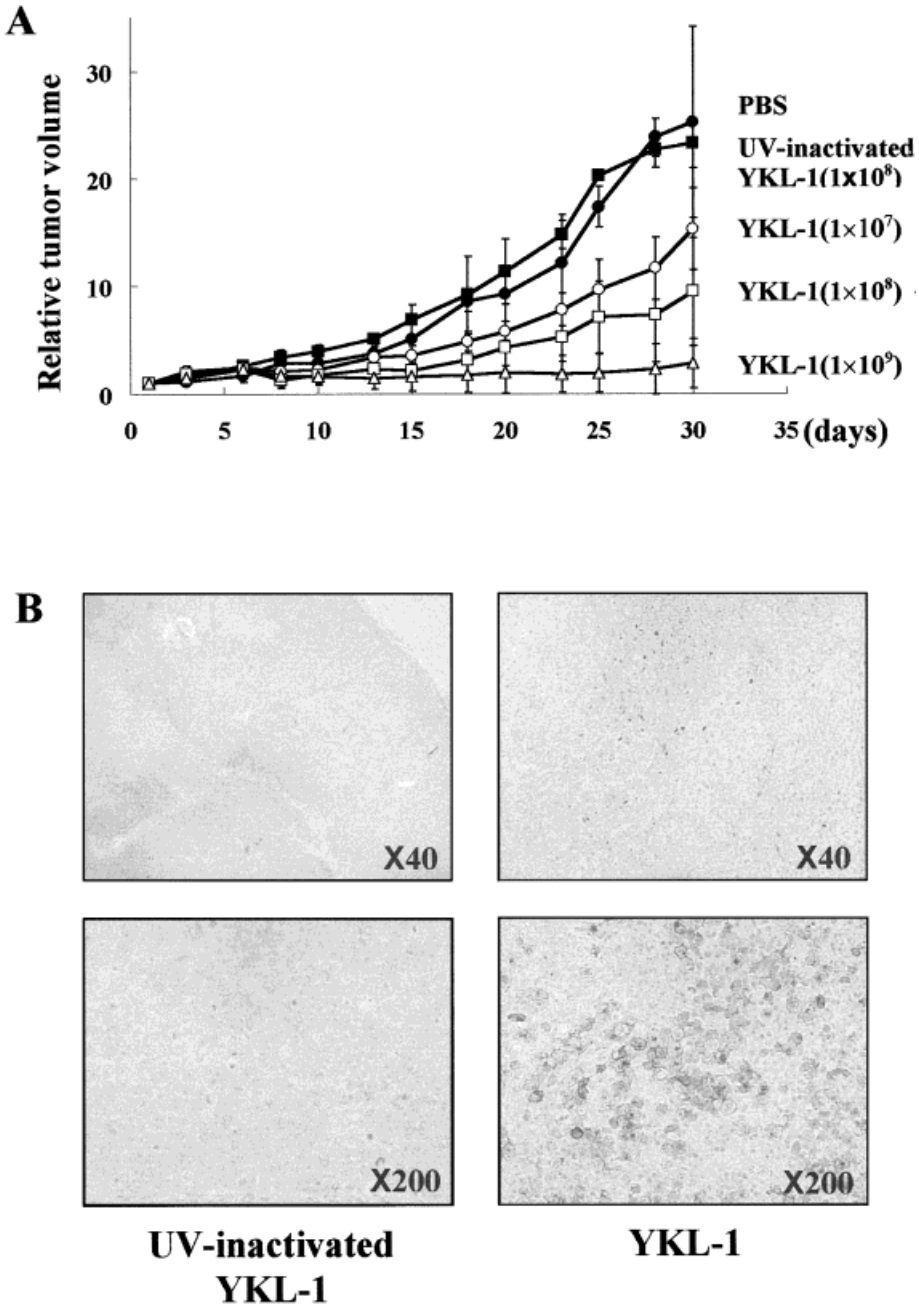

FIGURE 7 – Effect of YKL-1 on Hep3B xenograft. Hep3B xenograft was prepared and live or UV-inactivated YKL-1was administrated as described in Figure 6. (*a*) Relative tumor volumes by differently administered viral doses. (*b*) Tunnel assay of Hep3B human tumor xenografts. Fifteen days after the injection of live (right panel) or UV-inactivated YKL-1 (left panel), animals were scarified and TUNEL assay was conducted in formalin-fixed paraffin-embedded tumors. The right panel demonstrates numerous apoptotic cells.

kDa genes into E1/E3-deleted adenoviral vectors, which resulted in a larger deletion in E1B 55kDa than ONYX-015 (1,260 bp; 84% deletion of E1B 55 kDa *vs.* 827 bp). In addition, YKL-1 contains a 1.7 kb additional deletion in the E3 region, which provides higher capacity for the insertion of a foreign gene (4.9 kb *vs.* 2.7kb), and thereby provides enough space for various therapeutic gene expression cassettes. Finally, therapeutic genes can be easily inserted into YKL-1 vector at the BglII site, which was created at the 3′ end of restored E1.

The virus replication and cytotoxicity of YKL-1 was markedly attenuated in all 3 normal human cells examined in our study, which corresponds to at least a 2–3 log difference compared with that of wild-type adenovirus (Fig. 3). By contrast, under identical conditions, YKL-1 replicated and induced a cytopathic effect in all cancer cells with a broad spectrum of sensitivity (Fig. 2). These results indicated that the oncolytic potential of the YKL-1 is a reminder of a preferential cytotoxicity of ONYX-015, which confirms previously published reports concerning the ability of an E1B 55 kDa-deleted adenovirus to selectively replicate in most human cancer cells (Heise et al., 1997; Vollmer et al., 1999). In addition, the growth inhibition of both C33A and Hep3B xenograft in *nu/nu* mice was evident in a dose-dependent manner, by the intratumoral injection of YKL-1. H/E staining implied that severe necrosis was caused by live YKL-1, and TUNEL staining in the Hep3B xenograft indicated that active apoptosis on the periphery of the necrotic area was also induced by live YKL-1 (Fig. 6). This

result indicated that YKL-1 retaining E1B 19 kDa (which is well known for its anti-apoptotic properties) can drive apoptosis and initialize Hep3B cell death in the absence of functional p53 (Shen and Shenk, 1994). E1A oncogene has been identified to induce cellular apoptosis via p53 dependent and independent mechanisms (Shenk, 1996; Nakajima *et al*., 1998; Cook *et al*., 1999). Furthermore, E1A induced apoptosis via the p53 independent pathway was found to be resistant to blockage by E1B 19 kDa (Cook *et al*., 1999).

Recently, there has been a number of reports, which have raised questions about the use of E1B 55 kDa-deleted adenovirus in cancer gene therapy, by demonstrating a weak correlation between the p53 status of the cells and virus production, virus gene expression and the cytopathic effect of E1B 55 kDa-deleted adenovirus agent (Hall *et al*., 1998; Goldsmith *et al*., 1998; Goodrum and Ornelles, 1998; Rothmann *et al*., 1998; Harada and Berk, 1999; Hay *et al*., 1999; Turnell et al., 1999). However, there are several points, which should be addressed. Firstly, studies on the selectivity by p53 in most cases have relied on sequence information to determine the status of p53 in cancer cells, but it is widely accepted that as a multifunctional transcription modulator, p53 is at least partially nonfunctional in most cancer cells beyond the p53 sequence (Chang *et al*., 1995; Leach *et al*., 1993; Marchetti *et al*., 1995; Scheffner *et al*., 1990; Joseph and Vogt, 1996). In fact, our data supports the notion that the oncolytic potential of E1B 55 kDa-deleted adenovirus seems to be significantly related to the endogenous p53 functional status in both human normal cells and cancer cells (Figs. 2–5). All 3 different types of normal human cells examined in our study with functional p53 exhibited a resistant phenotype to YKL-1 in terms of both virus replication and resultant cytolysis (Figs. 3– 5). Moreover, Hep3B and C33A cancer cells, with aberrant p53, which is indicated by the lack of induction of p53 after treatment with camptothecin or YKL-1, exhibit the most sensitive phenotype to YKL-1. HepG2 and H460 were found to possess indistinguishably functional p53, compared with that of human normal cells in current study (Figs. 4,5). Yet, these cancer cells were more sensitive to YKL-1 than normal human cells (Figs. 2,3). These results may imply that p53 is partially inactivated in HepG2 and H460, which we were not able to detect in our study. The other possibility is that human cancer cells are more sensitive to YKL-1 by some other mechanism. Secondly, most studies on the potential of E1B-deleted adenovirus have been carried out on cancer cells only and focused on cytotoxicity or virus replication at a very early stage of infection with high MOI. This condition may not allow enough time or a proper environment for the virus to selectively replicate and result in a cytopathic effect. Thereby, this may cause limitations in terms of properly representing the practical situation in the tumor mass with infections induced at low MOIs, due to an unevenly distributed virus load. In fact, Vollmer *et al*. (1999) recently reported that p53 selective replication of an E1B-deleted adenovirus in hepatocellular carcinoma occurs only at a low viral dose. Nevertheless, the correlation between p53 functional status and the oncolytic potential of E1B 55 kDa-deleted adenovirus needs to be explored more extensively and the use of E1B 55 kDa-deleted adenovirus should be carefully verified *in vitro* and *in vivo* in each cancer model.

Recently, Wildner *et al*. generated E1B 55 kDa-deleted adenovirus expressing herpes simplex virus-thymidine kinase (HSV-TK) and exhibited much-improved therapeutic efficacy in a human cancer xenograft model (Wildner *et al*., 1998, 1999). In addition, Freytag et al. suggested the possibility of combining oncolytic potential, suicidal gene therapy (HSV-TK and CD) and radiotherapy (Freytag *et al*., 1998). Finally, Heise *et al*. showed that the therapeutic efficacy of E1B 55 kDa-deleted adenovirus could be augmented by standard chemotherapeutic agents, such as cisplatin or 5-FU (Heise *et al*., 1997). YKL-1 infection also enhanced the cytotoxicity of cancer cells to chemotherapeutic agents, including adriamycin, but not in normal cells (data not shown).

Coupled with the data presented here, E1B 55 kDa-deleted adenovirus YKL-1 seems to offer promising potential as an oncolytic agent and as a selectively replication-competent adenovirus vector capable of delivering therapeutic genes preferentially into tumor cells.

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