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Midkine promoter-driven suicide gene expression and mediated adenovirus replication produced cytotoxic effects to immortalised and tumour cells

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Abstract

We examined possible application of a regulatory region of *midkine (MK)* gene, which is frequently upregulated in a number of human tumours but not in normal cells, to cancer gene therapy. We examined transcriptional activity of the *MK* genomic fragments in paired cell lines, immortalized cells and their parental normal fibroblasts, and found that the *MK* fragments activated a fused reporter or a suicide gene preferentially in the immortalized cells. Recombinant adenoviruses (Ad), in which the *MK* fragment was inserted upstream to the *E1A* gene (AdMK), replicated preferentially in the immortalized cells and were cytotoxie to them. Human hepatocellular carcinoma cells were significantly susceptible to AdMK compared with human normal fibroblasts *in vitro* and the replication of AdMK was less than that of wild-type Ad in the infected fibroblasts. Hepatocellular carcinoma cells infected with AdMK did not form tumours in immunocompromised mice and intratumoural injection of AdMK into the hepatocellular carcinoma developed in mice retarded the subsequent tumour growth. Expression of E1A and necrosis of tumours were detected in AdMK-injected but not control Ad-injected cases. The MK promoter-driven suicide gene therapy and -mediated replicative Ad can thereby produce cytotoxic effects to immortalized and tumour cells with minimal damage to normal cells.

Keywords: Midkine; Promoter; *HSV-TK*; Adenovirus

1. Introduction

Selective expression of foreign genes in tumours contributes to the safety and the efficacy of cancer gene therapy. Regulatory regions of the genes that are preferentially activated in tumours enable regulated gene expression in tumours and can produce cytotoxic effects specific to target tumours. Previous studies showed that tumour or tissue-specific promoters, such as α-fetoprotein and c-erbB-2 promoter, transcribed an exogenous gene and could induce cell death in the targets by the restricted expression of a suicide gene such as the *herpes simplex virus-thymidine kinase (HSV-TK)* gene [1,2]. Recent studies also demonstrated that recombinant adenoviruses (Ad) type 5, in which the *E1A* gene expression is controlled by an exogenous promoter, killed the infected cells and released Ad consequently damaged surrounding cells [3]. The ElA of Ad is an immediate early gene product responsible for initiating S-phase entry and viral replication in the infected cells; therefore, regulated E1A expression by such tumour or tissue-specific promoters results in preferential Ad replication in directed cells. However, the promoters characterized

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were often lineage-specific and has a narrow range of applicable tumours.

The regulatory region of the *midkine (MK)* gene, which is frequently upregulated in a number of tumours despite its limited expression in normal adult tissues [4,5], could activate a fused exogenous gene in a variety of tumours. The MK promoter can thereby be applicable to a broader range of tumours in contrast to tissue-specific promoters. Although MK is a heparin-binding secretory protein with multiple functions, such as cell transformation and angiogenesis [6,7], the mechanism of upregulated gene expression in tumours is not well characterized. We previously demonstrated that transfer of the *HSV-TK* gene, linked with a 2.3-kb *MK* regulatory region, could Kil tumour cells but not normal fibroblasts [8]; however, the upregulated promoter activity found in tumour cells could be due to histological difference between tumourous and non-tumourous regions tested. In this study, we examined the transcriptional activity of the MK promoter using paired cell lines, immortalised cells and parental normal fibroblasts, to exclude the possible histological difference, and investigated cytotoxicity induced by the MK promoter-mediated suicide gene expression. We also prepared recombinant Ad whose E1A expression was controlled by the MK promoter and investigated whether the Ad replicated preferentially in tumours and produced antitumour effects *in vivo*.

2. Materials and methods

2.1. Cells and mice

Immortalised OUMS-24/P6X cells, which were established from human normal fibroblasts OUMS-24 cells by the expression of a dominant-negative *p53* gene and irradiation [9,10], human hepatocellular carcinoma (HCC), HuH-7 (from Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) HLE and HLF cells, human normal fibroblasts HFF, MRC-5 and CDC-18Co, and HEK293 cells were cultured in Reswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) fetal calf serum. Six-week old female BALB/c *scid/scid* severe combined immunodeficient mice (SCID) mice were purchased from CLEA Japan (Tokyo, Japan).

2.2. Dual luciferase assay

The 2335-bp genomic DNA fragment of the *MK* gene (MK2.3kb) ($-2285/+50$, $+1$ corresponds to the transcription start site) [11,12] was cloned into the pGL2-basic vector (Promega, Madison, WI) that contained the *firefly luciferase* gene without any promoter sequences (MK2.3kb-luc). MK2.3kb-luc DNA was digested with *Xho*I/*Not*I and *Xho*I/*Eco* 47 III to produce the 1041-bp and the 609-bp fragment-linked *firefly luciferase* genes (MK1.0kb-luc, MK0.6kb-luc), respectively. The 319-bp ($-285/+34$) and the 70-bp ($-20/+50$) MK fragments were produced by polymerase chain reactions (PCRs) and both fragments were ligated into the pGL2-basic vector (MK0.3kb-luc and MK70bp-luc). The transcriptional activity mediated by each genomic fragment was measured with the luciferase reporter assay system (Promega). Plasmid DNA containing respective MK genomic fragments was transfected into target cells with a lipofectin reagent (Life Technologies, Gaithersburg, MD). Cells were lysed 2 days later and the luciferase activities were measured according to the manufacturer's protocol. The firefly luciferase activity of each cell lysate was calculated based on the amount of luminescence per microgram of cell lysate. All the values were expressed as a mean of three independent experiments. The statistical analysis was performed by a one-way analysis of variance.

2.3. Preparation of Ad

Recombinant Ad type 5 containing the 0.6-kb MK promoter (AdMK) were prepared according to Rodriguez's method [13]. A unique *Age* I restriction site (at Ad nucleotide 522) was created in the pXC1 vector (Microbix Biosystems, Toronto, Canada) and the MK fragment was inserted into the *Age* I site. The DNA was transfected into HEK293 cells with the pBHGE3 vector (Microbix Biosystems) to produce recombinant Ad by homologous recombination. The Ad were propagated by repeated infection to HEK293 cells. Wild-type Ad (AdWT) and *E1A*-deleted, replication-defective Ad containing the *green fluorescent protein* gene (AdGFP) (Dr. H. Hamada, Sapporo Medical University, Japan) were also propagated with HEK293 cells.

2.4. In vitro cytotoxicity test

The 0.6kb-MK fragment was ligated into the pcDNA3 vector (Invitrogen, San Diego, CA) from which the cytomegalovirus promoter was removed. The *HSV-TK* gene was cloned downstream to the MK promoters (*MK-TK*). Cells were transfected with the MK-TK DNA and G418 (Life Technologies)-resistant cells were selected. The pooled cells, as well as parental cells, were seeded in to 96-well plates at a density of 5×10^3 cells/well and cultured with various concentrations of the prodrug ganciclovir (GCV). After 5 days, viable cell numbers in each well were measured using a cell-counting kit (Wako, Osaka, Japan). The amount of formazan produced in each well was determined from the absorbance at 450 nm. For Ad-mediated cytotoxicity, cells seeded in 96-well plates (1×10^3 cells/well) were infected with Ad at various multiplicities of infection

(MOI). Viable cell numbers on day 6 were measured with the kit.

2.5. Replication of Ad

Cells were infected with Ad at a MOI of 5 for 3 h and then the virus was removed. The cells were cultured for 2 days and the cell lysate was prepared with three cycles of freezing and thawing. HEK293 cells in 96-well plates (3×10^3 cells/well) were infected with serially-diluted lysate. After 8 days, wells in which more than 50% of the cells showed cytopathic effects were counted and 50% tissue culture infectious doses ($TCID_{50}$) [14] was calculated. The number of viral particles was expressed as $TCID_{50}$.

2.6. Animal study

For the tumorigenesis analysis, HuH-7 (7.5×10^6) cells were incubated AdMK or AdGFP at a MOI of 100 for 30 minutes and then subcutaneously (S.C.) inoculated into SCID mice. Phosphate buffered-saline (PBS)-treated HuH-7 cells were also inoculated as controls. Tumour volume was calculated according to the formula [$1/2 \times \text{length} \times \text{width}^2$]. In treatment experiments, SCID mice were S.C. inoculated with HuH-7 (1×10^7) cells. When the tumours became 4–5 mm in diameter (tumour volume was approximately 20–30 mm^3), Ad (1×10^9 plaque forming units (pfu), 0.1 ml/mouse) or PBS (0.1 ml/mouse) was injected into the tumours on days 7–9, 13 and 14. The mice were sacrificed when the tumour volume reached 4000 mm^3.

2.7. Western blotting and histological examination

HuH-7 tumours developed in SCID mice were injected with AdMK or AdGFP (1×10^9 pfu, 0.1 ml). The tumours and muscle tissues beneath the tumours were lysed after 24 hr and the lysate was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Expression of E1A was analysed with anti-Ad *E1A* antibody (Santa Cruz Biotech, Santa Cruz, CA) followed by horseradish peroxidase-conjugated anti-mouse IgG antibody (Southern Biotech, Birmingham AL). The membrane was developed with the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK). The sections of 4- or 5-μm thickness from the frozen tumour masses were stained with haematoxylin-eosin.

3. Results

3.1. Transcriptional activity and MK promoter-mediated cytotoxicity

We previously examined the transcriptional activity of the MK regulatory regions in tumours and found that the 0.3-kb genomic fragment contained *cis*-acting element(s) capable of transcribing a fused gene [15]. We examined the activity of the MK fragments in paired fibroblast lines, normal parental OUMS-24 and immortalised OUMS-24/P6X cells, to clarify the region which transcribed a reporter gene least in the normal cells. Comparison of the transcriptional activity showed that the MK promoter activity was greater in the OUMS-24/P6X cells, and that the ratio of the transcriptional activity between the paired cells was higher with the 0.3- and the 0.6-kb MK fragments than with other fragments ($P < 0.05$) (Fig. 1(a)). The upregulated MK promoter activity in tumours is thus not due to the histological differences between tumours and normal tissues, but correlates with cell immortalisation. The transcriptional activity of the 0.6-kb MK fragment was comparable to that of the Simian Virus40 (SV40) promoter ($142\% \pm 12.1$ SEM (standard error of mean)) in the OUMS-24/P6X cells.

We transfected OUMS-24 and OUMS-24/P6X cells with MK-TK DNA harboring the 0.6-kb *MK* fragment, and G418-resistant cells were examined for their sensitivity to various concentrations of GCV (Fig. 1(b)). Susceptibility to GCV did not differ between the untransfected and transfected OUMS-24 cells, while OUMS-24/P6X cells transfected with the MK-TK DNA were significantly sensitive to GCV compared with the untransfected cells. The sensitivity of vector DNA-transfected cells to GCV was not different from that of the respective untransfected cells (data not shown). The HSV-TK/GCV system mediated by the MK promoter was therefore cytotoxic in immortalised, but not in normal cells.

3.2. Cytotoxic activity of Ad containing the MK promoter

We constructed Ad containing the 0.6-kb MK promoter in the 5' region of the *E1A* gene and examined their cytotoxic activity (Fig. 2(a)). AdMK was cytotoxic to OUMS-24/P6X, but not to OUMS-24 cells, whereas AdWT killed OUMS-24 more effectively than OUMS-24/P6X cells. Viral replication was assayed by the number of viral particles released from the infected cells (Fig. 2(b)). OUMS-24/P6X cells produced larger amounts of virus particles than OUMS-24 cells irrespective of the Ad, and the production of AdMK was greater than that of AdWT. In contrast, OUMS-24 cells infected with AdWT produced larger amounts of Ad than those infected with AdMK ($P = 0.013$).

We also examined the cytotoxic activity of AdMK in human HCC cells (HuH-7, HLE and HLF) and normal fibroblasts (Fig. 3(a)). All the HCC cells tested were susceptible to AdMK, but normal fibroblasts were relatively resistant to AdMK. Viral production in the HCC cells did not differ between the AdMK- and AdWT-infected cases (Fig. 3(b), $P > 0.05$); however, fibroblasts

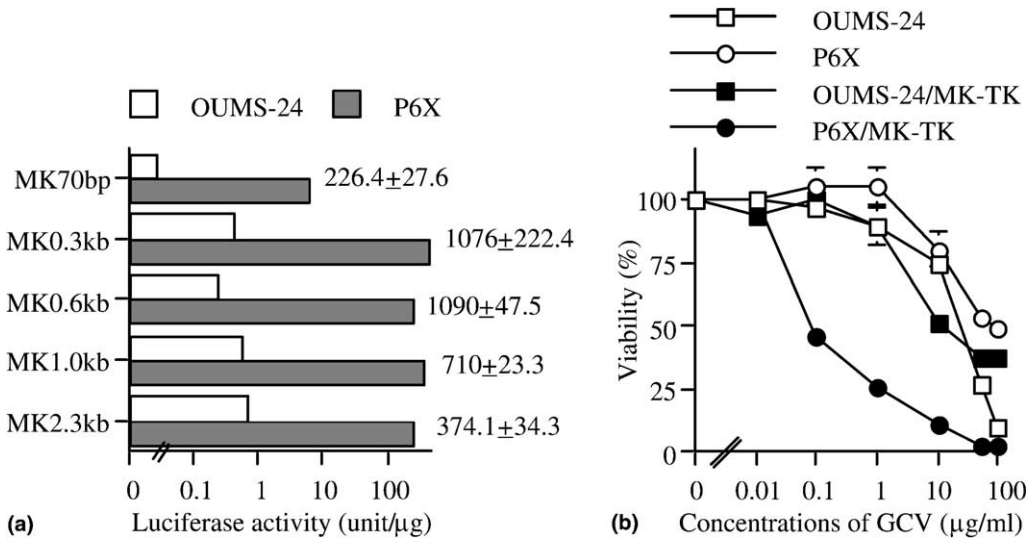

Fig. 1. (a) Transcriptional activity of the MK genomic regions. Luciferase activities were assayed in OUMS-24 and OUMS-24/P6X (P6X) cells transfected with the MK fragment-linked *luciferase genes*. The activity ratios of OUMS-24/P6X to OUMS-24 cell lysates are also shown with standard errors. (b) Cytotoxic activity of the HSV-TK/GCV system to OUMS-24 and OUMS-24/P6X cells. Sensitivity to various concentrations of GCV was examined in the untransfected and MK-TK-transfected cells. Standard error bars are also shown.

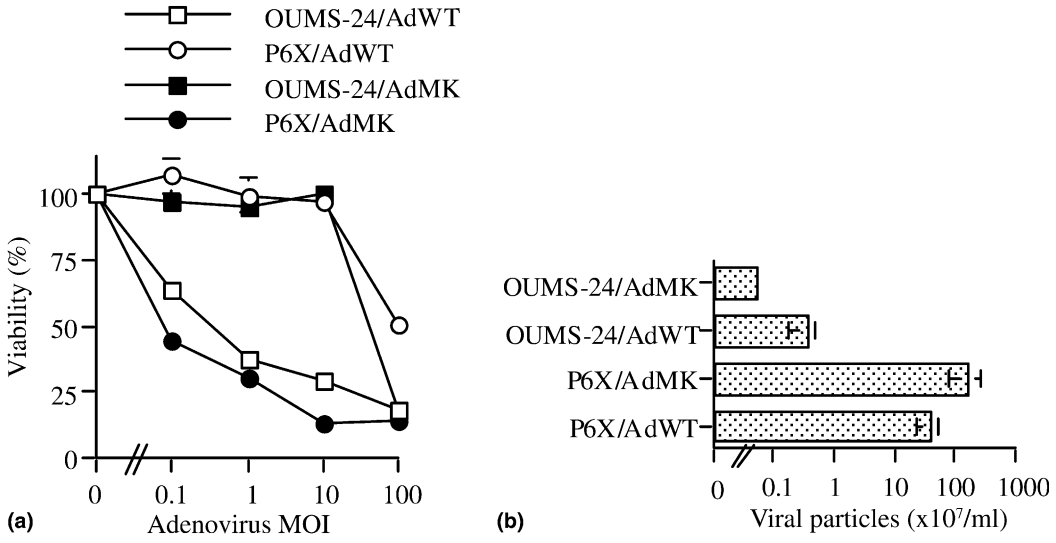

Fig. 2. (a) Cytotoxicity of AdMK and AdWT to OUMS-24 and OUMS-24/P6X (P6X) cells. Cells were infected at various multiplicities of infection (MOI). Standard error bars are also shown. (b) Viral replication in OUMS-24 and OUMS-24/P6X cells that were infected with either AdMK or AdWT. The numbers of viral particles were assayed with $TCID_{50}$ using HEK293 cells.

infected with AdMK produced less amounts of Ad than those with AdWT in all the cell types (Fig. 3(c), $P < 0.05$). Replication of AdMK in normal fibroblasts was thereby restricted whereas AdMK replication in HCC was not augmented compared with AdWT replication.

3.3. Antitumour effects of AdMK

We examined AdMK-mediated antitumour effects using two methods. HuH-7 cells were treated with AdMK, AdGFP or PBS and then inoculated into SCID mice ($n = 12$). Mice injected with AdMK-treated HuH-7 cells did not develop tumours (11/12 cases), whereas mice injected with AdGFP-treated (11/12) or PBS-treated (12/12) HuH-7 cells developed tumours (Fig. 4(a)). Antitumour effects *in vivo* was also investigated by intratumoral administration of Ad into S.C. HuH-7 tumours in SCID mice (Fig. 4(b)). Injection of both AdMK and AdGFP retarded subsequent tumour growth compared with the PBS-injected group and growth retardation was greater in the AdMK-injected than in the AdGFP-injected group ($P < 0.01$, after day 16). Expression of E1A in the tissues that had been in-

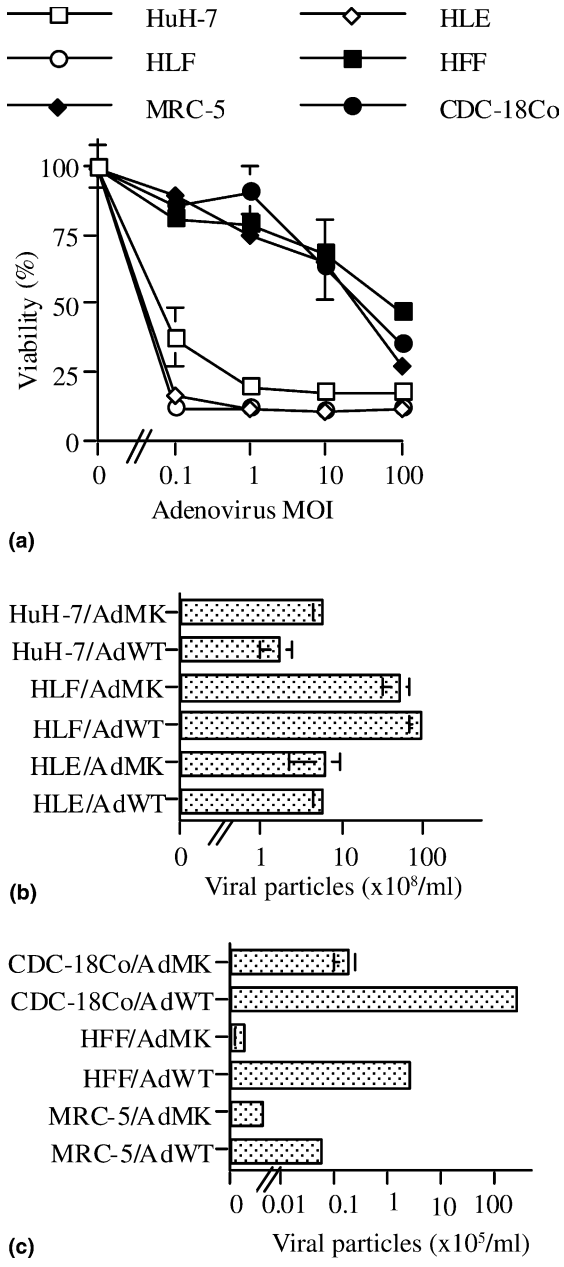

Fig. 3. (a) Cytotoxicity of AdMK to HCC cells (open symbols) and normal fibroblasts (closed symbols). Cells were infected at various MOI. Standard error bars are also shown. Viral replication in HCC cells (b) and normal fibroblasts (c) that were infected with either AdMK or AdWT. The numbers of viral particles was assayed as a $TCID_{50}$ using HEK293 cells.

jected with AdMK was examined with Western blot analysis (Fig. 5). The E1A protein was detected in the AdMK-injected, but not in the AdGFP-injected HuH-7 tumours nor in the muscle tissue beneath the AdMK-injected tumours. We also examined histologically HuH-7 tumours injected with either AdMK or AdGFP and found massive necrosis and pyknotic changes in the AdMK-injected but not in the AdGFP-injected tumours (Fig. 6).

4. Discussion

We demonstrated that the MK promoter was preferentially activated in immortalised, but not in parental fibroblasts, and that the promoter-mediated HSV-TK/GCV system and replicative Ad produced cytotoxic effects.

Previous studies showed that tumour- or tissue-specific promoters could activate cytotoxic genes in target cells and produce antitumour effects [1,2,16]; however, relatively weak transcriptional activity or narrow target spectrums for these cellular promoters can be a drawback in their wider application in cancer gene therapy. In contrast, the MK promoter is active in a number of tumour types and its transcriptional activity is stronger than that of the SV40 promoter [8,15]. Recently, we examined the *MK* gene expression in breast cancer and surrounding non-tumours specimens of the same patients, and found that upregulated MK expression in tumours was more frequent than the *c-erbB-2* gene [17]. Our present data showed that the increased MK promoter activity was related to cell immortalisation. Our previous report also suggested that the transcriptional activation of the MK promoter correlated partly with cell growth and partly with the *p53* gene status in the cells tested [15]. In fact, the cell proliferation rate of OUMS-24/P6X cells was greater than in the parental OUMS-24 cells (data not shown).

MK promoter-mediated *HSV-TK* gene expression showed that OUMS-24/P6X, but not OUMS-24, cells were susceptible to lower concentrations of GCV. In contrast, OUMS-24/P6X and OUMS-24 cells were susceptible to AdMK and AdWT, respectively. The differential susceptibility to Ad could be attributable to viral replication in the infected cells and to the cellular sensitivity to virus infection. Viral production in OUMS-26/P6X cells was greater in the AdMK-infected than in the AdWT-infected cells, while AdWT-infected OUMS-24 cells produced larger amounts of Ad than AdMK-infected cells. Although viral production of AdWT was greater in OUMS-24/P6X cells than in OUMS-24 cells, OUMS-24/P6X cells were more resistant to AdWT than OUMS-24 cells. This differential cytotoxicity is favourable to cancer gene therapy, since AdMK was cytotoxic to immortalised but not normal cells in contrast to AdWT. The selective cytotoxicity of AdMK was also demonstrated in HCC cells and normal fibroblasts; all the HCC cells tested were susceptible to AdMK, but fibroblasts were relatively resistant. Viral replication varied in the cells tested; nevertheless, all the fibroblasts produced less AdMK than AdWT, but the HCC cells produced comparable amounts of AdMK to AdWT. The numbers of AdMK particles produced were greater in HCC than in normal fibroblasts. These data suggest that the specificity of the MK promoter in the context of recombinant Ad allowed lower cytotoxicity to the

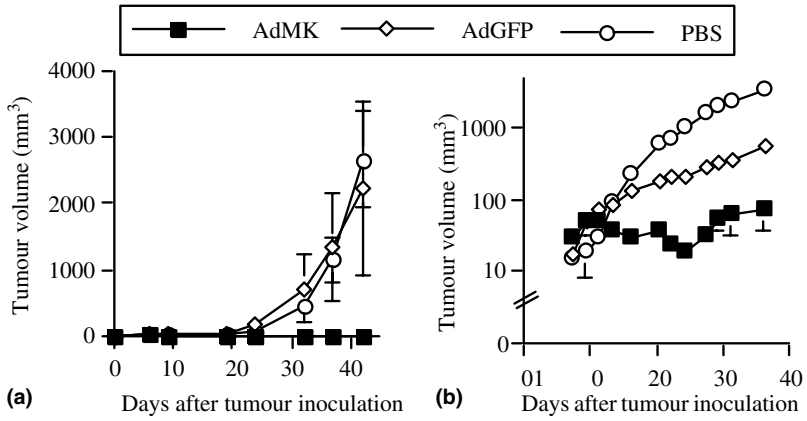

Fig. 4. (a) Tumorigenicity of HuH-7 cells in SCID mice after the cells were treated with AdMK, AdGFP or PBS ($n = 12$ per group). (b) Growth of HuH-7 tumours in SCID mice ($n = 6$ per group). When tumours became 4–5 mm in diameter, AdMK, AdGFP or PBS was injected intratumourally on day 7–9, 13 and 14.

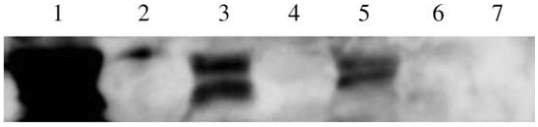

Fig. 5. Expression of E1A examined with Western blot analysis: AdWT-infected 293 cells (1, as a positive control), muscle tissue beneath the AdMK-injected HuH-7 tumours (2, 4), HuH-7 tumours injected with AdMK (3, 5) or AdGFP (6) and uninjected HuH-7 tumours (7). Two E1A bands migrated about at 48 and 38 kDa. The samples of muscle and tumour (2 and 3; 4 and 5) were obtained from the same mice.

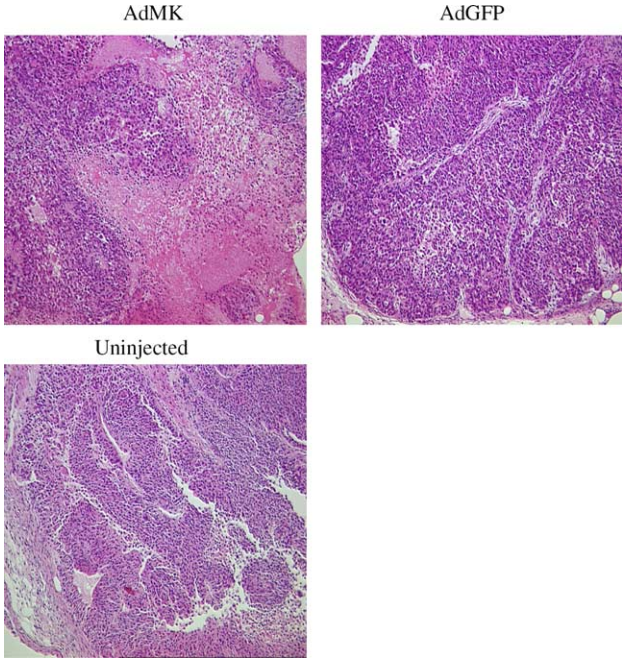

Fig. 6. Histological analysis of HuH-7 tumours that were injected with AdMK or AdGFP and uninjected HuH-7 tumours. (Haematoxylin-eosin-stained, original magnification × 200.)

normal cells rather than augmented cytotoxicity to tumour cells. Administration of AdMK produced antitumour effects *in vivo*, demonstrating possible clinical use of AdMK.

The promoter position in the context of replication-competent Ad influences its oncolytic activity. Transcriptional activity and specificity of an exogenous promoter localized downstream to the E1A regulatory region could be altered. In particular, read-through transcription initiated upstream to the integrated promoter could result in loss of specificity of the promoter [18]. We found small amounts of the E1A transcript initiated upstream to the MK promoter (data not shown), but the specificity of the MK promoter was maintained because most *E1A* transcripts were initiated by the MK promoter. Recent studies have shown that the Ad E3 region plays a role in augmenting Ad-mediated cell killing [19]. We therefore compared cytotoxicity induced by the AdMK either containing or lacking the E3 region, and found that AdMK with this region were more cytotoxic (data not shown).

Recently, promoters with a wider tumour target spectrum, such as the regulatory region of the *telomerase reverse transcriptase* gene or *hypoxia-inducible factor* α gene have been used for replication-competent Ad; these replication-competent Ad were effective for a variety of tumours, but relatively harmless in normal cells [14,20,21]. Another study demonstrated that the replication-competent Ad containing the 2.3-kb *MK* promoter killed several paediatric tumours without inducing liver damage [22], which is the major adverse reaction of Ad type 5-mediated gene transfer and cytotoxicity. We confirmed the relative safety of the untargeted tissues with a reverse transcription-PCR; the *E1A* gene was not expressed in the liver, spleen and kidney of the SCID and nude mice that were injected with AdMK (data not shown). Moreover, the antitumour effects produced by the replication-competent Ad were en-

hanced by combination with radiotherapy or chemotherapy [23,24]. However, the spread of the replication-competent Ad was hampered due to connective tissues and matrix within and around tumour masses [25]. Downregulated expression of the coxsackievirus and adenovirus receptor (CAR) in tumours has a negative impact on the effectiveness of replication-competent oncolytic Ad. Use of Ad with different tropisms, such as Ad type 35, can partly circumvent these disadvantages, because Ad type 35 infected cells of more effectively than Ad type 5 in CAR-independent manner [26]. In summary, replication-competent AdMK had reduced cytotoxicity to normal cells compared with immortalised and tumour cells. Further modifications, such as replacing the CAR-binding Ad type 5 fibre/knob structure with a non-CAR-binding Ad type 35 structure [27], could potentiate clinical feasibility of the MK promoter-mediated replication-competent Ad.

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