

Adenovirus-mediated Transfection of Caspase-8 Augments Anoikis and Inhibits Peritoneal Dissemination of Human Gastric Carcinoma Cells¹

Susumu Nishimura, Masaaki Adachi,² Tadao Ishida, Takahiro Matsunaga, Hiroaki Uchida, Hirofumi Hamada, and Kohzoh Imai

First Department of Internal Medicine [S. N., M. A., T. I., T. M., K. I.] and Department of Molecular Medicine [H. H.], Sapporo Medical University School of Medicine, Sapporo, 060-8543, and Department of Surgical Oncology, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655 [H. U.], Japan

Abstract

Caspase-8 is a member of the cysteine protease family that modulates apoptosis induced by a variety of cell death signals and has recently been found to be activated during the process of anoikis, which is a form of apoptosis caused by loss of anchorage in epithelial cells. We previously demonstrated that the inhibition of anoikis promotes peritoneal dissemination of human gastric carcinoma MKN45 cells, which are anchorage dependent. This suggests that augmentation of anoikis may suppress dissemination of carcinoma cells. To determine whether extrinsic overexpression of caspase-8 can augment anoikis in MKN45 cells, we transfected them with the *caspase-8* gene using an adenoviral (Adv) vector (Adv-caspase-8). Here we demonstrate that Adv-caspase-8 infection, at 15 multiplicity of infection (MOI), can augment anoikis in MKN45 cells and suppresses MKN45 peritoneal dissemination in SCID mice. The inhibitory effect on peritoneal dissemination resulted in a prolonged survival compared with that in control mice. In contrast, the Adv-caspase-8 (15 MOI) had no distinct effect on cell viability or growth either of attached MKN45 cells or of s.c. tumor growth in SCID mice. Thus, Adv-mediated overexpression of caspase-8 suppressed peritoneal dissemination mainly through augmentation of anoikis. In addition, Adv-caspase-8-mediated augmentation of anoikis was similarly observed in another gastric carcinoma MKN74 cell line. In contrast, Adv-p53 could not augment anoikis in MKN45 cells. These results imply that Adv-mediated gene transfer of caspase-8 can selectively induce apoptosis in detached carcinoma cells and, thus, shows potential as a novel cancer therapy against dissemination of gastric and probably other carcinoma cells originating from epithelial tissues.

Introduction

Proliferation and survival of epithelial cells depend on integrin receptor-mediated interaction with ECM³ proteins. This interaction triggers survival signals that are mediated mainly through PI 3-kinase/Akt- and focal adhesion kinase-mediated pathways (1, 2). However, once cells detach from the ECM, survival signals are down-regulated, and the cells gradually cease to proliferate, which leads to apoptosis as a terminal event. This form of apoptosis has been termed anoikis (3). Anoikis prevents epithelial cells from aberrant proliferation at inappropriate locations and is, thus, essential for the maintenance of physiological epithelial organization. In addition, anoikis may also suppress expansion of oncogenic transformed cells. However, the

ability of many carcinoma cells to grow in any location in the body suggests a defect in the mechanism of anoikis. In fact, we previously demonstrated that the suppression of anoikis enhances metastasis and peritoneal dissemination of malignant cells (4, 5).

Loss of cell anchorage not only abrogates survival signals but also induces cell death signals. Activation of caspase-8 plays a central role in cell death signaling during the process of anoikis. Suspension culture of dog kidney epithelial MDCK cells rapidly increases caspase-8 activity, and conversely, inhibition of caspase-8 activity dramatically blocks anoikis. Importantly, the loss of anchorage-mediated caspase-8 activation does not require activation of an executioner caspase-3, thereby indicating that caspase-8 activation is an initiating event in anoikis (6, 7). These data strongly suggest that caspase-8 activation is central to the process of anoikis.

Caspase-8 belongs to a family of caspases characterized as activators of executioner caspases, and it also activates a proapoptotic Bcl-2 family member Bid (8, 9). Caspase-8 precursor protein is activated at the initial step in the cascade of CD95 (Fas receptor)-induced apoptosis (10). When Fas ligands bind to CD95, caspase-8 precursor proteins are recruited to the DISC and form large receptor complexes with the adaptor protein FADD (11, 12). Thereafter, the caspase-8 precursor proteins become activated by self cleavage at internal aspartate residues. Active caspase-8 can directly activate an executioner caspase-3 and cause cell death (13). As mentioned above, suspension culture activates caspase-8 in MDCK cells. This activation occurs independently of Fas-mediated signals but depends on FADD oligomerization (6, 7). Although it remains unknown how caspase-8 precursors and FADD are activated during suspension culture, ligand-independent activation of caspase-8 raises the possibility that overexpression of caspase-8 precursor proteins may increase sensitivity to anoikis, because FADD is ubiquitously expressed in a variety of cell types (14).

Gene therapies for the treatment of carcinomas are now being established. For example, introduction of *p53* or Fas ligand genes into a large number of carcinoma cells and patients with various carcinomas shows promise for anticancer therapy (15–18). Among the expression vectors available for gene therapy the Adv vector is a powerful vehicle for the mediation of gene transfer into epithelial cells. Recently, we have established a caspase-8 expression system using an Adv vector in which caspase-8 expression is under the control of the chicken β -actin (CA) promoter (Adv-caspase-8). The Adv-caspase-8-mediated gene transfer strongly induced apoptosis in U251 human glioma cells and suppressed the *in vivo* growth of U251 xenografts (19). This system, therefore, shows potential for the therapy of other carcinomas and may be developed into a useful anticancer gene therapy. However, one problem that must be overcome before Adv-caspase-8 can be used for therapy is the problem of the level of caspase-8 expression in cells. Overexpression of caspase-8 molecules is known to result in self-oligomerization and subsequent activation in the absence of any apoptotic signal (20). Thus, overexpression of caspase-8 may induce apoptosis in all Adv-caspase-8-infected cells rather than selective inhibition of detached cells. For the application

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² To whom requests for reprints should be addressed, at First Department of Internal Medicine, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan. Phone: 81-11-611-2111, extension 3213; Fax: 81-11-611-2282; E-mail: adachi@sapmed.ac.jp.

³ The abbreviations used are: ECM, extracellular matrix (proteins); Adv, adenovirus; MOI, multiplicity/multiplicities of infection; FADD, Fas-associated death domain (protein); DISC, death-inducing signal complex; SCID, severe combined immunodeficiency; FLICE, FADD-like interleukin-1 β -converting enzyme; FLIP, FLICE (caspase-8) inhibitory protein; PE, phosphatidylserine externalization.

of this system to anticancer therapy, it is, therefore, necessary to reduce nonspecific cell death from overexpression of caspase-8. For this purpose, the expression level of caspase-8 from the Adv vector must be lower than that which results in self-oligomerization of caspase-8 precursor proteins. Low levels of caspase-8 precursor proteins produced by Adv-caspase-8 are expected to be inactive until the cells lose anchorage, at which time the caspase-8 precursor proteins are likely to be activated. Thus, a low expression level of caspase-8 would be expected to be useful for the augmentation of anoikis in adenocarcinoma cells.

To explore the possibility of *caspase-8* gene transfer for gene therapy of carcinomas, we investigated whether Adv-mediated *caspase-8* gene transfer could selectively affect the growth and survival of human gastric carcinoma cells in suspension culture. We report here that overexpression of low levels of caspase-8 (MOI 15) strongly induces apoptosis in detached gastric carcinoma cells but not in attached MKN45 cells. Moreover, caspase-8 overexpression significantly reduces the peritoneal dissemination of these cells in SCID mice and extends mouse survival compared with control mice. These data indicate that caspase-8 precursor proteins produced by Adv-caspase-8 are activated by the loss of anchorage and that activated caspase-8 molecules augment anoikis. Extrinsic introduction of the caspase-8 precursor protein had a limited effect on cell viability in attached MKN45 cells, which indicates that this system may selectively induce apoptosis in detached cells.

Our findings, therefore, outline a new concept for the sensitization of carcinoma cells to anoikis by the introduction of caspase-8 via an Adv-caspase-8 vector. Because augmentation of anoikis is expected to inhibit metastasis or dissemination of carcinoma cells, our findings have potential for the development of a new gene therapy against cancer progression.

Materials and Methods

Reagents and Cell Lines. MKN45, MKN74, and JRST cell lines were established from human gastric adenocarcinoma and were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were grown in RPMI 1640 supplemented with 10% FCS at 37°C in 5% CO₂. To evaluate cell viability, cells were stained with 0.4% trypan blue solution and immediately examined for dye exclusion by light microscopical observation. The anti-caspase-8 monoclonal antibody was purchased from MBL (Nagoya, Japan), and anti-FLIP and anti-HSC70 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-FADD and anti-p53 monoclonal antibodies were from Transduction Lab. (Lexington, KY). PolyHEMA (2-hydroxyethyl methacrylate) was from Sigma Chemical Co. (St. Louis, MO).

Adenoviral Vectors. Construction and propagation of the recombinant human FLICE (caspase-8) Adv (Adv-caspase-8) and the recombinant Adv (Adv-Cre) carrying the *Cre* recombinase gene have been described previously (19). In brief, the human caspase-8 precursor proteins can be expressed via the CA promoter but are silenced by a polyadenylate sequence flanked by a pair of loxP sites (21). In the presence of the *Cre* recombinase, the loxP sites are excised and the CA promoter is juxtaposed with the caspase-8 gene resulting in constitutive activation of the gene. For control experiments, we also used Adv-p53, which was described previously (16). The recombinant Adv was partially purified, and their viral titers were determined by plaque assay on 293 cells. Purified recombinant viruses were kept at -80°C until use.

Cell Culture and Infections with Recombinant Adv. MKN45 or MKN74 cells were cultured as adherent cells on polyHEMA-uncoated 100-mm Petri-dish or 6-well tissue culture plates. For Adv-mediated gene transfer, they were incubated with the partially purified recombinant Adv for 24 h, washed twice with completely fresh medium, and further incubated in either suspension or adhesion. For suspension culture, the infected cells were placed into poly-HEMA-coated dishes as described previously (3). Adv-mediated *caspase-8* gene transfection was performed by coinfection of AxCALNL-hFLICE and AxCACre (Adv-caspase-8) at a MOI of 2:1. For control experiments, cells were incubated with AxCACre alone (Adv-Cre) at the same total MOI.

Western Blotting. After washing with ice-cold PBS, cells were lysed by the addition of 50 μ l of radioimmunoprecipitation assay buffer containing 100 mM

NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% NP40, and 50 mM Tris-HCl (pH 7.2). Protein concentration of total cell lysates was evaluated with a Protein Assay kit (Bio-Rad, Melville, NY). The lysates (80 μ g/lane) were separated by 10–15% SDS-PAGE gels and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 18 V for 70 min. After blocking with 5% BSA (Sigma Chemical Co.) in washing buffer containing 140 mM NaCl, 25 mM Tris-HCl (pH 7.8) and 0.05% Tween 20, the membranes were incubated with primary antibodies overnight at 4°C and, thereafter, were incubated with the corresponding peroxidase-linked secondary antibody (Amersham, Arlington Heights, IL) for 1 h at room temperature. Signals were developed by a standard enhanced chemiluminescence (ECL) method following the manufacturer's protocol (Amersham).

Annexin V-FITC Staining Assay. Annexin V-FITC staining assay was performed following the manufacturer's protocol (ApoAlert Annexin V Apoptosis Kit; Clontech); 1×10^6 cells were incubated with Annexin V at room temperature for 15 min in the dark. The cells were then analyzed by flow cytometry (FACSCalibur; Becton Dickinson Immunocytometry Systems, San Jose, CA) using a single-laser-emitting excitation light at 488 nm. The data were converted to histogram (FL1) plots using CellQuest software.

DNA Fragmentation Assay. As described previously (22), low-molecular weight DNA was extracted with 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.4; treated with 400 μ g/ml RNase A and Proteinase K for 1 h at 37°C; ethanol-precipitated; and subjected to 1% agarose gel electrophoresis. The gels were stained with 1 μ g/ml ethidium bromide.

Caspase-3 Colorimetric Protease Assay. Caspase-3 colorimetric protease assay was performed following the manufacturer's protocol (Caspase-3 Colorimetric Protease Assay Kit; MBL). In brief, 5×10^6 cells were lysed in 250 μ l of chilled cell lysis buffer, and total cell lysates (100 μ g) were incubated with 4 mM VETD-pNA Substrate (200 μ M final concentration) at 37°C for 1 h. Caspase-3 activity was measured by colorimetric reaction at 400 nm.

Animal Experiments. Six to 8-week-old female SCID mice (CB/ICR crj-scid) were housed in specific pathogen-free units. For s.c. tumor generation studies, MKN45 cells, infected with the recombinant Adv, were washed twice with PBS, and 1×10^6 viable cells in 0.2 ml of PBS were inoculated s.c. into the femur of SCID mice. Tumors were visible after several weeks, and their sizes were measured at 3 and 7 weeks after inoculation. Tumor volumes were calculated by $[(1/2) \times (\text{longest diameter}) \times (\text{shortest diameter})^2]$ as described previously (5). In order to evaluate the *in vivo* growth of the Adv-infected MKN45 cells, we also measured the weight of s.c. tumors at 7 weeks after inoculation. Evaluation of peritoneal dissemination ability of carcinoma cells has been described previously (5). In brief, Adv-infected cells (1×10^6) in 0.5 ml of PBS were injected into the peritoneal cavity of SCID mice. After inoculation of MKN45 cells, mice were carefully observed everyday until they died. To investigate the tumor dissemination ability of the Adv-infected MKN45 cells, several mice were killed at 25 days after injection, and total and disseminated tumor weights were measured.

Results

Adv-mediated Expression of Caspase-8 in MKN45 Cells. We investigated a number of gastric carcinoma cell lines with respect to their suitability as a model system for determining the effect of caspase-8 on anoikis. We first investigated the expression levels of procaspase-8, FLIP, and FADD in three human gastric carcinoma cell lines: MKN45, MKN74, and JRST. Caspase-8 precursor proteins were expressed in all of these cell lines, but their expression levels varied among the cells. The highest expression level of caspase-8 was observed in the JRST cells with a relatively low level of expression observed in MKN45 cells (Fig. 1A). FADD was also expressed in all of the cell lines, with MKN45 cells expressing FADD at the highest level. FLIP was highly expressed in MKN74 cells (Fig. 1A). All of these cell lines were susceptible to Adv, because >75% of the cells strongly expressed green fluorescent protein (GFP) when they were infected with Adv-GFP at 15 MOI for 48 h (data not shown). We previously demonstrated that MKN45 and MKN74 cells are anchorage-dependent cell lines (5, 22), but in MKN74 cells, caspase-8 may be negatively regulated by FLIP, because MKN74 cells express FLIP abundantly (Fig. 1A). MKN45 cells appeared to be the most suitable

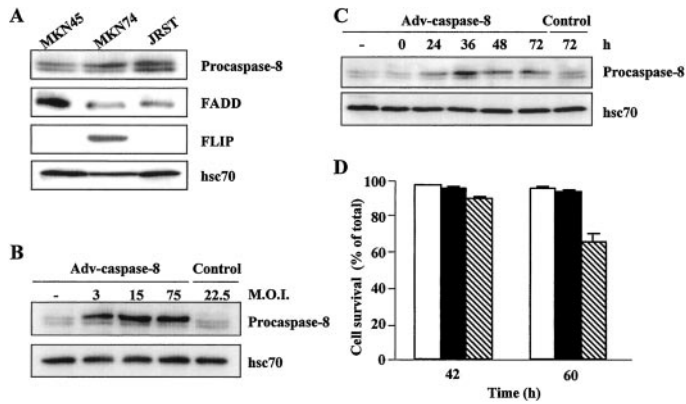


Fig. 1. Adv-mediated induction of procaspase-8 in MKN45 cells. *A*, expression of procaspase-8, FADD, and FLIP in the human gastric carcinoma cell lines MKN45, MKN74, and JRST. *B*, dose-dependent procaspase-8 expression. MKN45 cells were infected with Adv-caspase-8 or Adv-Cre (Control) at the indicated MOI. Cell lysates were harvested at 24 h postinfection. *C*, time course of procaspase-8 expression after Adv-caspase-8 infection. MKN45 cells were infected with Adv-caspase-8 or Adv-Cre (Control) at 15 MOI and the lysates were harvested at the indicated times. For all (*A*, *B*, and *C*) total cell lysates were subjected to 10% SDS-PAGE and Western blot analysis using the specified antibodies. The hsc70 protein is an indicator of protein loading in each lane. *D*, time course of cell death after Adv-caspase-8 infection. MKN45 cells were infected with Adv-caspase-8 at 3 (open bars), 15 (closed bars), and 75 MOI (hatched bars). Cell viability was evaluated at the indicated times by trypan blue exclusion assay. Each column displays the mean \pm SD of data from three separate experiments.

cell line for our study. Furthermore, MKN45 cells produced extensive peritoneal dissemination after inoculation into the mouse peritoneal cavity.⁴ We thus chose MKN45 cells to investigate the effect of caspase-8 overexpression on anoikis.

We first characterized the expression of procaspase-8 from the Adv vector in MKN45 cells. Infection of MKN45 cells with AxCALNL-hFLICE and AxCACre (Adv-caspase-8) or AxCACre alone (Adv-Cre) at MOIs ranging from 3 to 75 for 24 h induced increased intracellular expression levels of caspase-8 in a dose-dependent manner (Fig. 1*B*). We next investigated the time course of caspase-8 expression after infection with Adv-caspase-8 at 15 MOI. An increase in the caspase-8 expression level was visible at 24 h, peaked at 36 h after infection, and, thereafter, gradually declined to basal levels (Fig. 1*C*). The effect of Adv-caspase-8 expression in MKN45 cells on the viability of adherent cells was then determined. Infection of MKN45 cells with Adv-caspase-8 at 3, 15, or 75 MOI, did not markedly affect cell viability at 42 h after infection. However, the viability of MKN45 cells infected with Adv-caspase-8 (75 MOI) was substantially lost at 60 h after infection (Fig. 1*D*). From these experiments, we conclude that the viability of attached MKN45 cells was not significantly affected by the introduction of the *caspase-8* gene at 24–60 h after the Adv infection (15 MOI).

Adv-caspase-8 Augmented Anoikis in MKN45 Cells. To investigate the effect of Adv-mediated caspase-8 expression on anoikis, MKN45 cells were infected with Adv-caspase-8 or the control Adv-Cre. After incubation for 24 h, Adv recombinants were removed, and infected cells were subsequently cultured either in suspension or as adherent cells. Untreated MKN45 cells in suspension culture gradually lost viability in a time-dependent manner but were not significantly dead at 24 h, as reported previously (22). Overexpression of caspase-8 strongly enhanced cell death in suspension culture. Approximately 30% of the total number of cells were dead at 18 h and >50% of the cells in suspension were dead at 36 h (Fig. 2*A*). However, only ~12% of MKN45 cells that were infected with the Adv-Cre control were dead at this time point, which is a similar level of cell death observed in untreated MKN45 cells (data not shown). Furthermore, attached MKN45 cells that were infected with

Adv-caspase-8 did not show a significant enhancement of cell death (Fig. 2*A*). To evaluate the mechanism of cell death, we next investigated the effect of Adv-caspase-8 infection on PE, which occurs in apoptotic cells at an early stage. Overexpression of caspase-8 clearly increased PE in suspended but not in adherent cells (Fig. 2*B*), which indicated that Adv-caspase-8-mediated overexpression of caspase-8 increased apoptosis in MKN45 cells when they lost anchorage. We further investigated whether caspase-8 overexpression affected DNA fragmentation, which is a characteristic marker of apoptotic cell death. The Adv-caspase-8 infected cells in suspension exhibited substantial DNA fragmentation at 18 h. However, neither the control, nor the suspended Adv-Cre infected MKN45 cells, nor the attached Adv-caspase-8-infected MKN45 cells exhibited DNA fragmentation (Fig. 2*C*). Because similar levels of caspase-8 precursor proteins were expressed in the Adv-caspase-8-infected MKN45 cells regardless of their culture condition (Fig. 2*D*), these data suggest that suspension stress is activating the caspase-8 precursor proteins. To explore the mechanism(s) by which procaspase-8 was activated in cells in suspension culture we investigated whether cleavage of the caspase-8 precursor proteins were enhanced by suspension culture. When MKN45 cells infected with Adv-caspase-8 were cultured for 6–12 h in suspension, the level of the p18 cleavage product of the caspase-8 precursor clearly increased in a time-dependent manner. However, the cleavage product was only faintly detected in the anchored cells (Fig. 2*D*). Thus, suspension stress enhanced the cleavage of Adv-introduced caspase-8 precursor proteins. We further investigated the contribution of the cleaved caspase-8 to anoikis. As described above (13), enzymatically active caspase-8 can activate caspase-3 either directly or indirectly, which results in apoptosis. We, thus, examined caspase-3 activity in the MKN45 cells infected either with Adv-caspase-8 or with Adv-Cre during suspension culture. When the Adv-caspase-8-infected MKN45 cells were cultured in suspension, caspase-3 activity clearly increased in a time-dependent manner, whereas the Adv-Cre infected MKN45 revealed much less increase of caspase-3 activity (Fig. 2*E*). In addition, caspase-3 activity was not significantly increased when the Adv-caspase-8 infected cells were anchored. These results indicate that the cleavage of Adv-mediated caspase-8 precursor proteins was enhanced by suspension culture and that the enhanced caspase-8 activity appears to augment anoikis through activation of caspase-3 in MKN45 cells.

Adv-p53 Could Not Augment Anoikis in MKN45 Cells. Adv-p53 has been extensively studied to evaluate its clinical application for gene therapy against various carcinomas, and overexpression of p53 strongly induced apoptosis in these carcinomas (15, 16). We thus investigated whether Adv-p53 augmented anoikis in MKN45 cells. After incubation with Adv-p53 or the control Adv-Cre for 24 h, Adv recombinants were removed, and infected cells were subsequently cultured either in suspension or as adherent cells. Although Adv-p53 infection greatly elevated p53 expression levels in MKN45 cells in a dose-dependent manner (Fig. 3*A*), p53 overexpression could not augment anoikis in the cells (Fig. 3*B*). Thus, p53 appears not to contribute to the process of anoikis, signifying the selective contribution of caspase-8 to anoikis.

Adv-caspase-8 Similarly Augmented Anoikis in Another MKN74 Carcinoma Cell Line. To explore whether the observed augmentation of anoikis by Adv-caspase-8 infection in MKN45 cells is general, we investigated the effect of Adv-caspase-8 on anoikis in MKN74 cells. MKN74 cells originate from a human well-differentiated adenocarcinoma and are also anchorage dependent (22). As observed in MKN45 cells, Adv-caspase-8 infection strongly increased cell death (Fig. 3*C*) and caspase-3 activity (Fig. 3*D*) in MKN74 cells when they were cultured in suspension. Thus, Adv-caspase-8-mediated overexpression of caspase-8 proteins is likely to overcome FLIP and augment anoikis in MKN74 cells.

⁴ Unpublished observation.

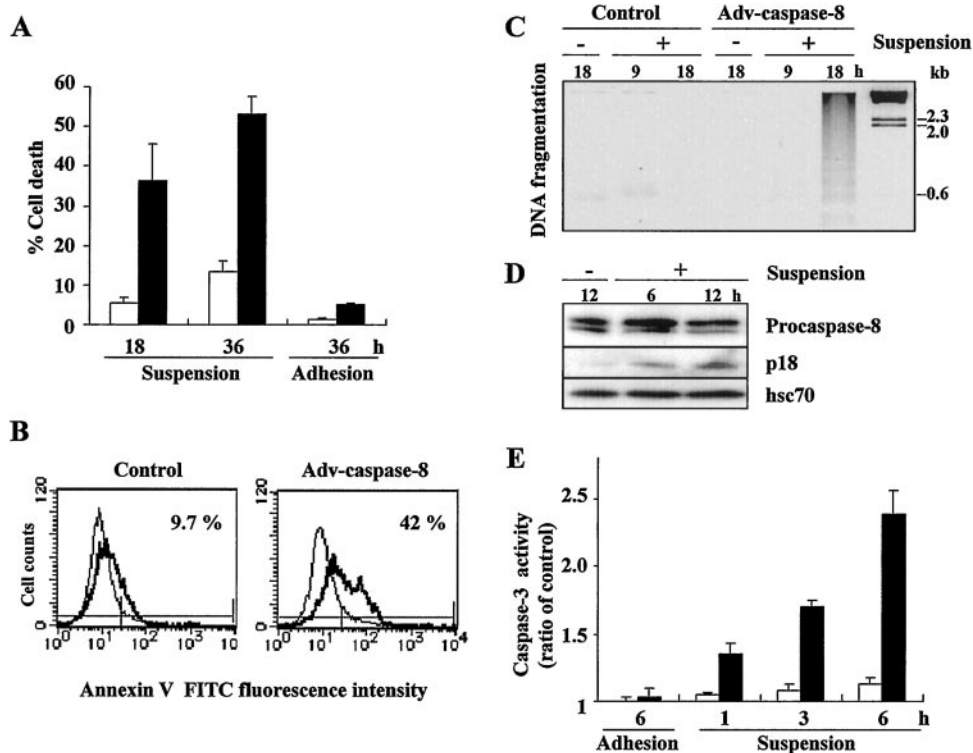


Fig. 2. Augmentation of anoikis by Adv-caspase-8 infection. *A*, cell death of MKN45 cells overexpressing procaspase-8. MKN45 cells were infected with Adv-caspase-8 (15 MOI; closed bars) or Adv-Cre (22.5 MOI; open bars) for 24 h and were washed and further incubated for the indicated times either as suspended or adherent cells. Cell viability was evaluated at the indicated times by trypan blue exclusion assay. Each column displays the mean \pm SD of data from three separate experiments. *B*, PE on the plasma membrane of Adv-caspase-8 (15 MOI)-infected MKN45 cells in suspension. MKN45 cells were infected with either Adv-Cre (Control) or Adv-caspase-8 for 24 h and further incubated for 9 h either as suspended (thick lines) or as adherent (thin lines) cells. Annexin V binding capability was evaluated using the ApoAlert Annexin V-FITC kit and is shown in histogram plot of FL-1 fluorescence intensity. *C*, DNA fragmentation in Adv-caspase-8-infected MKN45 cells in suspension. DNA fragmentation of low-molecular-weight DNA, prepared from the cells at the indicated periods after culture as suspended (+) or adherent (-) cells is displayed. Cells were infected with either Adv-caspase-8 (15 MOI) or Adv-Cre (Control) for 24 h and were washed and further incubated for 9 or 18 h as suspended or attached cells as indicated. Molecular size markers (kb) are shown. *D*, effect of suspension culture on cleavage of caspase-8 precursor proteins. After infection with Adv-caspase-8 (15 MOI), MKN45 cells were further incubated for the indicated times as suspended or adherent cells. Procaspase-8 and its cleavage product p18 were detected by Western blotting of the total cell lysate with the anti-caspase-8 monoclonal antibody. The hsc70 protein levels indicate an equal amount of protein loaded in each lane. *E*, caspase-3 activities of infected MKN45 cells. Caspase-3 activities of MKN45 cells infected with Adv-caspase-8 (15 MOI; closed bars) or Adv-Cre (open bars) were measured in suspended and adherent cells at the indicated times using the CPP32/Caspase-3 colorimetric protease assay kit. Each column displays the mean \pm SD of data from three separate experiments.

Adv-caspase-8 Did Not Affect Either *in Vitro* or *in Vivo* Cell Growth. Caspase-8 precursor proteins introduced by Adv-mediated gene-transfer (15 MOI) were not significantly cleaved in adherent MKN45 cells and, thus, did not significantly affect cell viability. To evaluate the effect of the caspase-8 precursor proteins on cell growth, adherent MKN45 cells were incubated with either Adv-caspase-8 or Adv-Cre for 24 h and cultured for 5 days after removal of the recombinant Adv vectors by extensive washing. The cell growth of adherent MKN45 cells was similar whether the cells were infected with Adv-caspase-8 or with the control Adv-Cre (Fig. 4A). When the Adv-infected MKN45 cells were s.c. inoculated into SCID mice, s.c. tumors appeared at 3 weeks after inoculation. Tumor sizes revealed no significant difference between MKN45 cells infected with Adv-caspase-8 or those infected with Adv-Cre (Fig. 4B). Furthermore, total weights of these s.c. tumors showed no significant difference in these two groups (Fig. 4C). Thus, overproduction of caspase-8 precursor proteins alone did not affect cell growth either *in vitro* or *in vivo*, when the cells could interact with ECM proteins and become anchored.

Adv-caspase-8 Reduced Peritoneal Dissemination and Prolonged Survival. We previously demonstrated that the inhibition of apoptosis by overexpression of Bcl-2 or BAG-1 led to an increase of peritoneal dissemination of MKN74 cells in nude mice (5). This observation raised the possibility that the introduction of proapoptotic genes into these carcinoma cells may reduce their peritoneal dissemination and protect mice from carcinoma dissemination. To explore this possibility, we first investigated tumor generation in the mouse abdominal cavity

after inoculation of MKN45 cells into the cavity. When MKN45 cells that were infected with Adv-Cre alone were injected into the cavity, tumors were observed at 25 days after injection. On the other hand, MKN45 cells infected with Adv-caspase-8 generated much fewer tumors in the cavity. More importantly, we observed that peritoneal dissemination of MKN45 cells was greatly reduced when the cells were infected with Adv-caspase-8 (Fig. 5A). Microscopic investigation revealed that the tumors arising from Adv-infected MKN45 cells were composed mostly of poorly differentiated adenocarcinoma cells rather than of fibroses or granuloma regardless of Adv-Cre or Adv-caspase-8 infection (Fig. 5B). We thus compared the total weight of the tumors in these two groups. Mean total tumor weight of the Adv-Cre-infected MKN45 cells was $\sim 0.79 \pm 0.39$ g, but that of the Adv-caspase-8-infected MKN45 cells was slightly reduced to 0.56 ± 0.29 g (Fig. 5A). However, when we compared the disseminated tumor weights in the two groups, a much greater difference was observed. Mean disseminated tumor weight of the Adv-Cre group was 0.43 ± 0.28 g, whereas that of Adv-caspase-8 was only 0.18 ± 0.11 g (Fig. 5A). These data indicate that Adv-mediated overexpression of caspase-8 significantly reduced peritoneal dissemination of MKN45 cells ($P = 0.04$; Adv-Cre versus Adv-caspase-8). To explore whether Adv-mediated caspase-8 introduction is applicable for therapy of disseminating gastric carcinomas, we next investigated the effect of Adv-mediated introduction of caspase-8 on murine survival after inoculation of MKN45 cells into the abdominal cavity. SCID mice inoculated in this way were carefully observed until they died. The median survival of mice inoculated with parental MKN45 cells was 29

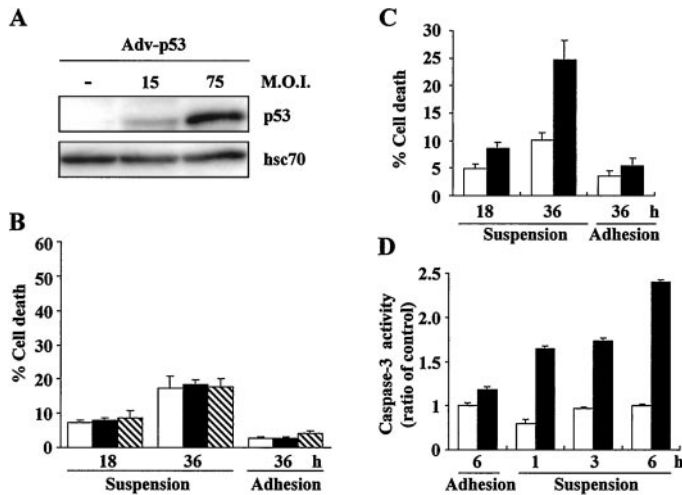


Fig. 3. Marginal effect of Adv-p53 infection on anoikis. A, dose-dependent p53 expression. MKN45 cells were infected with Adv-p53 at the indicated MOI and were harvested at 48 h after infection. Expression levels of p53 were evaluated by Western blot analysis. The hsc70 protein is an indicator of protein loading in each lane. B, cell death of MKN45 cells overexpressing p53. MKN45 cells were infected with Adv-p53 at 15 (closed bars) and 75 MOI (hatched bars) or with Adv-Cre at 75 MOI (open bars) for 24 h and were washed and further incubated for the indicated hours either as suspended or as adherent cells. Cell viability was evaluated at the indicated times by trypan blue exclusion assay. C, cell death of MKN74 cells overexpressing procaspase-8. MKN74 cells were infected with Adv-caspase-8 (15 MOI; closed bars) or Adv-Cre (22.5 MOI; open bars) for 24 h and were washed and further incubated for the indicated times either as suspended or as adherent cells. Cell viability was evaluated at the indicated times by trypan blue exclusion assay. D, caspase-3 activities of infected MKN74 cells. Caspase-3 activities of MKN74 cells infected with Adv-caspase-8 (15 MOI; closed bars) or Adv-Cre (open bars) were measured in suspended and adherent cells at the indicated times using the CPP32/Caspase-3 colorimetric protease assay kit. Each column displays the mean \pm SD of data from three separate experiments.

days, which is similar to that of mice inoculated with the Adv-Cre infected cells (31 days; Fig. 5C). Importantly, inoculation of mice with MKN45 cells in which the *caspase-8* gene was introduced by Adv greatly enhanced the survival of the mice. The median survival of these mice was 43 days ($P = 0.01$, versus parental MKN45 group; $P = 0.04$, versus Adv-Cre infected MKN45 group). Thus Adv-mediated introduction of caspase-8 into MKN45 cells significantly reduced their peritoneal dissemination after inoculation and resulted in prolonged survival of the SCID mice.

Discussion

Anoikis results in the removal of disordered epithelial cells that have lost the survival signals induced by interaction with ECM proteins. Anoikis thus contributes to the physiological maintenance and organization of epithelial structure. Conversely, prolonged survival of epithelial cells detached from ECM proteins may facilitate their growth at inappropriate regions, and these events may allow them to establish metastasis or infiltration into various tissues. In this regard, we previously demonstrated that the inhibition of anoikis augments pulmonary metastasis and peritoneal dissemination of malignant melanoma and gastric carcinoma cells (4, 5). It is also known that some carcinomas have lost appropriate apoptotic signaling pathways (23) and can, therefore, escape from anoikis. This defect appears to be tightly linked not only to carcinogenesis but also to the behavioral properties of carcinoma cells, *i.e.*, metastatic potential or invasive ability. Thus, induction of apoptotic signals in these malignant cells may be a promising therapy against metastasis and dissemination. Here, we present evidence that Adv-mediated *caspase-8* gene transfer can augment anoikis in human gastric carcinoma MKN45 and MKN74 cells, thereby significantly reducing peritoneal dissemination in SCID mice and prolonging their survival. To our knowledge, this is the first study to outline a model of gene therapy

that uses the enhancement of anoikis to reduce peritoneal dissemination of carcinoma cells.

Caspase-8 is a member of a cysteine protease family that facilitates apoptotic signals (8). As has been determined for other caspases, caspase-8 is synthesized as an inactive precursor protein and is activated by proteolytic cleavage at aspartic acid residues to form heterotetrameric complexes of cleaved products. Activation of caspase-8 is observed after exposure of cells to a variety of apoptotic stimuli and may occur via a variety of different mechanisms. Among the apoptotic stimuli examined, Fas-mediated signals have been the most extensively studied with regard to activation of caspase-8 precursors. The accumulated data demonstrate that caspase-8 is activated by rapid formation of the DISC after Fas-L stimulation (10–12). The large heterotetrameric complexes of DISCs enable self-cleavage of caspase-8 (12). In addition, caspase-8 can be activated by γ -irradiation, chemotherapy, and calcium ionophore independently of Fas-L stimulation, although the precise mechanisms for its activation remain obscure. Abundant expression of caspase-8 precursor proteins results in self-oligomerization and in subsequent self-cleavage and activation in the absence of any external apoptotic signal (20). During anoikis, caspase-8 is activated independently of Fas-mediated signals, but its activation requires FADD oligomerization (6, 7). Consistent with these previous studies, our study shows that Adv-produced caspase-8 precursor proteins are cleaved and activated by suspension culture of infected cells. This Adv-caspase-8 cleavage could potentially occur by either self-oligomerization and subsequent activation or by the FADD-dependent mechanism that occurs during anoikis. To investigate the former possibility, we determined whether large complexes of oligomerized caspase-8 could be detected in detached MKN45 cells infected with Adv-caspase-8. Our preliminary experiments, however, indicated that distinct large complexes in the Adv-caspase-8-infected MKN45 cells were not observed either in suspended or in adherent cells (data not shown). Thus, activation of extrinsic caspase-8 by self-oligomerization seems an unlikely possibility. The most likely mechanism of extrinsic caspase-8 activation is, therefore, that an increase of caspase-8 expression levels may enhance accessibility to FADD in the process of anoikis leading to enhanced cleavage and activation of caspase-8 and subsequent augmentation of anoikis.

We present evidence that Adv-mediated gene transfer of caspase-8 augments anoikis in human gastric carcinoma MKN45 and MKN74 cells. Although malignant cells tend to proliferate and survive without physiological interaction with ECM proteins (24), some carcinoma cells continue to depend on anchorage for their growth or survival (25). For metastasis to occur, these carcinoma cells must be able to

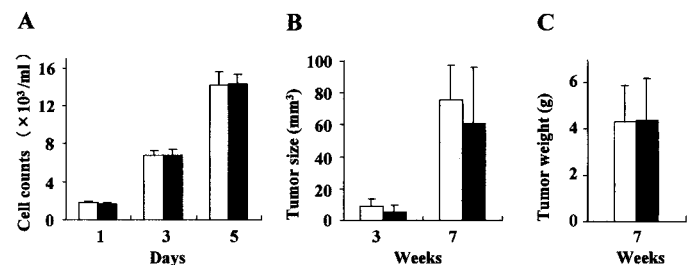


Fig. 4. Effect of procaspase-8 overexpression on *in vitro* and *in vivo* cell growth. A, effect of Adv (15 MOI)-mediated overexpression of procaspase-8 on *in vitro* cell growth. MKN45 cells were infected either with Adv-caspase-8 (closed bars) or with Adv-Cre (open bars) for 24 h and were washed and, thereafter, cultured as adherent cells for the indicated times. Cell counts display the mean \pm SD of data from three separate experiments. B and C, effect of Adv-mediated overexpression of procaspase-8 on *in vivo* cell growth. MKN45 cells infected either with Adv-caspase-8 (closed bar) or with Adv-Cre (open bar) were s.c. inoculated into nude mice, and the size (B) and weight (C) of growing s.c. tumors were measured. Bars, the means \pm SE of whole tumors ($n = 3$ per each group).

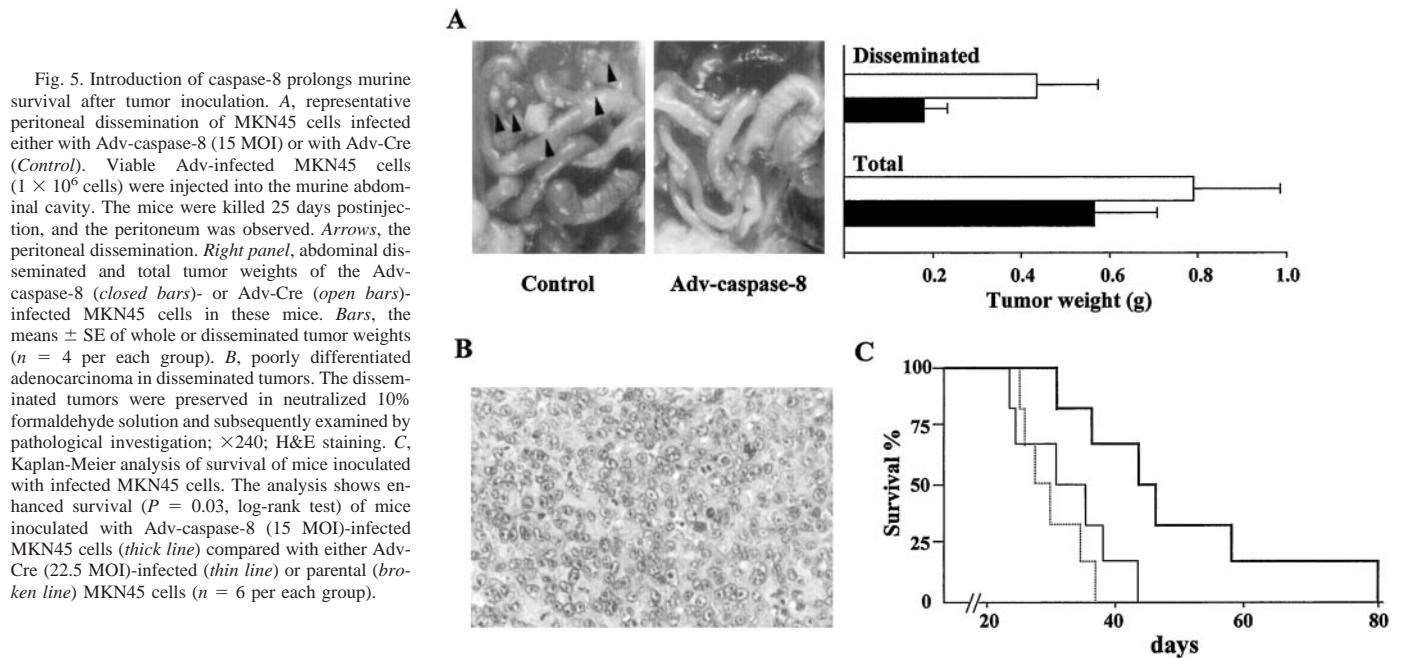


Fig. 5. Introduction of caspase-8 prolongs murine survival after tumor inoculation. **A**, representative peritoneal dissemination of MKN45 cells infected either with Adv-caspase-8 (15 MOI) or with Adv-Cre (Control). Viable Adv-infected MKN45 cells (1×10^6 cells) were injected into the murine abdominal cavity. The mice were killed 25 days postinjection, and the peritoneum was observed. *Arrows*, the peritoneal dissemination. *Right panel*, abdominal disseminated and total tumor weights of the Adv-caspase-8 (closed bars)- or Adv-Cre (open bars)-infected MKN45 cells in these mice. *Bars*, the means \pm SE of whole or disseminated tumor weights ($n = 4$ per each group). **B**, poorly differentiated adenocarcinoma in disseminated tumors. The disseminated tumors were preserved in neutralized 10% formaldehyde solution and subsequently examined by pathological investigation; $\times 240$; H&E staining. **C**, Kaplan-Meier analysis of survival of mice inoculated with infected MKN45 cells. The analysis shows enhanced survival ($P = 0.03$, log-rank test) of mice inoculated with Adv-caspase-8 (15 MOI)-infected MKN45 cells (thick line) compared with either Adv-Cre (22.5 MOI)-infected (thin line) or parental (broken line) MKN45 cells ($n = 6$ per each group).

survive without anchorage throughout the process of metastasis. In these anchorage-dependent carcinomas, the Adv-mediated *caspase-8* gene therapy described here could strongly decrease the survival rate during metastasis. In addition, Adv-mediated *caspase-8* gene therapy for anticancer dissemination could be further enhanced by combination with other treatments, such as those with synthetic peptides, that inhibit cell-ECM interaction (26). Because of strong adverse side effects it has been difficult to develop gene therapy that involves the transfer of proapoptotic genes. However, the approach described in this article represents a new method of reducing nonspecific cell death. Moderate expression levels of caspase-8 precursor proteins may selectively induce apoptosis of carcinoma cells that have lost anchorage. We believe that our findings should facilitate the development of a novel therapy to prevent carcinoma dissemination.

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Susumu Nishimura, Masaaki Adachi, Tadao Ishida, et al.

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