Advances in Brief

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

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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 ECM 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 Bel-2 family member Bcl-2 (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...
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 induction 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 of carcinomas, we investigated whether Adv-mediated 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.

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cell line for our study. Furthermore, MKN45 cells produced extensive peritoneal dissemination after inoculation into the mouse peritoneal cavity. 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 AxCArCre (Adv-caspase-8) or AxCArCre 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. 1A). 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. 1C). 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. 1D). 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. 2A). 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. 2A). 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. 2B), 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. 2C). Because similar levels of caspase-8 precursor proteins were expressed in the Adv-caspase-8-infected MKN45 cells regardless of their culture condition (Fig. 2D), 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. 2D). 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. 2C). 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-3 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. 3A), p53 overexpression could not augment anoikis in the cells (Fig. 3B). 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. 3C) and caspase-3 activity (Fig. 3D) 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.

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*Unpublished observation.*
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 ~0.79 ± 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
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

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
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 prosapoptotic 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.

References

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