Cross Talk between Apoptosis and Invasion Signaling in Cancer Cells through Caspase-3 Activation

Mutsuko Mukai, Toshiyuki Kusama, Yukou Hamanaka, Takumi Koga, Hiroko Endo, Masaharu Tatsuta, and Masahiro Inoue

Departments of Biochemistry and Gastrointestinal Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Nakamichi, Higashinari-ku, Osaka, Japan; Shinko Hospital, Kobe, Japan; First Department of Surgery, School of Medicine, University of Yamanashi, Kofu, Japan; Department of Regenerative Medicine, Osaka University Graduate School of Medicine, Suita, Japan; and Japan Applied Microbiology Research Institute Ltd., Tamaho, Japan

Abstract
In solid tumors, cancer cells are exposed to various microenvironmental stresses such as hypoxia, nutritional depletion, and low pH. Cancer cells adapt to these stresses and circumvent cell death. When the ant apoptotic signals overcome the stress, cancer cells might acquire physiologic functions, such as invasiveness, instead of cell death. Here, we report that tumor cells acquire an invasive capacity from apoptotic signals through caspase activation. We treated rat ascites hepatoma MM1 cells with an apoptosis-inducing drug, etoposide, or hypoxia, and assessed the invasion capacity with an in vitro bioassay. Although MM1 cells rarely showed invasiveness in serum-free medium, under stress conditions, invasive capacity accompanied with morphologic change was induced with caspase-3 activation. Such stress-induced invasion as well as morphologic change was suppressed by blocking caspase-3 activity with caspase inhibitors or by RNA interference of caspase-3. In contrast, lysophosphatidic acid–induced invasiveness was not affected by caspase-3 inhibition. These results suggest that caspase-3 activation contributes to the stress-induced invasive capacity of these cancer cells. (Cancer Res 2005; 65(20): 9121-5)

Introduction
Tumors are exposed to stress microenvironments, such as hypoxia, acidosis, and free radical overproduction, and cancer cells respond to these stresses in various ways (1). Hypoxia, for example, can directly increase tumor cell invasiveness through multiple pathways such as inductions of urokinase receptor, interleukin 8 (2), and c-met proto-oncogene (3). Therapeutic challenges also have been reported to promote invasive capacity. We and others have reported that some chemotherapeutics induce invasive capacity in cancer cells (4). Sublethal irradiation of cancer cells has also been shown to promote invasive capacity (5). As of yet, the mechanisms of these therapy-induced stresses have not been clarified. Although these stresses also induce apoptosis under some conditions, stress-induced invasiveness and apoptosis have been considered as two unrelated phenomena. Cancer cells are generally resistant to apoptosis (6). It is thought that the activation of survival pathways, such as the expression of inhibitor of apoptosis proteins, may rescue cells from an apoptotic nuclease attack initiated by transient caspase activation (7). Indeed, some of the cancer cells have high basal levels of activated caspase-3 without evidence of apoptosis (8). These observations indicate that if stress stimulates caspase activation without accomplishing cell death, apoptotic signals might activate another function other than cell death. Here, we investigate whether activated caspase-3 induced by stress conditions contributes to the invasive capacity of the cancer cells other than apoptosis.

Materials and Methods
Cells and cell culture. MM1 cells were derived from rat ascites hepatoma AH130 cells. MM1 cells were cultured in normoxic or hypoxic conditions (1% O2) for 22.5 hours and with etoposide (VP-16) for 24 hours in DMEM with 10% fetal bovine serum (FBS), and assayed in serum-free medium. MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). MDA-MB-231 cells were grown in RPMI 1640 supplemented with 10% FBS.

Invasion assay. The invasion bioassay was previously described (9). Briefly, mesothelial cells from rat mesentery were cultured to confluency in 35-mm dish and 2 × 10^5 MM1 cells were seeded onto the mesothelial cell monolayer. After 20 hours of coculture, the number of invasive foci was counted with a phase-contrast microscope. Matrigel invasion assay was previously described (10). Briefly, the upper compartment of Transwell culture chambers was coated with 5% Matrigel. One hundred thousand cells were placed in the upper compartment, and serum-free medium containing 0.1% bovine serum albumin and the indicated ligand was placed in the lower compartment. After 16 hours of incubation, the membranes were stained with May-Grünwald and Giemsa solution. The cells that had migrated through the membrane to the lower surface were counted under a light microscope. Each experiment was done in triplicate and repeated thrice.

Apoptosis and cell death assay. The cultured cells were stained for nucleus morphology with Hoechst 33342 dye, and for intracellular active caspase-3 with the Caspase-3 Intracellular Activity Assay Kit II (PhiloLux G2D2, Calbiochem-Novabiochem, San Diego, CA). Caspase-3 activity from the cell lysate was assayed with a CPP32 Colorimetric Protease Assay Kit (BioVison Research Products, Mountain View, CA).

Immunoblot and immunocytochemistry analysis. Western blot analysis was done as previously described (11). Primary antibodies used were against caspase-3, cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), ROCKI (BD Transduction Laboratories, San Jose, CA), or actin (clone AC-74, Sigma, St. Louis, MO). Secondary antibodies used were goat anti-mouse immunoglobulin G and goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated with horseradish peroxidase. Immunocytochemistry was done as previously described (12). Focal adhesions were visualized by staining vinculin with antivinculin antibody, VIN-11-5 (Sigma). The images were obtained with a confocal microscopy (Radiance 2000, Bio-Rad, Hercules, CA).

Reagents. VP-16 was purchased from Wako (Osaka, Osaka, Japan). Y-27632 and C3 were from Calbiochem. Lysophosphatidic acid (LPA) was obtained from Sigma and the caspase inhibitors DEVD and zVAD were obtained from the Peptide Institute (Mino, Osaka, Japan).

Requests for reprints: Masahiro Inoue, Department of Biochemistry, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan. Phone: 81-6-6972-1181; Fax: 81-6-6972-7749; E-mail: inoue-ma@mc.pre.osaka.up.

©2005 American Association for Cancer Research.

DOI: 10.1158/0008-5472.CAN-04-4344

This article can be found at cancerres.aacrjournals.org.
Gene silencing of the rat caspase-3 gene. Gene silencing was done using the pSuperRetro plasmid purchased from Oligoengine (Seattle, WA). In brief, a duplex oligonucleotide corresponding to the rat caspase-3 gene (13) was subcloned into a pSuperRetro plasmid. As a control, a scrambled duplex oligonucleotide, in which the corresponding sequence (ACGTCGTAGCACACTACG) replaced those of caspase-3, was synthesized and subcloned. All clones were verified by sequencing. The recombinant plasmids were transfected into plat-E packaging cells using FuGENE 6. Forty-eight hours after transfection and after the addition of 8 μg/mL polybrene, the cell culture medium was used to infect MM1 cells. Infected cells were selected with puromycin (10 μg/mL).

Statistical analysis. Statistical analysis was done with GraphPad Prism 4 (GraphPad Software, San Diego, CA). The statistical significance of the results was determined with an unpaired t test, one-way ANOVA. P < 0.05 was considered to be statistically significant.

**Figure 1.** VP-16 and hypoxia treatment induced invasive capacity and apoptosis in parallel. A and B, invasive capacity was assessed by in vitro invasion bioassay and displayed as the number of invasive foci. A, comparison at different doses of VP-16. B, comparison with cultures under normoxic and hypoxic conditions (1% O2). Columns, mean of three results; bars, SD. *P < 0.001, when compared with DMSO only (VP-16 dose, 0 μmol/L; A) or normoxia (B). C, phase-contrast images of in vitro invasion bioassay. The images are from a representative experiment stimulated with none (a), LPA (b), VP-16 alone (c), and VP-16 with DEVD (d). Arrowheads, invasive foci. D, immunocytochemistry images showing vinculin immunoreactivity. The images are from a representative cell stimulated with none (a), LPA (b), VP-16 alone (c), and VP-16 with DEVD (d). E, various types of cells which have active caspase-3 after VP-16 treatment for 24 hours. Top, images from a cell with an intact nucleus, without a membrane bleb, and with a round cell shape. Middle, images from a cell with an intact nucleus, exhibiting membrane blebs and a round cell shape. Bottom, images from a cell with nucleus fragmentation, exhibiting a membrane bleb and an irregular cell shape.
Results and Discussion

Two types of stress were studied to determine whether stress conditions induce invasive capacity in rat ascites hepatoma MM1 cells. First, MM1 cells were treated with VP-16 at the indicated concentrations for 24 hours. Invasive capacity was significantly increased depending on the dose (Fig. 1A and C). Second, the cells were cultured under hypoxic conditions (1% O2) for 22.5 hours. The cells cultured under hypoxic conditions showed a significantly higher number of invasive foci than those under normoxic conditions (Fig. 1B). We have previously reported the correlation between motility and morphology of MM1 cells (12). When challenged with VP-16 and hypoxia (data not shown), a number of MM1 cells showed morphologic change from round shape (Fig. 1D, a) to fusiform shape with pseudopodia and focal adhesions detected by vinculin staining (Fig. 1D, c), which was typically observed with LPA stimulation (Fig. 1D, b). It is well acknowledged that VP-16 and hypoxia induce apoptosis. Therefore, to examine the correlation of invasion and cell death, a morphologic analysis was done using the VP-16 (2 μmol/L)-treated cells. With Hoechst 33342 dye staining, few of the cells showed apparent nucleus fragmentation; the majority had intact nuclei. The cells were double stained with an antibody against active caspase-3, which only detects the cleaved form of caspase-3. Among the caspase-3-activated cells, some nuclei were intact and others were fragmented. Some of the cells that had intact nucleus morphology with active caspase-3 showed membrane blebbing (Fig. 1E).

To investigate the mechanism of the invasion under stress conditions, the kinetics of invasive capacity and caspase-3 activity were assessed. When the cells were cultured with 2 μmol/L of VP-16 for the periods indicated (Fig. 2A), invasive capacity and caspase-3 activity were elevated in parallel for 24 hours. Cultured under hypoxic conditions, invasive capacity and caspase-3 activity also increased over time (Fig. 2B). Caspase-3, an executioner protease, is activated by the upstream caspase cascade and cleaves multiple vital substrates in apoptosis (14). However, there are plenty of reports showing that caspase-3 activation, independent of apoptosis, is involved in some cellular functions, such as growth and differentiation of muscle, cytokine maturation, cell movement, and receptor internalization (15). Because cancer cells are generally resistant to apoptosis, activated caspase-3 might exert another function, such as invasiveness.

Although the correlation between caspase-3 activity and elevation of invasive capacity in this experiments suggests the causative role of caspase-3 in stress-induced invasion, it is possible that these two events are independent. To investigate the direct cross talk between signals of apoptosis and invasion, we examined the effect of caspase-3 inhibition on invasive capacity. First, caspase-3 activity was inhibited by peptide caspase inhibitors, DEVD for caspase-3 and zVAD for broad caspase inhibition. These inhibitors partially suppressed invasive capacity (Figs. 3A, a and b, and 1C, d) as well as morphologic change (Fig. 1D, d), induced by both VP-16 and hypoxia. In contrast, LPA-induced invasive capacity (11) was not affected by DEVD or zVAD (Fig. 3A, c). Next, we suppressed caspase-3 expression using RNA interference (Fig. 3C). When the cells were treated with either VP-16 or hypoxic stress, the caspase-3 knockdown cells, which had a lower level of caspase-3 protein, showed less invasive capacity compared with control cells (Fig. 3B, a and b), whereas caspase-3 knockdown cells did not affect LPA-induced invasive capacity (Fig. 3B, c). These data suggest that stress-induced, but not LPA-induced, invasive capacity was mediated, at least partially, by caspase-3 activation. To validate the above results in a different cell line with a different assay, the human breast cancer cell line MDA-MB-231 was examined with Matrigel invasion assay (Fig. 3D). Indeed, invasive capacity was increased after incubation under hypoxic conditions, which was partially suppressed by caspase-3 inhibitor DEVD.

As shown in Fig. 1E, the membrane bleb was formed on the cells under stress conditions. Dynamic membrane blebbing is one characteristic of the apoptosis execution phase. We previously reported that a membrane bleb is also formed at LPA-induced motility without any sign of apoptosis (16). These blebs that formed at apoptosis and motility are morphologically similar in which phosphorylation of the myosin light chain by ROCK1 and LIMK2 is necessary (17–19). The role of ROCK1 in bleb formation

Figure 2. Invasive capacity correlates with the level of caspase-3 activity induced by VP-16 and hypoxia. Numbers of invasive foci shown by the column graph (left y-axis) and caspase-3 activity shown by the line graph (right y-axis). X axis, culture periods with VP-16 (2 μmol/L; A) or under hypoxic conditions (B). Columns, mean of three results; bars, SD. *, $P < 0.001$, when comparing two groups indicated both for caspase-3 activity and invasive foci.
at apoptosis and motility (12, 20) suggests that ROCK1 might be a cross-point of stress-induced apoptosis and invasiveness. To assess the contribution of the Rho/ROCK signal in the VP-16- and/or hypoxia-induced invasiveness, we blocked the signal with specific inhibitors for Rho and ROCK1. A Rho inhibitor, C3, and a ROCK inhibitor, Y-27632, partially suppressed the stress-induced as well as the LPA-induced invasive capacity (Fig. 4).

Taken together, the Rho/ROCK signal is involved in the stress-induced as well as the LPA-induced invasiveness. We further examined whether the ROCK fragment cleaved by caspase-3 (18, 19) appeared during the stresses which induced invasive capacity. Indeed, under hypoxia, the cleaved form of ROCK1 appeared along with active caspase-3, and the level was suppressed by DEVD but not by C3 (Fig. 4B).

Although an inhibitor of Rho, which is known as a direct upstream regulator of ROCK, also suppressed the stress-induced invasion, it is still possible that the caspase-3–mediated invasive signal feeds directly into ROCK1 because positive feedback loop of Rho/ROCK signal was shown to exist in MM1 cells (20).

Acknowledgments

Received 12/21/2004; revised 7/13/2005; accepted 8/8/2005.


The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank T. Yasuda and A. Fukano for technical assistance.

Figure 3. Hypoxia- and VP-16–induced invasion is partially suppressed by caspase-3 inhibition. A, the cells were cultured under stress conditions, VP-16 (2 μmol/L) for 24 hours (a) and under hypoxic conditions (H) for 22.5 hours (b), and were subjected to invasion assay. C, LPA was added. Number of invasive foci of the cells which were treated with DMSO, DEVD (50 μmol/L), or zVAD (50 μmol/L). B, the cells transfected with a vector of caspase-3 RNA interference were cultured with VP-16 (a), under hypoxia (b), or with LPA (c), and were subjected to invasion assay. Cont and casp3, scrambled sequence and caspase-3 sequence for RNA interference, respectively. Columns, mean number of invasive foci from three results; bars, SD. * and †, P < 0.001 and P < 0.005, respectively, when comparing two groups as indicated. C, Western blotting of caspase-3 (upper panel) in the cells transfected with empty vector (Mock), with a shRNA vector of the scrambled sequence (cont RNAi), and with a shRNA vector of caspase-3 (casp-3 RNAi). Lower panel, actin. Columns, mean number of invasive foci from three results; bars, SD. * and †, P < 0.001, when comparing two groups as indicated.

References

Cross Talk between Apoptosis and Invasion Signaling in Cancer Cells through Caspase-3 Activation

Mutsuko Mukai, Toshiyuki Kusama, Yukou Hamanaka, et al.


**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/20/9121

**Cited articles**
This article cites 20 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/20/9121.full#ref-list-1

**Citing articles**
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/20/9121.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.