Abstract. Malignant mesothelioma is an aggressive tumor arising from mesothelial cells of serous membranes, and forms spheroid-like cell aggregates in pleural and peritoneal effusions. We examined the levels of anoikis, apoptosis induced by the detachment of cells from the extracellular matrix, in suspension culture in the human mesothelioma cell line NCI-H2052. NCI-H2052 cells were adherent in conventional monolayer cultures, but were found to form spheroids in suspension cultures using dishes with ultra-low cell binding capacity. NCI-H2052 cells proliferated in both cultures, but the proliferation rate was markedly lower in suspension cultures than in monolayer cultures. In addition, NCI-H2052 cells in suspension cultures showed little apoptosis, suggesting that the suspension culture induces anoikis resistance. Western blot analysis revealed that suspension cultures induced activation of Src family kinases (SFK) after spheroid formation. Dasatinib, an inhibitor of multi-tyrosine kinases including SFK, abolished anoikis resistance in suspension cultures, indicating that SFK activated by spheroid formation are responsible for anoikis resistance. Cisplatin induced apoptosis in NCI-H2052 cells, but the apoptotic rate was significantly lower in suspension cultures than in monolayer cultures, suggesting that spheroid formation is involved in cisplatin resistance. Furthermore, a combination of dasatinib and cisplatin induced apoptosis more significantly than either alone in suspension cultures. These results suggest that spheroid formation induces resistance to anoikis and to cisplatin through SFK activation and that dasatinib facilitates cisplatin-induced apoptosis in human mesothelioma cells.

Introduction

Malignant mesothelioma is an aggressive tumor arising from mesothelial cells of serous membranes, including the pleura, peritoneum and pericardium (1-3). Mesothelioma is highly resistant to most chemotherapeutic drugs and radiation therapy (3), and surgical therapy generally show limited efficacy (3-5). So far a combination of cisplatin and pemetrexed appears to be the best chemotherapy regimen for mesothelioma, but the median survival of patients with mesothelioma remains at <12 months (5). Thus, new approaches for the mesothelioma treatment are urgently required.

Anoikis, a Greek word meaning ‘homelessness’, is defined as the subset of apoptosis triggered by cell-cell or cell-extracellular matrix (ECM) detachments (6,7). Apoptosis is programmed cell death in which caspases relay messages through so-called initiator caspases to effector caspases that mediate apoptotic processes, such as externalization of phosphatidylserine, membrane blebbing and nuclear fragmentation (8). These apoptotic features are all observed during anoikis (9). Anoikis plays a crucial role in defense mechanisms by preventing the re-adhesion of detached cells to incorrect locations and their unregulated growth (10,11). Anoikis resistance is emerging as a hallmark of cancer cells and contributes to invasion and metastasis formation of many types of tumors including mesothelioma (12). Three-dimensional tissue culture methods, including the formation of multicellular aggregates (spheroid) using suspension culture, have been adopted with the superiority over conventional monolayer culture to mimic the tumor behavior in vivo (13). Spheroid formation has been reported to be involved in anoikis resistance (13,14).
Src family kinases (SFK) are non-receptor and cytoplasmic tyrosine kinases that have a pivotal role in cell adhesion, proliferation, survival and apoptosis. Among the SFK, Src, Yes and Fyn show ubiquitous expression, whereas others including Lyn, exhibit more restricted tissue localization (15,16). There have been many studies showing that Src protein level or its kinase activity is increased in a variety of human tumors (16). Our previous study clarified that human mesothelioma cells express Lyn in addition to Src, Yes and Fyn (17).

Mesothelioma cells form spheroid-like cell aggregates in pleural and peritoneal effusions (14). Spheroid formation in other tumor types such as osteosarcoma appears to play a role in chemoresistance (18), but whether the aggregates of mesothelioma cells are associated with chemoresistance remains unclear. In this study, we examined the association between anoikis and chemoresistance in the human mesothelioma cell line NCI-H2052. We found that suspension cultures induced spheroid formation, resulting in the development of resistance to anoikis and to cisplatin. Further clarification of cell growth-related signal transduction revealed that suspension culture induces SFK activation and that inhibition of the SFK activation abolishes anoikis resistance, which in turn facilitates cisplatin-induced apoptosis in NCI-H2052 cells.

Materials and methods

Cell lines and reagents. A non-malignant transformed human pleural mesothelial cell line, Met5A and two human mesothelioma cell lines, NCI-H28 and NCI-H2052, were obtained from the American Type Culture Collection (Rockville, MD, USA). Conventional tissue-culture dishes (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) for monolayer cultures and non-adherent dishes with ultra-low cell binding capacity (HydroCell, Celsseed Inc., Tokyo, Japan) for suspension cultures were used. Cells were cultured in culture medium composed of RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Moregate, Brisbane, Australia), 5 µg/ml penicillin, 5 µg/ml streptomycin, and 10 µg/ml neomycin. Cells were incubated at 37°C under 95% air and 5% CO₂. Cisplatin (LKT Lab., Saint Paul, MN, USA) and dasatinib (Biovision, Mountain View, CA, USA) were prepared in dimethylsulfoxide (DMSO) using the following stock solutions: 125 mM cisplatin and 5 mM dasatinib. DMSO was used as a vehicle control as appropriate. All western blot analyses were performed three times and representative data are shown. Other chemicals were purchased from Sigma.

Cell proliferation analysis. Cell proliferation was analyzed as described earlier with slight modifications (19). Briefly, NCI-H2052 cells were seeded at 1x10⁵ per 5 ml in 60-mm dishes. The cells were incubated for 24-96 h and harvested by trypsinization. Cell numbers were measured with a Coulter Counter Z1 (Coulter Japan, Tokyo, Japan).

Morphological observation. Morphological observation was performed as described (20). Briefly, NCI-H2052 cells were incubated for 72 h as described above. For suspension cultures, the cells were spun down and the cell culture supernatant was aspirated. The cells were observed under a light microscope (Nicon, Tokyo, Japan) for phase contrast images.

Treatment with cisplatin and dasatinib. In experiments using cisplatin, cells were seeded at 2x10⁵ per 9 ml culture medium in 100-mm dishes or at 2x10³ per 90 µl culture medium in each well of a 96-well plate, and cultured for 24 h. After the incubation, 1 ml and 10 µl of fresh culture medium containing cisplatin or vehicle (DMSO) were added to the dishes and the wells of the plate, respectively, and incubation was continued for an additional 24-72 h. In experiments using dasatinib, cells were seeded at 2x10⁵ per 10 ml culture medium containing dasatinib or DMSO in 100-mm dishes, and then cultured for 24-96 h. The final concentration of dasatinib was chosen based on a prior study (17). In the combination experiments of cisplatin and dasatinib, cells were seeded at 2x10⁵ per 9 ml culture medium containing dasatinib or DMSO in 100-mm dishes, and cultured for 24 h. After the incubation, cisplatin or DMSO was added to 1 ml of fresh culture medium supplemented with dasatinib or DMSO, the medium was then added to the dishes, and incubation was continued for an additional 72 h.

Cell viability analysis. Cell viability analysis was performed as described (21). Briefly, cells were seeded in each well of a 96-well plate (Becton-Dickinson Labware) as described above. Cell viability was analyzed by a colorimetric assay using Cell Counting Kit-8 (CCK-8) (Dojin Chemical Institute, Kumamoto, Japan) according to the manufacturer's protocol.

Flow cytometric analysis of apoptosis. Apoptosis was analyzed by flow cytometry using an Annexin V (Ax)-FITC Kit (Medical and Biological Laboratories, Nagoya, Japan) as described (21). Briefly, 1x10⁵ cells treated with cisplatin or dasatinib were trypsinized, washed with phosphate-buffered saline (PBS) and then labeled with Ax-FITC and propidium iodide (PI). Fluorescence intensity was measured using a Cytomics FC 500 flow cytometer and CXP software (Beckman Coulter, Fullerton, CA, USA).

Western blotting and antibodies. Western blotting was performed as described (21). All antibodies used were purchased from Cell Signaling Technology (Beverly, MA, USA). All western blot analyses were performed three times and representative data are shown.

Statistical analysis. All data are presented as the means ± standard errors (SEs) of 3-4 independent experiments. Comparisons between two groups were performed using Student's unpaired t-test (p<0.05, **p<0.005).

Results

NCI-H2052 cells form spheroids and develop anoikis resistance in suspension cultures. The human mesothelioma NCI-H2052 cells adhered to the tissue-culture dish in conventional monolayer cultures (Fig. 1A, left panel), but were found to form multicellular aggregates (spheroids) in suspension cultures using a non-adherent dish with ultra-low cell binding capacity (HydroCell) after a 24-h incubation period (Fig. 1A, right panel). Anoikis, a subset of apoptosis,
is known to be induced by cell-cell or cell-ECM detachment and has been reported to be induced in suspension cultures of mesothelial cells (14). NCI-H2052 cells in suspension culture proliferated in a time-dependent manner, although the proliferation rate was markedly lower in suspension cultures than in monolayer cultures (Fig. 1B). Furthermore, flow cytometric analysis using double staining with Annexin V (Ax) and propidium iodide (PI) revealed that the suspension cultures as well as monolayer cultures showed little apoptosis in NCI-H2052 cells (Fig. 1C). These results suggest that suspension culture induces spheroid formation, resulting in the development of anoikis resistance in NCI-H2052 cells.

**SFK activation induced by suspension culture is responsible for anoikis resistance in NCI-H2052 cells.** We examined cell growth-related signal transduction in NCI-H2052 cells in monolayer and suspension cultures. Compared to monolayer cultures, suspension cultures suppressed Akt phosphorylation after a 24-h incubation, but induced SFK phosphorylation.
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after a 48-h incubation in a time-dependent manner (Fig. 2A). We then investigated the effects of dasatinib, an inhibitor of multi-tyrosine kinases including SFK, on NCI-H2052 cells in monolayer and suspension cultures. Dasatinib inhibited SFK phosphorylation in monolayer and suspension cultures (Fig. 2B). Dasatinib slightly induced apoptosis in monolayer cultures, but apoptosis was markedly induced by dasatinib in suspension cultures (Fig. 2C). In addition, dasatinib markedly induced caspase-3 cleavage in suspension cultures (Fig. 2D). Thus, inhibition of SFK activation by dasatinib abolished anoikis resistance induced by spheroid formation. Collectively, these results suggest that SFK activation induced by spheroid formation is responsible for anoikis resistance in NCI-H2052 cells.

Spheroid formation in suspension cultures develops resistance to cisplatin in NCI-H2052 cells. Subsequently, we investigated the effects of cisplatin on the human mesothelial cell line Met5A, and the human mesothelioma cell lines NCI-H2052 and NCI-H28. In monolayer cultures, cisplatin reduced cell viability in Met5A and NCI-H2052 cells to a much higher degree than in NCI-H28 cells (Fig. 3A). In addition, cisplatin induced apoptosis in these cell lines in a concentration-dependent manner, but 10 µM cisplatin did not induce apoptosis in NCI-H28 cells compared to Met5A and NCI-H2052 cells (Fig. 3B). These results suggest that NCI-H2052 cells as well as Met5A cells are more sensitive to cisplatin than NCI-H28 cells in monolayer cultures. Intriguingly, suspension cultures suppressed cisplatin-induced apoptosis compared to monolayer cultures in NCI-H2052 cells (Fig. 3C). These results suggest that spheroid formation in suspension culture induces cisplatin resistance in NCI-H2052 cells.

Dasatinib facilitates cisplatin-induced apoptosis in suspension culture in NCI-H2052 cells. To further investigate whether dasatinib facilitates cisplatin-induced apoptosis in suspension culture, we treated NCI-H2052 cells with dasatinib together with cisplatin. Treatment with the combination of dasatinib and cisplatin induced apoptosis more significantly
and to cisplatin. The spheroids activate SFK, which leads to the development of resistance to anoikis in mesothelioma cells. Mesothelioma cells form spheroids in suspension similar to those observed in pleural and peritoneal effusions. and to cisplatin in mesothelioma cells. Mesothelioma cells form spheroids in suspension similar to those observed in pleural and peritoneal effusions. The spheroids activate SFK, which leads to the development of resistance to anoikis and to cisplatin.

Discussion

As most tissues have three-dimensional structures composed of multiple types of cells, suspension culture that induces spheroid formation has been increasingly used in the field of cancer research to mimic the in vivo condition. In the present study, we adopted a suspension culture system by using a non-adherent dish with ultra-low cell binding capacity, which enabled NCI-H2052, a mesothelioma cell line, to form spheroids (Fig. 1). We further used this culture system to analyze the sensitivity of these mesothelioma cells to anoikis and drug-induced apoptosis.

Anoikis is the subset of apoptosis triggered by the detachment of cells from other cells or the ECM, and anoikis resistance is associated with cancer metastasis. We found that NCI-H2052 cells in suspension cultures formed spheroids, which led to the induction of anoikis resistance (Fig. 1). Mesothelioma cells form spheroid-like cell aggregates in pleural and peritoneal effusions, and mesothelioma cell lines, but not the non-malignant mesothelial cell line Met5A, resist anoikis as multicellular aggregates in suspension culture (14). Some tumors, including lung and ovarian cancers, have shown a similar correlation between spheroid formation and anoikis resistance (22,23). These results suggest that spheroid formation of malignant tumors favors the induction of anoikis resistance.

Akt is a serine/threonine kinase that plays a crucial role in multiple cellular processes such as cell proliferation, survival and apoptosis, and especially inactivates pro-apoptotic factors, leading to cell survival (24). In anoikis resistant osteosarcoma cells, Akt activity was upregulated in suspension cultures (25). However, in the present study, Akt activity in suspension cultures was lower than that in monolayer cultures (Fig. 2). Similarly, Barbone et al reported that Akt activity is downregulated in suspension cultures in mesothelioma cells (26). Presently, we have no rational explanation for this difference, but given the multifactorial nature of cancer, it is possible that Akt downregulation in anoikis resistance may be mesothelioma-specific. In addition, this Akt downregulation may be associated with the observed decrease in growth rate of NCI-H2052 cells in suspension cultures.

Malignant mesothelioma is refractory to conventional chemotherapy, which is related to resistance to the apoptosis induced by chemotherapeutic drugs. It has been reported that spheroid cells in osteosarcoma and ovarian cancer are more resistant to chemotherapeutic drugs than their parental cells (18,27). In the present study, spheroid-forming NCI-H2052 cells developed cisplatin resistance (Fig. 3). Thus, it seems to be a general phenomenon that spheroid formation of malignant tumors induces chemoresistance.

v-Src transformation in MDCK cells induces anoikis resistance (6), but how SFK including endogenous Src (c-Src) are involved in anoikis resistance remains unclear in malignant mesothelioma. It has been reported that suspension culture induces Src activation in anoikis resistant osteosarcoma cells (25). Similarly, we found that suspension cultures induced SFK activation and anoikis resistance in mesothelioma cells (Fig. 2). Our previous study also showed that the Fyn-expressing mesothelioma NCI-H2052 cells, are more insensitive to SFK inhibitors including dasatinib than Fyn-deficient mesothelioma cells, NCI-H28, in monolayer cultures (17). However, we observed that the rate of NCI-H2052 cell apoptosis induced by dasatinib in suspension cultures was more prominent than that in monolayer cultures (Fig. 2). These results suggest that inhibition of SFK activation abolishes the anoikis resistance of spheroid-forming mesothelioma cells. Furthermore, the present study shows for the first time that inhibition of SFK activation in spheroid cultures of a mesothelioma cell line facilitates cisplatin-induced apoptosis (Fig. 4). Phase II clinical trials have shown that dasatinib alone has been ineffective in unselected mesothelioma patients (28), but our study suggests that combination therapy using dasatinib and cisplatin may be more effective than dasatinib alone in the treatment of mesothelioma.

In conclusion, mesothelioma cells form spheroids in suspension culture that induces SFK activation, resulting in developing resistance to anoikis and to cisplatin (Fig. 5). This study also suggests that the combination of dasatinib
and cisplatin is potentially useful for treatment of malignant mesothelioma.

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