A biochemical mechanism for resistance of intervertebral discs to metastatic cancer: Fas ligand produced by disc cells induces apoptotic cell death of cancer cells

Jong-Beom Park · Jin-Kyung Lee · Sung-Tae Cho · Eun-Young Park · K. Daniel Riew

Received: 16 July 2007 / Accepted: 23 July 2007 / Published online: 8 August 2007
© Springer-Verlag 2007

Abstract Metastatic spinal cancer is characterized by the maintenance of normal disc structure until the vertebral body is severely destroyed by cancer cells. Anatomic features of the discs have been thought to be the main factor which confer the discs their resistance to metastatic cancer. However, little is known about the biochemical mechanism to prevent or attenuate the local infiltration of cancer cells into the discs. The purpose of this study was to investigate whether Fas ligand (FasL) produced by disc cells can kill Fas-bearing breast cancer cells by Fas and FasL interaction. Two human breast cancer cells (MCF-7 and MDA-MB-231) were obtained and cultured (1×10^6 cells/well), and the expression of Fas was investigated by western blot analysis. Annulus fibrosus cells were isolated and cultured, and the presence of FasL was quantified in the supernatants of three different numbers of annulus fibrosus cells ($1 \times$, $2 \times$, and 4×10^6 cells/well) by ELISA assay. The MCF-7 and MDA-MB-231 cancer cells were cultured with supernatants of annulus fibrosus cells for 48 h. As controls, MCF-7 and MDA-MB-231 cancer cells were also cultured by

themselves for 48 h. Finally, we determined and quantified the apoptosis rates of MCF-7 and MDA-MB-231 cancer cells by Annexin V–FITC and PI and TUNEL at 48 h, respectively. The expression of Fas was identified in MCF-7 and MDA-MB-231 cancer cells. The mean concentrations of FasL in supernatants of annulus fibrosus cells ($1 \times$, $2 \times$, and 4×10^6 cells/well) were 10.8, 29.6, and 56.4 pg/mL, respectively. After treatment with the supernatant of three different numbers of annulus fibrosus cells, the mean apoptosis rate of MCF-7 cancer cells was increased (2.8%, $P < 0.01$; 6.7%, $P < 0.001$; 31.0%, $P < 0.001$) in a dose-dependent manner of FasL compared to that of control (1.1%). The mean apoptosis rate of MDA-MB-231 cancer cells was also increased (5.7%, $P < 0.01$; 11.1%, $P < 0.001$; 25.3%, $P < 0.001$) in a dose-dependent manner of FasL compared to that of control (2.1%). TUNEL also demonstrated direct evidence of apoptoses of MCF-7 and MDA-MB-231 cancer cells. Our results demonstrate that Fas-bearing cancer cells undergo apoptosis by FasL produced by disc cells, which may be considered as a potential biochemical explanation for the disc's resistance to metastatic cancer.

This is the EuroSpine Winning Paper 2007 on Basic Science.

J.-B. Park (✉) · S.-T. Cho · E.-Y. Park
Department of Orthopaedic Surgery,
The Catholic University of Korea School of Medicine,
Uijongbu-si, Kyunggi-do 480-717, South Korea
e-mail: spinejb@catholic.ac.kr

J.-K. Lee
Department of Laboratory Medicine,
Korea Cancer Center Hospital, Seoul, South Korea

K. Daniel Riew
Department of Orthopaedic Surgery,
Washington University School of Medicine, St Louis, MO, USA

Keywords Intervertebral disc · Resistance · Metastatic cancer · Fas and Fas ligand · Apoptosis

Introduction

Despite the frequency of vertebral metastases, the intervertebral disc is usually spared and preserves its normal structure even after extensive collapse of the vertebral body by neoplastic tissue [1, 4, 10, 13]. A number of possible explanations for the disc's resistance to metastatic cancer have been proposed [2, 3, 32]. The most common one is

that the disc is avascular, limiting its exposure to metastatic cells. However, it is well known that with aging process, neovascularization of the annulus fibrosus gradually occurs [12, 21, 30]. Although this might serve as hematogenous route for metastatic cancer cells that reach the intervertebral discs through systemic circulation, the intervertebral discs of older patients are still resistant to metastatic cancer. Another explanation is that the abundant collagen content and high intradiscal pressure of the discs act as mechanical barriers against tumor invasion. However, metastatic cancer cells secrete matrix metalloproteinases, such as collagenase, that can destroy extracellular matrix components of the discs during metastasis or progression [5, 11, 17, 23]. Therefore, these findings suggest that there may be a mechanism other than the disc's anatomic characteristics that confer its resistance to metastases.

When Fas ligand (FasL), a protein belonging to the tumor necrosis factor family, binds to the Fas receptor, the cells die within hours [22, 24, 27]. While the Fas receptor is expressed in a wide variety of cells, FasL expression is tightly restricted to activated T cells, natural killer cells, and stromal cells of certain immune-privileged sites such as the eye, testis, and brain [7, 8]. The interaction between Fas receptor and FasL plays a major role in the regulation of immune responses. In general, FasL expressed by immune cells binds to Fas receptor on the surface of target cells and induces apoptosis of the target cells by activation of downstream caspases. However, in immune-privileged sites, the immune cells become the target of destruction. In these organs, the stromal cells express FasL, which binds to Fas receptor on the surface of immune cells that are infiltrating into the organs. This induces apoptosis of the infiltrating immune cells, so as to maintain the organ's immune-privileged status.

It has been reported that the intervertebral disc cells including nucleus pulposus and annulus fibrosus cells express FasL, which is a prerequisite of immune-privileged site [19, 25]. In addition, Nishida et al. [18] have demonstrated long-term expression of adenovirus-mediated transgene by disc cells. One possible explanation for such long-term expression is that the FasL produced by disc cells prevents infiltration by Fas-bearing activated T cells or natural killer cells that would normally destroy such cells. A similar mechanism may explain how the intervertebral discs resist infiltration by metastatic tumor cells that bear Fas receptors on their membranes. To date, however, no study has directly demonstrated apoptosis of either immune or cancer cells by FasL-expressing disc cells. We therefore performed the current study to investigate the hypothesis that FasL-expressing disc cells can kill Fas-bearing malignant tumor cells by Fas and FasL interaction.

Materials and methods

Cell culture

Five lumbar intervertebral discs (L1–L6) were harvested from two male Sprague Dawley rats (age 4 weeks) immediately after killing. We carefully dissected the discs under the microscope to obtain annulus fibrosus tissue and cultured them for 12 h. To isolate the cells, annulus fibrosus tissues were digested for 4 h, filtered, and washed. After three-passage, the cells were trypsinized, and subcultured into six-well plates at 1×10^6 cells/well. Two breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased and cultured. After three-passage, the cells were trypsinized and subcultured into six-well plates at 1×10^6 cells/well.

Expression of Fas in two breast cancer cells

Expression of Fas was determined in lysates of MCF-7 and MDA-MB-231 breast cancer cells (1×10^6 cells/well) by western blot analysis according to the manufacturer's instructions. Primary antibody for Fas was purchased from Santa Cruz Biotechnology (Santa Cruz, California). β -Actin was used as an internal control for protein loading. Rat thymus was used as positive control for Fas.

Presence of FasL in supernatant of annulus fibrosus cells

The presence of FasL in the supernatant of three different numbers of cultured annulus fibrosus cells ($1 \times$, $2 \times$, and 4×10^6 cells/well) was determined and quantified by enzyme-linked immunosorbent assay (ELISA) kit with antibody that recognize rat FasL (R and D Systems, Minneapolis, MN) according to the manufacturer's instructions. For calibration, we used natural rat FasL provided by the supplier to construct a standard curve and to obtain absolute values. The concentration of FasL was measured three times in each sample, and the average of the three measurements was considered to be the final concentration.

Apoptosis of MCF-7 and MDA-MB-231 breast cancer cells

MCF-7 and MDA-MB-231 breast cancer cells were subcultured into six-well plates at 1×10^6 cells/well and treated with 300 μ l supernatants that were obtained from three different numbers of cultured annulus fibrosus cells

($1\times$, $2\times$, and 4×10^6 cells/well) for 48 h, respectively. Apoptosis rates of three different concentrations of FasL groups in two breast cancer cells were quantified by staining cells with both Annexin V–FITC (PharMingen, San Diego, CA) and propidium iodide (PI; PharMingen, San Diego, CA) following the manufacturer's instructions, and then analyzed with FACScan flow cytometry (Becton Dickinson, San Jose, CA) at 48 h. The apoptosis rates of the breast cancer cell lines that were cultured for 48 h by themselves were used as controls. All experiments were performed three times for each sample, and the average of the three measurements was considered to be the final apoptosis rate.

Direct evidence of in situ apoptosis of two breast cancer cells was determined by staining the cells with 15 μ l AP-OPercentage dye incubated for 30 min. After syringing off the culture medium and dye mixture the cells were gently washed twice with 500 μ l/well PBS, and were transferred to an inverted microscope and photographs were obtained. Two breast cancer cell lines that were cultured for 48 h by themselves were used as controls.

Statistical analysis

Statistical analysis was determined by the paired samples *t* test. Asterisk (***) $P < 0.001$, (***) $P < 0.01$, (*) $P < 0.05$) was considered to be statistically significant.

Results

Expression of Fas in MCF-7 and MDA-MB-231 breast cancer cells

Western blot analysis clearly demonstrated the expression of Fas in the lysates of MCF-7 and MDA-MB-231 breast cancer cells (Fig. 1).

Concentration of FasL in supernatants of annulus fibrosus cells

The mean concentrations of FasL in supernatant of annulus fibrosus cells were 10.8 ± 2.0 pg/mL (1×10^6 cells/well),

Fig. 1 Western blot analysis showing expressions of Fas in MCF-7 and MDA-MB-231 breast cancer cells. Cont = positive control; rat thymus was used as positive controls for Fas

29.6 ± 4.5 pg/mL (2×10^6 cells/well), and 56.4 ± 7.2 pg/mL (4×10^6 cells/well) (mean $\pm$ SD).

Apoptosis of MCF-7 breast cancer cells

The mean percentage of apoptotic cell death in MCF-7 cancer cells treated with supernatant of annulus fibrosus cells containing three different concentrations of FasL was significantly increased ($2.8 \pm 0.9\%$, $P < 0.01$; $6.4 \pm 2.1\%$, $P < 0.001$; $31.0 \pm 5.3\%$; $P < 0.001$) compared with control ($1.1 \pm 0.4\%$) in a dose-dependent manner (Fig. 2a). TUNEL also clearly demonstrated significantly increased apoptotic cell death of MCF-7 cancer cells treated with the supernatant of annulus fibrosus cells containing three different concentrations of FasL (Fig. 2b), respectively.

Apoptosis of MDA-MB-231 breast cancer cells

The mean percentage of apoptotic cell death in MDA-MB-231 cancer cells treated with the supernatant of annulus fibrosus cells containing three different concentrations of FasL was significantly increased ($5.7 \pm 1.7\%$, $P < 0.01$; $11.1 \pm 3.6\%$, $P < 0.001$; $25.3 \pm 6.2\%$, $P < 0.001$) compared with control ($2.1 \pm 0.6\%$) in a dose-dependent manner (Fig. 3a). TUNEL also clearly demonstrated significantly increased apoptotic cell death of MDA-MB-231 cancer cells treated with the supernatant of annulus fibrosus cells containing three different concentrations of FasL (Fig. 3b), respectively.

Discussion

Many cancers have been found to exhibit de novo expression of FasL, which plays an important role in local tissue destruction, metastatic spread, and immune escape of cancer cells [15, 20, 31]. The expression of FasL by cancer cells enables them to kill Fas-bearing immune cells and stromal cell of the target organs or tissues. Therefore, FasL expression by cancer cells has been thought to facilitate the establishment of cancer metastasis in organs, such as liver, lung, colon, and breast. However, in the spine the normal anatomic structure of the intervertebral disc is often preserved even in the face of severe vertebral body destruction by cancer cells. The intervertebral disc is separated from vertebral body by cartilaginous endplates. The tissue constituting the cartilaginous endplates contains protease inhibitors and other substances that inhibit tumor vascularization [14]. Moreover, with aging process, the cartilaginous endplates become sclerotic, calcific, and bony plates that can inhibit vascular invasion of the cancer

Fig. 2 After treatment of MCF-7 breast cancer cells with supernatants of annulus fibrosus cells ($1 \times$, $2 \times$, and 4×10^6 cells/well) containing Fas ligand for 48 h, apoptotic cell death was assayed by flow cytometry after double staining with Annexin V-FITC and propidium iodide (PI) (**a**) and TUNEL (**b**). Asterisk (***) $P < 0.001$, ** $P < 0.01$ was considered to be statistically significant

cells and diffusion of nutrients into the discs [9, 29]. On the contrary, with increasing age, neovascularization of the annulus fibrosus increases and may enhance hematogenous invasion of the metastatic cancer cells, which reach the intervertebral discs via systemic circulation, into the discs [12, 21, 30]. However, Fujita et al. [6] reported that although many metastatic cancer cells are frequently found at the outer portion of the annulus fibrosus, none of them could infiltrate into the inner portion of the annulus fibrosus. On the basis of what is known about the characteristics of metastatic spinal tumors and Fas and FasL interaction, we hypothesized that FasL-expressing intervertebral disc cells kill Fas-bearing cancer cells infiltrating into the discs to maintain the normal intervertebral disc structure.

In the current study, we found a significantly increased apoptosis of Fas-bearing cancer cells by the supernatant of annulus fibrosus cells containing FasL in a dose-dependent

manner. These findings suggest that local expression of FasL by the intervertebral disc cells may function as a biochemical mechanism, which contributes to the resistance of the disc to local invasion by cancer cells. It is well known that the human FasL is known to be highly homologous to that of mouse and rat, demonstrating cross-reactivity with cell-surface Fas from either species [16, 26, 28]. The amino acid sequences of human, mouse, and rat FasL are highly similar and no species-specificity is observed among human, mouse, and rat FasL. Therefore, both human or rat FasL can induce apoptosis in the cells expressing either human Fas or rat Fas. In the current study, we used FasL of rat annulus fibrosus cells to induce apoptosis of Fas-positive human breast cancer cells. The results of our study were in accordance with those of previous studies that demonstrated no species-specificity of Fas and FasL interaction [16, 26, 28].

Fig. 3 After treatment of MDA-MB-231 breast cancer cells with supernatants of annulus fibrosus cells ($1 \times$, $2 \times$, and 4×10^6 cells/well) containing Fas ligand for 48 h, apoptotic cell death was assayed by flow cytometry after double staining with Annexin V-FITC and propidium iodide (PI) (**a**) and TUNEL (**b**). Asterisk (***) $P < 0.001$, ** $P < 0.01$) was considered to be statistically significant

We cannot be positive that it was just the FasL in the supernatant that increased the apoptosis of malignant tumor cells. It is possible that there could be another biochemical compound that disc cells release into the supernatant and increase apoptosis of cancer cells. While there may be some unknown substance, it is known that FasL is sufficient to induce apoptosis of Fas-positive cancer cells.

In the current study, while FasL of annulus fibrosus cells induced apoptotic cell death of Fas-positive cancer cells, the range of percentage of cancer cells undergoing apoptosis was very wide (3–31%) depending on the concentration of FasL produced by different numbers of annulus fibrosus cells. However, we do not know how many metastatic cancer cells and disc cells might encounter at the outer surface of the intervertebral disc

during the metastasis at any given time in vivo situation. Further, we are uncertain which dosage of FasL that we used is similar to what is present in the normal disc tissue.

In conclusion, our results demonstrate that Fas-bearing cancer cells undergo apoptotic cell death by FasL produced by disc cells, which may be considered as a potential biochemical explanation for the disc's resistance to metastatic cancer. We believe that it works in conjunction with anatomic factors to confer the intervertebral disc its resistance to local invasion by cancer cells. We are currently investigating whether such interactions also occur in other types of tumors that commonly metastasize to the spine. Further research in the interaction between Fas and FasL may yield novel treatment for limiting spinal metastatic disease.

References

- Asdourian PL, Weidenbaum M, DeWald RL, et al (1990) The pattern of vertebral involvement in metastatic vertebral breast cancer. *Clin Orthop* 250:164–170
- Batson OV (1940) The function of the vertebral veins and their role in the spread of metastases. *Ann Surg* 112:138–149
- Berrettoni BA, Carter JR (1986) Current concepts review: mechanisms of cancer metastasis to bone. *J Bone Joint Surg Am* 68:308–312
- Boland PJ, Lane JM, Sundaresan N (1982) Metastatic disease of the spine. *Clin Orthop* 169:95–102
- Dunne AA, Grobe A, Sesterhenn AM, et al (2005) Influence of matrix metalloproteinase 9 (MMP-9) on the metastatic behavior of oropharyngeal cancer. *Anticancer Res* 25:4129–4134
- Fujita T, Ueda Y, Kawahara N, et al (1997) Local spread of metastatic vertebral tumors: a histologic study. *Spine* 22:1905–1912
- Griffith TS, Brunner T, Fletcher SM, et al (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 270:1189–1192
- Griffith TS, Ferguson TA (1997) The role of FasL-induced apoptosis in immune privilege. *Immunol Today* 18:240–244
- Gruber HE, Ashraf N, Kilburn J, et al (2005) Vertebral endplate architecture and vascularization: application of micro-computerized tomography, a vascular tracer, and immunocytochemistry in analyses of disc degeneration in the aging sand rat. *Spine* 30:2593–2600
- Harrington KD (1986) Metastatic disease of the spine. *J Bone Joint Surg Am* 68:1110–1115
- Kamat AA, Fletcher M, Gruman LM, et al (2006) The clinical relevance of stromal matrix metalloproteinase expression in ovarian cancer. *Clin Cancer Res* 12:1707–1714
- Kauppila LI (1995) Ingrowth of blood vessels in disc degeneration. Angiographic and histological studies of Cadaveric spines. *J Bone Joint Surg Am* 77:26–31
- Krishnamurthy GT, Tubis M, Hiss J, et al (1977) Distribution pattern of metastatic bone disease: a need for total body skeletal image. *JAMA* 237:2504–2506
- Langer R, Brem H, Falterman K, et al (1976) Isolation of a cartilage factor that inhibits tumor neovascularization. *Science* 193:70–72
- Müllauer L, Mosberger I, Grusch M, et al (2000) Fas ligand is expressed in normal breast epithelial cells and is frequently up-regulated in breast cancer. *J Pathol* 190:20–30
- Muneta Y, Shimoji Y, Inumaru S, et al (2001) Molecular cloning, characterization, and expression of porcine Fas ligand (CD95 Ligand). *J Interferon Cytokine Res* 21:305–312
- Nikkola J, Vihinen P, Vuoristo MS, et al (2005) High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. *Clin Cancer Res* 11:5158–5166
- Nishida K, Kang JD, Gilbertson LG, et al (1999) Modulation of the biologic activity of the rabbit intervertebral disc by gene therapy: an in vivo study of adenovirus-mediated transfer of the human transforming growth factor beta 1 encoding gene. *Spine* 24:2419–2425
- Park JB, Chang H, Kim KW (2001) Expression of Fas ligand and apoptosis of disc cells in herniated lumbar disc tissue. *Spine* 26:618–621
- Reichmann E (2002) The biological role of the Fas/FasL system during tumor formation and progression. *Cancer Biol* 12:309–315
- Roberts S, Evans H, Trivedi J, et al (2006) Histology and pathology of the human intervertebral disc. *J Bone Joint Surg Am* 88:S10–S14
- Schneider P, Holler N, Bodmer JL, et al (1998) Conversion of membrane-bound Fas (CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J Exp Med* 187:1205–1213
- Sprague JE, Li QP, Liang K, et al (2006) In vitro and in vivo investigation of matrix metalloproteinase expression in metastatic tumor models. *Nucl Med Biol* 33:227–237
- Suda T, Nagata S (1994) Purification and characterization of the Fas-ligand that induces apoptosis. *J Exp Med* 179:873–879
- Takada T, Nishida K, Doita M, et al (2002) Fas ligand expression on intervertebral disc cells: a potential molecular mechanism for immune privileged of the disc. *Spine* 27:1526–1530
- Takahashi T, Tanaka M, Inazawa J, et al (1994) Human Fas ligand: gene structure, chromosomal location and species specificity. *Int Immunol* 6:1567–1574
- Tanaka M, Itai T, Adachi M, et al (1998) Downregulation of Fas ligand by shedding. *Nat Med* 4:31–36
- Tsuyuki S, Kono M, Bloom ET (2002) Cloning and potential utility of porcine Fas ligand: overexpression in porcine endothelial cells protects them from attack by human cytolytic cells. *Xenotransplantation* 9:410–421
- Urban JP, Smith S, Fairbank JC (2004) Nutrition of the intervertebral disc. *Spine* 29:2700–2709
- Yasuma T, Yamauchi Y, Arai K, et al (1989) Histopathologic study on tumor infiltration into the intervertebral disc. *Spine* 14:1245–1248
- Younes M, Schwartz MR, Ertan A, et al (2000) Fas ligand expression in esophageal carcinomas and their lymph node metastases. *Cancer* 88:524–528
- Yuh WTC, Quets JP, Lee HJ, et al (1996) Anatomic distribution of metastases in the vertebral body and modes of hematogenous spread. *Spine* 21:2243–2250