Enhanced progression of human prostate cancer PC3 cells induced by the microenvironment of the seminal vesicle

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The objective of this study was to characterise the mechanism mediating the prostate cancer progression induced by the microenvironment of seminal vesicle (SV). The invasive potential of PC3 cells significantly increased after treatment with extract from SV of NOD/SCID mouse. Among several growth factors and cytokines that were present in the SV extract, transforming growth factor-β1 (TGF-β1) significantly enhanced the invasive potential of PC3 cells; however, the additional treatment with neutralising antibody against TGF-β1 suppressed the enhanced invasive potential induced by the SV extract. Changes in the invasive potential in PC3 cells after treatment with the SV extract and/or TGF-β1 were in proportion to those in the production of urokinase-type plasminogen activator (uPA) by PC3 cells. Tumour growth as well as the incidence of lymph node metastasis in NOD/SCID mice after the injection of PC3 cells into the SV were significantly greater than those after the injection into the prostate. These findings suggest that the microenvironment of SV enhances the progression of prostate cancer through a stimulated invasive potential, and that enhanced uPA production in prostate cancer cells induced by TGF-β1 could therefore be one of the most important mechanisms involved in the progression of prostate cancer after SV invasion.

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Invasion of prostate cancer cells into the seminal vesicle (SV) is an adverse prognostic factor in patients undergoing radical prostatectomy. Contemporary series analysing outcomes of radical prostatectomy reported that biochemical recurrence occurred in more than 50% of patients with SV invasion (Sofer et al, 2003; Bloom et al, 2004). However, SV invasion has been shown to lack a systematic relationship with other potential pathological factors indicating a poor prognosis, and there has not been any independent prognostic predictor in patients with SV invasion (Sofer et al, 2003; Masterson et al, 2005). These findings suggest that adverse features of prostate cancer with SV invasion may be due to an acquired aggressive phenotype rather than ‘volume effect’ as a result of disease progression.

The outcome of cancer progression depends on multiple interactions of cancer cells with various host factors; that is, the interaction of tumour cells with the organ environment modulates the tumorigenic properties by regulating their phenotypes, such as cell proliferation, motility and invasion (Fidler, 1990). A number of studies have demonstrated significant effects of organ microenvironment on the malignant potentials in several types of cancer cells, including prostate cancer, using in vivo experimental models (Gohji et al, 1997; Sato et al, 1997; Alencar et al, 2005). For example, Gohji et al reported that human renal cancer cells implanted in the subcutis of nude mice produced local nonmetastatic tumours, whereas the same cells orthotopically implanted in the kidney resulted in the formation of local tumours and metastases to the lungs. Furthermore, they clarified the important role of proteolytic enzymes whose production is influenced by the organ microenvironment, in the progression of implanted renal cell carcinoma cells (Gohji et al, 1997).

To date, the molecular mechanism mediating disease progression following SV invasion has remained largely unknown, and there is no study analysing whether the malignant phenotype of prostate cancer cells is affected by the microenvironment of SV. We, therefore, developed an implantation model of human prostate cancer cells to the SV of immunodeficient mice, resulting in systemic disease progression in vivo, and investigated the mechanism underlying the modulation of malignant potential of human prostate cancer cells induced by the microenvironment of SV.

MATERIALS AND METHODS

Reagents

Recombinant human transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor, hepatocyte growth factor, platelet-derived growth factor, granulocyte colony-stimulating factor, anti-human TGF-β1 antibody, and anti-human epidermal growth factor (EGF) antibody were from R&D Systems (Minneapolis, MN, USA). Recombinant human EGF, granulocyte monocyte colony-stimulating factor, tumour necrosis factor-α, interleukin-1β, interleukin-6, fibronectin, and anti-rat β-tubulin antibody were from Chemicon International (Temecula, CA, USA). Anti-human urokinase-type plasminogen activator (uPA) antibody and quantitative sandwich
enzyme immunoassay kit for human uPA were from American
Diagnostica (Greenwich, CT, USA). Horseradish peroxidase-
conjugated anti-mouse IgG antibody was from Amersham Life
Science (Arlington Heights, IL, USA). Biotinylated goat anti-mouse
IgG was from Vector Laboratories (Burlingame, CA, USA).

Tumour cell line

PC3, derived from human prostate cancer, was purchased from the
American Type Culture Collection (Rockville, MD, USA). Cells
were maintained in DMEM (Life Technologies Inc., Gaithersburg,
MD, USA) supplemented with 5% heat-inactivated fetal calf serum.

Preparation of extracts from the SV and prostate

After 12-week-old nonobese diabetic/severe combined immuno-
deficient (NOD/SCID) mice (CLEA Japan Inc., Tokyo, Japan) were
killed, SV and prostate were harvested, washed with PBS, and
disrupted using a sonicator (Ultrasonic Systems Inc., Haverhill,
MA, USA). Following centrifugation of the respective extracts of
SV and prostate, each supernatant was stored at −80°C until used.

In vitro cell growth assay

The effects of extract from the SV or prostate on the in vitro
growth of PC3 cells were assessed using MTT (Sigma Chemical Co.,
St Louis, MO, USA) as described previously (Yamanaka et al.,
2005). Briefly, 1 × 10⁴ cells were seeded in each well of 96-well
microtitre plates and allowed to attach overnight. Cells were then
treated with various concentrations of either SV or prostate extract
diluted with serum-free DMEM/F12. After 48 h of incubation, 20 µl
of 5 mg/mL MTT in PBS was added to each well, followed by
incubation for 4 h at 37°C. The formazan crystals were dissolved in
DMSO. The optical density was determined with a microculture
plate reader (Becton Dickinson Labware, Lincoln Park, NJ, USA) at
540 nm. Absorbance values were normalised to the values obtained
for vehicle-treated cells. Each assay was performed in triplicate.

In vitro tumour cell invasion assay

Tumour cell invasion was measured using cell invasion assay kit
(Chemicon) as described previously (Miyake et al., 1999a). Briefly,
we used polycarbonate filters with a pore size of 8 µm coated with
basement membrane Matrigel. The coated filters were placed in
Boyden chambers, in the upper compartment of which 1 × 10⁴ cells
were suspended in serum-free DMEM/F-12 followed by treatment
with extract from the SV or prostate and/or growth factor or
cytokine and in the lower compartment of which 25 mg/mL
fibronectin diluted with serum-free DMEM/F-12 were added as a
chemoattractant. After 48 h incubation at 37°C, cells on the top
side of the filter were removed, and cells that had migrated and
invaded the Matrigel through the filter and attached to the bottom
of the membrane were stained with crystal violet stain solution.
The crystal violet stain solution was eluted with 10% acetic acid
extraction buffer and transferred to wells of a 96-well microtitre
plate, and the absorbance was read with a microculture plate
reader (Becton Dickinson Labware) at 540 nm. Absorbance values
were normalised by the values obtained for the vehicle-treated
cells. Similarly, cell motility was also assessed using the Boyden
chambers without Matrigel. Each assay was performed in triplicate.

Measurement of uPA levels in conditioned media

The concentrations of uPA in conditioned media were determined
using a quantitative sandwich enzyme immunoassay kit for human
uPA as described previously (Miyake et al., 1999b). Briefly, PC3
cells were seeded in each well of 96-well microtitre plates and
allowed to attach overnight. Cells were then treated with extract
from the SV, TGF-β1, and/or anti-TGF-β1 antibody diluted with
serum-free DMEM/F-12. After 48 h of incubation, serum-free
DMEM/F-12 was collected. For each analysis, 100 µl of conditioned
media were added to microtitre plates coated with a purified
polyclonal antibody against human uPA. Bound uPA was detected
by an additional biotinylated anti-uPA antibody. After the addition
of streptavidin-conjugated horseradish peroxidase, peroxidase-
mediated conversion of 3,3′,5,5′-tetramethylbenzene was measured
with a microculture plate reader (Becton Dickinson Labware) at
450 nm. Each assay was performed in triplicate.

Western blot analysis

Western blot analysis was used to evaluate the expression level of
uPA protein in PC3 cells after treatment with extract from the SV
and/or anti-TGF-β1 antibody as described previously (Miyake et al.,
1999a). Briefly, samples containing equal amounts of protein (15 µg)
from lysates of the cultured PC3 cells were electrophoresed on an
SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The
filters were blocked in PBS containing 5% nonfat milk powder at
4°C overnight and then incubated for 1 h with a 1:400-diluted anti-
human uPA mouse antibody or 1:10 000-diluted anti-rat β-tubulin
mouse antibody. The filters were then incubated for 30 min with
horseradish peroxidase-conjugated anti-mouse IgG antibody, and
specific proteins were detected using an enhanced chemilumines-
cence western blotting analysis system (Amersham Life Science).

Assessment of in vivo tumour growth

Male NOD/SCID mice, 10- to 12-week-old, (CLEA Japan Inc.) were
housed in a controlled environment at 22°C on a 12-h light and
12-h dark cycle. Animals were maintained in accordance with
the National Institutes of Health Guide for the Care and Use of
Laboratory Animals. Each experimental group consisted of 10
mice. PC3 cells were trypsinised, washed twice with PBS, and
5 × 10⁵ cells suspended in 20 µl of PBS were directly injected into
the SV or the dorsal love of the prostate under the prostatic
capsule. Eight weeks after the injection of tumour cells, the mice
were killed and the presence of metastasis was macroscopically
examined in all abdominal organs, and the weight of each tumour
formed in the SV or prostate was measured.

Statistical analysis

Differences between the two groups were compared using the
χ²-test, unpaired t-test or Mann–Whitney U-test. All statistical
calculations were performed using Statview 5.0 software (Abacus
Concepts Inc., Berkley, CA, USA), and P-values < 0.05 were
considered significant.

RESULTS

Changes in the malignant phenotype of PC3 cells induced
by extract from the SV or prostate

We initially evaluated the effects of SV or prostate extract on the
malignant potential of PC3 cells. As shown in Figure 1, neither the
SV or prostate extract had any impact on cell growth or motility of
PC3 cells. However, despite the lack of significant effect of prostate
extract on the invasive potential of PC3 cells, treatment of PC3
cells with SV extract increased the invasive potential in a dose-
dependent manner.

Influence of growth factors and cytokines on the invasive
potential of PC3 cells

To identify candidate factor responsible for the enhanced invasive
potential of PC3 cells induced by SV extract, the abilities of 10
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analysed the role of uPA, one of the most important proteolytic enzymes involved in tumour cell invasion (Festuccia et al., 1998), in this process. Treatment of PC3 cells by TGF-β1 resulted in a dose-dependent increase in uPA production released in the culture medium (Figure 3A). Furthermore, the SV extract also induced increased uPA production by PC3 cells in a dose-dependent manner; however, this stimulated production of uPA by treatment with the SV extract was significantly inhibited by additional treatment with anti-TGF-β1 antibody (Figure 3B). Western blot analysis was used to measure changes in the expression levels of uPA protein in PC3 cells following treatment with SV extract and/or anti-TGF-β1 antibody. As shown in Figure 3C, uPA protein expression in PC3 cells was enhanced by treatment with SV extract in a dose-dependent manner; whereas treatment with anti-TGF-β1 antibody resulted in the suppression of enhanced uPA protein expression by the SV extract in PC3 cells.

Disease progression following the injection of PC3 cells into the SV in NOD/SCID mice

To compare the effects of organ microenvironment between SV and prostate on the disease progression of PC3 tumours in vivo, we injected PC3 cells into either the SV or prostate in NOD/SCID mice. The mice were killed 8 weeks after the tumour cell injection, during which we found that the weight of tumours in mice receiving SV injection was significantly greater than that in mice receiving prostate injection. Furthermore, the incidence of peritoneal lymph node metastases in mice receiving SV injection was significantly higher than that in mice receiving prostate injection (Table 1). In addition, haemorrhagic ascites was observed only in the mice following SV injection.

DISCUSSION

Although SV invasion has been regarded as one of the most potent factors related to an adverse prognosis in patients undergoing radical prostatectomy (Soffer et al., 2003; Bloom et al., 2004), the molecular mechanism mediating the progression of prostate cancer following the invasion of cancer cells into the SV remains largely unknown. To date, a number of studies have demonstrated a significant impact of organ microenvironment on disease progression of various types of human malignant tumours (Gohji et al., 1997; Sato et al., 1997; Alencar et al., 2005); however, there have not been any studies investigating the significance of the SV microenvironment as a factor influencing the progression of prostate cancer. In this study, therefore, we focused on the role of microenvironment of the SV, and evaluated its effects on changes in malignant phenotypes of human prostate cancer PC3 cells both in vitro and in vivo.

It was initially examined whether the SV or prostate extract influences the malignant potential of PC3 cells, and demonstrated that despite the lack of a significant effect of prostate extract, the invasive potential of PC3 cells was markedly enhanced by SV extract. To identify the factors in SV extract mediating the stimulation of invasive potential, we examined the effects of 10 different growth factors or cytokines that have been shown to be abundantly present in seminal plasma (Matalliotakis et al., 1998; Robertson, 2005) and/or associated with the invasion of prostate cancer cells (Barton et al., 2001; Bindukumar et al., 2005). Of the 10 factors examined in this study, TGF-β1 and EGF were found to significantly stimulate the invasive potential of PC3 cells; however, anti-TGF-β1 antibody, but not anti-EGF antibody, suppressed the enhanced invasive potential of PC3 cells induced by SV extract. These findings suggest that TGF-β1 may, at least in part, be involved in the increased invasive potential of PC3 cells induced by SV extract.

It is of interest to clarify the mechanism by which TGF-β1 induces the increased invasive potential of PC3 cells. Several
previous studies have shown that TGF-β1 enhances the secretion of proteolytic enzymes in prostate cancer cells, which helps degrade the connective tissue extracellular matrix and basement membrane components (Festuccia et al., 2000; Unlu and Leake, 2003). Among these enzymes involved in tumour cell invasion, uPA is one of the most predominant factors involved in the disease progression of malignant tumours (Choong and Nadesapillai, 2003). In prostate cancer as well, accumulating evidence strongly suggests the important role of uPA in the disease progression of prostate cancer (Pulukuri et al., 2005; Usher et al., 2005; Shariat et al., 2007).
For example, Pulukuri et al. (2005) reported that RNA interference-directed knockdown of uPA and its receptor in PC3 cells significantly reduced tumour cell viability and invasion, and ultimately resulted in the induction of apoptotic cell death. Considering these findings, in this study, we analysed the TGF-β1-induced stimulation of invasive potential in PC3 cells focusing on the role of uPA. Interestingly, treatment of PC3 cell with TGF-β1 enhanced their secretion of uPA in a dose-dependent manner. In addition, inhibition of TGF-β1 activity in the SV extract resulted in the suppression of uPA production in PC3 cells, which was proportional to their invasive potential. Collectively, these results indicated the potential role of uPA in TGF-β1-mediated enhanced invasive potential of PC3 cells.

To compare the different effects of organ microenvironment between the SV and prostate on disease progression in vivo, we directly injected PC3 cells into the SV or prostate in NOD/SCID

**Table 1** Comparison of disease progression in vivo following the injection of PC3 cells into the prostate or SV

|                          | Prostate injection | SV injection | P-value |
|--------------------------|--------------------|--------------|---------|
| Incidence of lymph node metastasis (%) | 2/10 (20)          | 7/10 (70)    | <0.05   |
| Incidence of haemorrhagic ascites (%) | 0/10 (0)           | 4/10 (40)    | <0.05   |
| Weight of the primary tumor (mg) | 22.8 ± 8.7        | 40.7 ± 10.6  | <0.01   |

SV = seminal vesicle. *No. of mice with lymph node metastases/no. of injected mice. **No. of mice with haemorrhagic ascites/no. of injected mice. *Mean ± s.d.
mice. A number of studies have demonstrated that cancer cells, including prostate cancer, can achieve favourable environments for disease progression in anatomically relevant (i.e., orthotopic) organs (Gohji et al., 1997; Sato et al., 1997; Alencar et al., 2005). In this study as well, lymph node metastases was observed in some mice following injection of PC3 cells into the prostate as described previously (Saffran et al., 2001); however, disease progression in mice following the SV injection of PC3 cells was more prominent than that in mice following intraprostatic injection. Furthermore, we performed in vivo experiments injecting androgen-dependent human prostate cancer LNCaP cells into the SV or the prostate of NOD/SCID mice, and demonstrated that tumour growth as well as the incidence of lymph node metastasis after the injection of LNCaP cells into the SV were significantly greater than those after the injection into the prostate (data not shown). To our knowledge, this is the first study clearly showing that SV rather than the orthotopic organ (i.e., prostate) provides a stimulating environment for the progression of prostate cancer cells.

Here, we would like to emphasise several limitations of this study. First, the phenomenon of uPA induction by TGF-β1 may not be entirely responsible for the enhanced invasive potential of PC3 cells by treatment with SV extract; that is, other molecules present in the SV may be involved in promoting the invasive potential. In addition, different mechanisms associated with the microenvironment of the SV, such as the regulated production of proteolytic enzymes by organ-specific fibroblasts (Gohji et al., 1997), may have a significant impact on the disease progression following the injection of PC3 cells into the SV. In fact, despite the lack of a stimulatory effect of SV extract on the proliferation of PC3 cells, injection of PC3 cells into the SV resulted in the enhanced growth of PC3 tumours, suggesting the involvement of a mechanism other than the SV extract-induced enhanced invasive potential of PC3 cells in the disease progression of PC3 tumours in vivo. It is also a limitation that the findings presented in this study may not be uniformly true for a wide variety of cancer cells, considering a divergent function of TGF-β1 in modulating the malignant phenotype of cancer cells (Desruisseau et al., 1996; Tuxhorn et al., 2002). Finally, we could not show in vivo data supporting the hypothesis of this study; that is, there were no characteristic findings on histological examinations of both tumours developed after the injection of PC3 cells into the SV and the prostate, such as those suggesting different metastatic potentials, and both tumours were shown to exhibit strong uPA expression by immunohistochemical staining (data not shown), which may be due to the originally high expression level of uPA in PC3 cells, resulting in the lack of evident difference in uPA expression between tumours in the SV and the prostate. Accordingly, to enhance the reliability of our findings, the outcomes of this study should be confirmed using different kinds of human prostate cancer model systems.

In conclusion, the findings presented in this study suggest that the microenvironment of SV enhances the progression of prostate cancer through a stimulated invasive potential, and that enhanced uPA production in prostate cancer cells induced by TGF-β1, which is abundantly present in seminal plasma, could therefore be one of the most important mechanisms involved in the progression of prostate cancer following SV invasion. To address the functional impact of TGF-β1-induced uPA production on the progression of prostate cancer in the microenvironment of SV, it would be absolutely necessary to perform further experiments characterising changes in malignant phenotype of PC3 cells both in vitro and in vivo before and after the inactivation of TGF-β1 and/or uPA.

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